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A Hybrid Model for a Hybrid Science

Those of you who know me personally will be surprised to see me on this masthead, as I have said that I would not edit a ‘nanomedicine journal.’ Despite initial appearances, I hope to convince you that this publication is something different: a living document, a hybrid that recognizes the diversity of the research that contributes to our field.

I have several concerns about the usefulness of current journal formats for nanomedicine and nanobiotechnology. First, I feel that, because this is such a cross-disciplinary field, traditional journals often are not able to serve their readers effectively. By necessity, most journals tend to be put together from a single perspective, be it medical, nanomaterials, or nanodevices, that does not address the needs of all interested readers. Second, I am concerned that the standard journal format does not offer an appropriate entrée to students and professionals attempting to enter the field, again from a variety of disciplines. This is because most journals publish research or review papers that are highly technical in nature, and such articles do not provide the type of perspective that a student, or a professional in an adjacent area of research, needs to help them find their place in a field. In contrast, a textbook for this rapidly evolving field would be extremely difficult to keep current.

So, if not a standard journal, what kind of publication is Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology? This will be a unique endeavor that will combine the best features of online reference works (it is a comprehensive, authoritative, frequently updated resource comprising invited contributions from leading researchers) and review journals (it offers high online visibility as well as journal-type abstracting and indexing that will confer appropriate professional credit on authors). This will be a truly living document that will exist primarily in electronic format but can also be produced in print versions periodically to provide access to those without the skill or inclination to use electronic references. Most importantly, this work is meant to provide an ongoing perspective on the development of the field. Several distinctive article types have been commissioned. Advanced reviews will survey specific areas in a citation-rich format suitable for graduate students and researchers. Opinions on issues related to nanomedicine and nanobiotechnology will be invited from thought leaders in the field. Overviews will allow people not entirely familiar with an area to rapidly gain a perspective on what might be important, new or innovative. Finally, we will include shorter contributions, known here as Focus articles, in which authors will offer perspectives on their own work in the field. This will provide them with an opportunity to place what they are doing in the context of the long-term development of nanomedicine and nanobiotechnology.

One of the key benefits of this publication is that it will be written from multiple viewpoints ranging from medical to material science. Importantly, we hope to make this content accessible to individuals in diverse disciplines in such a way as to provide a single source and platform to discuss and define the field. We will develop an interactive online forum that will allow editors and contributors to discuss published articles and offer input to the evolving knowledge base. In addition, the highly structured format of the publication will allow instructors to create class texts for different disciplines (such as medicine or bioengineering or material science) from the same set of articles. This will provide the novice with a means to enter the field and gain familiarity with many aspects that would have previously required diverse compilations of single articles from different sources.

Thus, I believe this evolving publication will not only foster new initiatives in nanomedicine and nanobiotechnology but will also bring new investigators to the field. As with any new endeavor, I am sure there are going to be growing pains, and it will take an ongoing effort to try and achieve our goals. Wiley-Blackwell is firmly committed to this concept and believes it will fundamentally change the separation between online journals and reference works. We look forward to your feedback and hope that you find this new publication of interest, and stay with us to see how it evolves.

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Opinion

The public acceptance of nanomedicine: a personal perspective
David M. Berube*

Limited understanding of a subject leads to limited perceptions, including misinformed biases and associations. In regard to the field of nanotechnology, prior biotechnologies have harmed public perception of nanotechnology through association alone. While public bias is slow to convert toward truth, it is likely that the medical applications of nanotechnology will foster a renewed interest and trust in the field through the prolonged escape from death.

This opinion piece is drawn from some work under consideration by the Public Communication on Science and Technology (PCOST) Project at North Carolina State University with which I am associated. It suggests lines of future research on the basis of observations from a review of the academic literature in the fields of applied nanoscience and risk studies.

It is a blessing as well as a curse. Applied nanoscience or nanotechnology brings great opportunities, but at the same time it scares the bejebus out of some people. Exactly how and why many people are apprehensive about nanotechnology remain difficult to discern at this time. Given the inherent ambiguity of the word ‘nanotechnology’ with its nearly infinite applications and the weaknesses of deliberative polling methodologies, any claim made regarding public attitude about nanotechnology should be critically, if not skeptically, evaluated. Nevertheless, this has not stopped some critics from speaking on the public’s behalf and voicing powerful concerns about precaution and uncertainty.

The question surfaces whether patient consumers (read as the public) will be reluctant to embrace nanomedicine. As argued below, there seems little likelihood that the field of medicine will have as much difficulty as nonhealth commercial industries. One of the reasons has to be the rigorous process drugs and devices undergo before they are marketed. While some may criticize the FDA for their failures in this area, comparative to other potential applications, FDA regulation is reasonably acceptable. When something slips through, there are always the lawyers. Companies in the drugs and devices world understand the potential liability issues associated with the products they market and generally act prudently. The public will embrace nanomedicine due to the sense of ease drawn from the regulatory purview of the FDA and potential litigation against those who market unsafe medical products. Below I argue there is an additional motivator: the fear of death.

My second argument detailed below is this: there is some likelihood that advances in the field of nanomedicine and the diagnosis and treatment of disease will ease the way for nonhealth-related commercial applications. A variation of the psychological concept of transference will be at work here.

First, we examine the biases associated with medical developments and their introduction into commerce. The feeling is generally called necrophobia and it is the fear of death and dead things. Fear of death is a dense concept and includes fear of the dying process, of the dead, of being destroyed; for significant others, of the unknown, of conscious death, for body after death, and of premature death. While excessive fear of death may be socio-pathological, some fear of death is a survival mechanism for the species. It ranks first of all the phobias and is shared in some sense by almost everyone. This fear instigates care and caution. For most of us, it animates our efforts to avoid unnecessary and excessively risky activities.

There is some evidence this fear may be intrinsic to our species, but more easily defended is the concept
that we are taught to fear death. Most religions separate life and afterlife into distinct categories and the transition from one to the other is mystical, if not traumatic. We see death portrayed by the media as sad and grief-laden, and some of us experience epiphanies following a disturbing death—often by someone very close to us.

Fear of death is an important variable in health care delivery. There is even some evidence fear and denial of death among medical professionals and the public is the basis of the belief that prolongation of life is the predominant goal of medicine.

The introduction of a new technology, exotic or familiar, which serves to prolong life taps into our fear of death and dying. Sating this apprehension makes the adoption of this technology, including nanomedicine, more likely against the same technology associated with some less essential application, like stronger automobile bumpers and better performing baseball bats.

Second, consider a troubling phenomenon often associated with the generation of polling data on preferences toward applications of nanotechnology. More respondents opine about nanotechnology than can define it.

This is not wholly surprising. We know from perception studies that populations wishing to provide positive feedback to a survey will offer opinions they feel are likely to be anticipated by the survey question. This produces a range of false positive and negative results depending on the survey question and general subject of the survey itself.

Moreover, when respondents become befuddled with a question, instead of admitting ignorance they draw from a set of sensibilities they feel are related to the subject of the question, a form of reasoning by analogy. Some have suggested that when asked about nanotechnology, respondents will equate it with biotechnology and transfer their feelings about biotechnology to nanotechnology. Others suggest they transfer sensibilities about health and safety issues. Still others suggest environmental risks as a source for the transference.

This form of transference is fundamental in lists of important heuristics in perception. These phenomena are derivative of research on representativeness and set theory, as well as the availability and anchoring biases. When making decisions, individuals over-relate to a set of classes, availability of instances and scenarios, and adjustment from an anchor.

Focalism is another name for this effect, and it is loosely related to the fallacy of composition in logic. Some might want to call it simply a hasty generalization.

Another phenomenon called contagion has been a part of the debate over nanotechnology for some time. Those of us who carefully watch public pronouncements about the risks associated with nanotechnology have noticed a rhetorical device surfacing aside demands for more research about the health and safety implications of nanotechnologies. Speakers and commentators have added a powerfully negative scenario. They claim the effect of a single serious health and safety event might be sufficient to produce a contagion effect. This effect assumes the entire gamut of businesses and industries engaged in nanotechnology applications will wither with attendant economic losses to a spate of stakeholders.

This phenomenon is incredibly dense and complex, whereby in some instances it is noticeable while in others it is not. In addition, there are some examples testing the power of the contagion effect. For example, contagion phenomena occurred neither with the release of Kleinman’s Magic Nano (which was responsible for over a hundred reported cases of respiratory distress in Europe last year) nor with the Samsung SilverCare product line allegedly related to waste treatment difficulties. However, both of these examples did not receive substantial media attention, hence they were not amplified. While the Johnson & Johnson Tylenol tampering case from 1982 is cited as a counter-example, the blame in this instance was not the business but a miscreant. Recently, the food and personal care industries seem to have weathered gales of their own: salmonella tainted fresh spinach, Escherichia coli-tainted green onions, melamine-tainted pet foods and fish feed, and ethyl glycol (an antifreeze ingredient) tainted toothpastes in 2006 and 2007 as well, though in these instances blame was equally unclear and dissipated across long supply lines.

Generally, contagion refers to the spillover of the effects of shocks from one or more firms to other firms. Most studies of contagion limit their analysis to how shock affects firms in the same industry, or ‘intra-industry’ contagion. Most of the studies on contagion attempt to differentiate between a ‘pure’ contagion effect and a signaling or information-based contagion effect. An example of a pure contagion effect would be the negative effects of a bank failure spilling over to other banks regardless of the cause of the bank failure. An example of a signaling contagion effect would be if a bank failure is caused by problems whose revelation is correlated across banks, and the correlated banks are impacted negatively.

Evidence supporting intra-industry contagion is fairly common and comes from studies of the credit default market and other financial institutions.
Evidence supporting inter-industry or extra-industry contagion is mostly limited to the financial industry as well. For example, studies on banks and life insurance companies demonstrate some cross-industry cascade phenomena. Evidence crossing industries as diverse as cosmetics and food production is nearly impossible to find, but nanotechnology may be the exception. While a contagion event across a diverse industry might be difficult to prove, it is clearly not impossible. Indeed, in terms of a newly emerging industry with unclear boundaries such as nanotechnology, it might be plausible.

We have already learned the deficit theory of science literacy is fallacious. Providing more information about science to a subject does not equate to a more positive feeling toward a scientific artifact, like nanotechnology per se. Noteworthy to some, a recent study suggested that more information actually reduces positive responses (see Kahan et al.). However, there is an equally strong case that information can affect feelings, especially when mediated. While Kahan et al.’s finding remains very consistent with the observation that merely mentioning risk of a phenomenon will increase apprehensions by raising its saliency may be true; on some level mediated information can attenuate some of the power of the effect.

One of the hypotheses we have been studying involves a variation on the anchoring bias. The first feeling one develops about a phenomenon or artifact tends to anchor subsequent assessments. A negative experience or anchor is very difficult to erase or reduce. A positive anchor is difficult to degrade, though less so. Essentially, there is a bias toward the negative. We argue when the public has a feeling, which is not based on understanding but drawn from an analog feeling (such as, nanotechnology is like biotechnology so how I feel about biotechnology is much like how I should feel about nanotechnology), we might be able to rehabilitate the antecedent. For the example mentioned above, if the public develops a positive feeling about nanotechnology, it might help rehabilitate a less than positive feeling about biotechnology. By extension, we sense that a positive experience with nanotechnology in the present will affect feelings about nanotechnology in the future as well.

While this hypothesis needs to be tested, we anticipate that some of the first authentic applications of nanotechnology will make this case. Many, if not most, of current product releases have involved the use of nanoparticles in coatings, e.g., paint, and as reinforcement when associated with another media, e.g., carbon composites. We expect nanomedicine will be perceived as actual nanotechnology and may be willing to go as far as suggesting nanomedicine products will be perceived as archetypal for nanotechnology based on public expectations of major and breakthrough technological development in the field of medicine in general.

Nanomedicine may help anchor public sentiment positively. This should have some positive effects on feelings toward the subsequent introduction of products in nanomedicine (intra-industry contagion). Given the public positive response to exotic health technologies, the positive public feelings for nanomedicine may transfer to subsequent applications of nanotechnology in health and even outside of health (inter-industry contagion). If the public embraces applications in nanomedicine, follow-up applications in the food industry may be positively affected as well.

In final extension, if respondents to surveys about nanotechnology opine without an understanding of nanotechnology and draw the warrant for their opinion from an analog, then a positive feeling toward nanomedicine might help to rehabilitate the feelings toward the relevant analog, such as biotechnology or environmental health and safety in general.

While this needs to be studied, there is some anecdotal evidence that this point of view is plausible. Global warming concerns are rehabilitating our feelings toward nuclear fission power generation, and advances in bioengineering less expensive pharmaceuticals may be rehabilitating our feelings toward genetically modified foods. Opposition to ‘introducing genetically modified foods into the US food supply’ has declined from 58% in 2001 to 47% today, an 11-point decrease. On the other hand, the public’s attitude toward genetically modified foods seems to be inversely related with the evolutionary ladder; hence efforts to genetically modify animals might exacerbate negative attitudes toward genetically modified crops.

In conclusion, we predict nanomedicine products will be welcomed by the public as a stay against the fear of death and we find some solace in this prognostication based on public responses toward biomedicine, especially recent opinion shifts toward stem cell research. Furthermore, we envision a positive feeling toward nanomedicine products will make later introductions of nanomedicine products even more welcomed by the public. Finally, we hypothesize a positive feeling towards nanomedicine products may carry over to nanoproduct lines from other industries, and a positive feeling toward nanomedicine products may rehabilitate past held beliefs toward analogical products and other related phenomena.
REFERENCES


RELATED ONLINE ARTICLES

Commercialization of nanotechnology.
Nanoparticles in food as potential health hazards.
Ethical issues in nanomedicine.
Nanotechnology and orthopedics: a personal perspective

Cato T. Laurencin,1,2,3∗ Sangamesh G. Kumbar2,3 and Syam Prasad Nukavarapu2,3

Bone is a nanocomposite material comprised of hierarchically arranged collagen fibrils, hydroxyapatite and proteoglycans in the nanometer scale. Cells are accustomed to interact with nanostructures, thus providing the cells with a natural bone-like environment that potentially enhance bone tissue regeneration/repair. In this direction, nanotechnology provides opportunities to fabricate as well as explore novel properties and phenomena of functional materials, devices, and systems at the nanometer-length scale. Recent studies have provided significant insights into the influence of topographical features in regulating cell behavior. Topographical features provide essential chemical and physical cues that cells can recognize and elicit desired cellular functions including preferential adhesion, migration, proliferation, and expression of specific cell phenotype to bring desired effects. The current article will address some of the nanotechnology implications in addressing issues related to orthopedic implants performance and tissue engineering approach to bone repair/regeneration.

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Nanotechnology, a new focus in the area of biomedical research, involves visualization, manipulation, and fabrication of materials on the smallest scales, in dimensions of 1 µm down to 10Å. This diverse new field draws from various disciplines: biology, medicine, materials science, physics, and manufacturing. At the nanoscale, materials possess several novel properties including extremely high surface area to volume ratio, tunable optical emission, enhanced mechanical properties, and super paramagnetic behavior, which contrast the properties that are deemed important when working with the bulk parent materials. Most of the current technical research in this area is focused on fabrication and evaluation of nanostructures, devices, and systems. Nanotechnology is rapidly gaining momentum and attracting large investments from both government and private sectors. The National Nanotechnology Initiative (NNI), a multidisciplinary strategy for development of science and engineering fundaments, has proposed a total investment budget of $8.3 b for the year 2008. The global market for nanotechnology products, such as nanoscale devices and molecular modeling systems, has seen an average annual growth rate of 27.5%, from $400 million in 2002 to $1.37 b in 2007.

Moreover, nanomedicine, which is an offshoot of nanotechnology, can be broadly defined as a technology that uses molecular tools and knowledge of the human body for diagnosis, treatment, and prevention of diseases and traumatic injury. Health care applications of nanomedicine can be roughly classified into diagnostics, imaging, nanobiomaterials, nanodevices/implants, novel drug delivery systems (NDDS) and issues related to toxicity. Applications and products dealing with NDDS dominate the nanomedicine market, and thus, there are currently more than 40 nanotechnology-based products available in the market. Several biomaterials in the form of self-assembled nanofibers/nanoparticles, electrospun nanofibers, nanocomposites, and hydroxyapatite are also being used as integral parts of biomedical devices to improve their in vivo performance (Figure 1).
Nanotechnology: Nanobiomaterials
- Topography
- Surface chemistry
- Wettability

Orthopaedic applications
- Nano-patterned implants
- Nano composite fillers
- Nano featured Scaffolds
- Novel drug delivery Systems
- Diagnostics

Toxicity issues
- Inflammation
- Cytotoxicity
- Nanoparticle clearance

**FIGURE 1** | Nanotechnology and orthopedic applications.

Nanohydroxyapatite (nHAp)-based products such as Ostim, Vitoss, and Perossal are now commercially available for bone filling. Additionally, some of the nanoparticle composites available for dental filling and repair applications include Filtek Supreme, Ceram X duo, Tetric EvoCeram, Premise and Mondial. Several other nanotechnology approaches being explored include coating of orthopedic implants with nHAp and bioactive signaling molecules to stimulate osteoblast proliferation and differentiation.

**BONE: A NANOCOMPOSITE**

The extracellular matrix (ECM) in natural bone tissue is principally composed of hierarchically arranged collagen fibrils, hydroxyapatite, and proteoglycans in the nanometer scale. The individual collagen helical chains are 10 nm in length and self-assemble into orientated collagen fibers measuring approximately 500 nm in length. The mineral or inorganic component of bone, hydroxyapatite, exists as plate-like nanocrystals, measuring 20–80 nm in length. Additionally, the triple helical structure of collagen provides bone with a structural framework, high tensile strength, and flexibility, while crystalline hydroxyapatite accounts for the stiffness and high compressive strength of bone. With a more in-depth investigation into the constituents of bone and their properties, researchers hope to develop scaffold and implants that closely mimic the physicochemical characteristics of natural bone.

**Nanobiomaterials and Cellular Recognition**

The success of orthopedic implants and tissue-engineered constructs greatly depends on the biocompatibility of the material. Furthermore, the biocompatibility of an entity is largely determined by its physicochemical properties. Many studies have clearly demonstrated that cell behavior can be easily manipulated by providing cells with suitable biochemical cues, surface topography and external stimuli. It has also been observed that minimal interactions between implant surfaces and surrounding tissue often result in poor tissue formation on the implant surface and can ultimately lead to failure of the treatment.

Researchers are currently investigating a variety of methods/techniques to combine both the structural and chemical components of natural ECM in novel materials for tissue engineering applications. The ECM, a self-assembled nanofibrillar complex three-dimensional (3D) dynamic structure, plays a vital role in determining the cell behavior. Thus, within the ECM, cells experience complex nanotopography and encounter a variety of chemical cues in the form of proteins and growth factors that regulate cell growth, differentiation and metabolic activity. Following implantation of a device, many proteins from bodily fluids adsorb onto the surface of the implant and subsequently control cell adhesion. In this case, the implanted materials are essentially playing a role similar to that played by the ECM. Initial cell adhesions to implant surfaces are determined by the presence of specific amino acid sequences available for binding to cell membrane integrin receptors. For instance, osteoblasts preferentially adhere to amino acid sequences such as heparin-sulfate and arginine-glycine-aspartic acid (RGD) regions in the adsorbed proteins. In addition to chemical cues, surface topography significantly alters cell behavior and can be responsible for changes in morphology, adhesion, motility, proliferation, endocytotic activity, and gene regulation. Cells existing on surfaces having the same topography but different chemistry, with different concentrations of protein adsorption also showed very similar cell behavior. Researchers, however, argue whether the observed cell behavior can be attributed to surface topography or differential protein adsorption on the surface. Furthermore, it is apparent that detailed understanding of implant surface properties, protein adsorption, and cell behavior can potentially circumvent the problems associated with current orthopedic implants.

**NANOBIOIMATERIALS**

Nanotechnology has revolutionized many fields of science including fabrication and characterization of various nanostructures. Several techniques for patterning
implant surfaces and for efficiently constructing scaffolds for tissue engineering have emerged; some of these techniques include: lithography, polymer demixing, phase separation chemical etching, electrospinning, and molecular self-assembly. The nanofeatures created by these methods can provide the bulk material with high surface area to volume ratio, tunable mechanical, electrical, optical emission, and super paramagnetic behavior, characteristics that have been successfully exploited for a variety of health care applications ranging from drug delivery to biosensors.

**Topography**

Nanotopographical features such as pores, ridges, grooves, fibers, nodes, and combinations of these features are known to influence cell behavior significantly. The emphasis in current research is on developing implant surfaces with a suitable surface topography to elicit desired cellular function. In one set of studies, cellular interaction with nanophasic Ti, Ti6Al4V, and CoCrMo alloys resulted in increased osteoblast adhesion due to the presence of more particle boundaries compared to conventional metals. For instance, nanopatterned polystyrene grooves with two different depths of 50 and 150 nm, with a periodicity of 500 ± 100 nm, showed a strong alignment and orientation of primary osteoblasts in the direction of the grooves. One other study showed that primary human osteoblasts migrated away from Ti oxide surfaces patterned with 110-nm-high hemispherical protrusions of varying topography densities. Another important finding reveals that sensitivity and cell behavior on nanotopographical features depends on the cell type. For example, with an increase in either carbon nanofiber surface energy or a simultaneous change in carbon nanofiber chemistry, enhanced osteoblast adhesion was observed on carbon nanofibers while smooth muscle cell, fibroblast, and chondrocyte adhesion decreased. At this state, it is not fully understood how cells detect and respond to nanofeatures. Thus, it is important to understand cell-nanotopography interactions, and that variability in results may exist among varying cell types.

**Surface Chemistry**

Implant surface chemistry plays a critical role in deciding the performance and success of the devices. Proteins and other biomolecules dynamically adsorb to biomaterial surfaces upon implantation. These complex molecules can trigger nonspecific inflammatory responses characterized by foreign body reaction and fiber capsule formation. Thus nonspecific inflammatory responses can limit integration of the device and influence in vivo performance. Limited success has been achieved through the delivery of anti-inflammatory agents and nonfouling of implant surfaces. Studies have demonstrated that self-assembled monolayers (alkanethiols on gold), having well-controlled surface properties and different terminal functionalities such as CH3, OH, COOH, and NH2, possess different affinities for fibronectin adsorption, and thus differentially influence integrin binding and cell adhesion. The binding of monoclonal antibodies and α5β1 integrin to adsorbed fibronectin affinities were in the order of OH > COOH = NH2 > CH3 while α5 integrin binding was in the order of COOH ≫ OH = NH2 = CH3, demonstrating the α5β1 integrin specificity for fibronectin adsorbed onto the NH2 and OH. Differences in integrin binding differentially regulate focal adhesion assembly and signaling which, in turn, modulate cellular functions in biomaterial and implant surfaces.

**Wettability**

It is evident from the literature that the hydrophobicity or hydrophilicity of a surface can significantly alter cell behavior. Moreover, the wettability of a material can allow for characterizing materials with regards to the hydrophobic/hydrophilic categories. Implant or biomaterial surface composition, surface treatment, surface roughness, immobilization of various chemical agents to the surface, and the presence of nanofeatures on the surface alter the surface wettability and affect cell behavior. For instance, the surface wettability of alumina can be improved by reducing the alumina grain size from 167 to 24 nm. In another study, ultrafine titanium crystals produced by high pressure torsion provided a high degree of surface wettability, and thus preosteoblasts showed enhanced attachment and proliferation rates. Additionally, improved wettability enhances adsorption of vitronectin and fibronectin on nanofeatures that stimulate the osteoblast adhesion. It is important to investigate the influence of surface topography, chemistry, and wettability of various biomaterials and implant surfaces on protein adsorption and receptor-mediated cell adhesion. Optimization of these parameters to improve adsorption of osteogenic proteins on the implant surface and enhance implant exposure time to cell integrin binding domains, may provide opportunities to develop implant surfaces which enhance the attachment, adhesion, and developmental response of osteoblast precursors leading to accelerated osteointegration.
NANOCOMPOSITES
Bone is a natural composite of nHAp and organic components such as collagen and proteoglycans. Hence, various tissue engineering strategies have adopted composite scaffold approaches to more closely mimic the bone in structure and composition. Nanoparticles including calcium triphosphate, bioactive glass, hydroxyapatite, and calcium-deficient HAp in combination with natural (PLGA, polycaprolactone, and polylactide) or synthetic (chitin, chitosan, and hyaluronic acid) biodegradable polymers have been fabricated into porous 3D scaffolds for bone repair/regeneration purposes. Not only does this approach allow for mimicking bone in composition but incorporation of nanoceramics enhances the materials mechanical strength and nanotopographical features. The presence of a mineral phase on the surface enhances the scaffold’s osteoconductivity, osteogenicity, and osteointegrative nature. Though most of these scaffold types showed mechanical properties in the range of human cancellous bone, fabricating scaffolds with mechanical performance close to compact bone is still a persisting challenge. In a bid to improve mechanical properties, researchers have begun to develop and evaluate composite polymeric matrices containing single or multilayered carbon nanotubes. In addition to the size, carbon nanotubes offer excellent properties, such as high tensile strength, high flexibility, and low density that can be exploited to develop more successful orthopedic implant materials.

NANOFIBERS
Cells are organized and trapped within organs and tissues by a nanofibrous 3D ECM. Electrospun nanofiber matrices closely mimic the structure of natural ECM and have shown great promise as scaffolds for tissue engineering applications. Polymeric nanofiber scaffolds are characterized by ultrathin continuous fibers, high surface-to-volume ratio, porosity and variable pore size distribution, making them ideal for tissue regeneration efforts. The nanofiber structure provides anchorage for cells while high porosity provides a means for the supply and removal of nutrients and metabolic wastes. Cell growth has been found to be significantly enhanced on nanofiber matrices. Additionally, the nanofiber scaffold environment was found to drive mesenchymal stem cells to differentiate along an osteogenic lineage, resulting in the formation of mineralized tissue. Current efforts in this area focus on coating biomedical implants with nanofibers to provide high surface area, achieve tissue compatibility and allow for selective delivery of bioactive agents.

NANOSTRUCTURE CYTOTOXICITY ISSUES
Degradation of nanocomposite or nanostructured scaffolds result in erosion of nanoparticles that are either retained or degraded and get excreted from the system. In the process of metabolism, nanoparticles pass through various organs such as blood, liver, and kidneys, and possibly cause oxidative stress and inflammation. Thus, it is important to establish the clearance rate and the cytocompatibility of these nanoparticles with hemocytes, stem cells, hepatocytes, and nephrocytes. Biodegradable nanoparticles cause less damage in contrast to biostable particles, provided the degradation products are physiologically compatible. The cytotoxic effects of traditionally used nHAp which are neither biodegradable nor same as the native bone apatite can be mitigated when replaced with biodegradable carbonated hydroxyapatite nanoparticles. Also, there are conflicting reports in literature about the safety of carbon nanotubes for human use. However, their clearance can be improved by functionalizing carbon nanotubes with hydrophilic groups such as –COOH, –OH, and –NH2.

CONCLUSION
Nanotechnology has revolutionized many research areas. Orthoped research, in particular, has now turned its focus to utilizing these developments to address the limitations associated with implant design and tissue regeneration. A current fabrication and design issue for nanobased implants is optimization of physicochemical properties. It is also important to address issues related to inflammatory response and toxicity. A combined approach involving multifunctional nanotopographic features that incorporate bioactive factors and a suitable cell population may be an alternative to aid in the rapid development of engineered organs. Recent studies have provided significant insights into the influence of topographic features in regulating cell behavior, including preferential adhesion, migration, proliferation, and expression of cell-specific phenotypes. It is critical to understand the molecular mechanisms governing cells and cell-material interaction to generate better scaffold and implant performance. With the advent of nanofabrication techniques several novel biomaterials can be fabricated into nanostructures that simulate the native

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Cells are organized and trapped within organs and tissues by a nanofibrous 3D ECM. Electrospun nanofiber matrices closely mimic the structure of natural ECM and have shown great promise as scaffolds for tissue engineering applications. Polymeric nanofiber scaffolds are characterized by ultrathin continuous fibers, high surface-to-volume ratio, porosity and variable pore size distribution, making them ideal for tissue regeneration efforts. The nanofiber structure provides anchorage for cells while high porosity provides a means for the supply and removal of nutrients and metabolic wastes. Cell growth has been found to be significantly enhanced on nanofiber matrices. Additionally, the nanofiber scaffold environment was found to drive mesenchymal stem cells to differentiate along an osteogenic lineage, resulting in the formation of mineralized tissue. Current efforts in this area focus on coating biomedical implants with nanofibers to provide high surface area, achieve tissue compatibility and allow for selective delivery of bioactive agents.

NANOSTRUCTURE CYTOTOXICITY ISSUES
Degradation of nanocomposite or nanostructured scaffolds result in erosion of nanoparticles that are either retained or degraded and get excreted from the system. In the process of metabolism, nanoparticles pass through various organs such as blood, liver, and kidneys, and possibly cause oxidative stress and inflammation. Thus, it is important to establish the clearance rate and the cytocompatibility of these nanoparticles with hemocytes, stem cells, hepatocytes, and nephrocytes. Biodegradable nanoparticles cause less damage in contrast to biostable particles, provided the degradation products are physiologically compatible. The cytotoxic effects of traditionally used nHAp which are neither biodegradable nor same as the native bone apatite can be mitigated when replaced with biodegradable carbonated hydroxyapatite nanoparticles. Also, there are conflicting reports in literature about the safety of carbon nanotubes for human use. However, their clearance can be improved by functionalizing carbon nanotubes with hydrophilic groups such as –COOH, –OH, and –NH2.

CONCLUSION
Nanotechnology has revolutionized many research areas. Orthoped research, in particular, has now turned its focus to utilizing these developments to address the limitations associated with implant design and tissue regeneration. A current fabrication and design issue for nanobased implants is optimization of physicochemical properties. It is also important to address issues related to inflammatory response and toxicity. A combined approach involving multifunctional nanotopographic features that incorporate bioactive factors and a suitable cell population may be an alternative to aid in the rapid development of engineered organs. Recent studies have provided significant insights into the influence of topographic features in regulating cell behavior, including preferential adhesion, migration, proliferation, and expression of cell-specific phenotypes. It is critical to understand the molecular mechanisms governing cells and cell-material interaction to generate better scaffold and implant performance. With the advent of nanofabrication techniques several novel biomaterials can be fabricated into nanostructures that simulate the native
hierarchical structure of the bone. Results of the preliminary studies mentioned are quite encouraging, and the next step should involve thorough evaluations of these structures in suitable animal models to make the transition to clinical use.

REFERENCES


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Overview

Prospects and developments in cell and embryo laser nanosurgery

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Recently, there has been increasing interest in the application of femtosecond (fs) laser pulses to the study of cells, tissues and embryos. This review explores the developments that have occurred within the last several years in the fields of cell and embryo nanosurgery. Each of the individual studies presented in this review clearly demonstrates the nondestructiveness of fs laser pulses, which are used to alter both cellular and subcellular sites within simple cells and more complicated multicompartmental embryos. The ability to manipulate these model systems noninvasively makes applied fs laser pulses an invaluable tool for developmental biologists, geneticists, cryobiologists, and zoologists. We are beginning to see the integration of this tool into life sciences, establishing its status among molecular and genetic cell manipulation methods. More importantly, several studies demonstrating the versatility of applied fs laser pulses have established new collaborations among physicists, engineers, and biologists with the common intent of solving biological problems.

Several studies have reported the application of femtosecond (fs) laser pulses as a precise scalpel tool for performing cellular surgery.1–17 In each study, fs laser pulses were produced from a titanium sapphire (Ti:Sapphire) laser oscillator or amplifier (700–900 nm) delivering a sub-10 fs to 250 fs pulse at a repetition rate of 76 MHz to 1 kHz. The fs laser pulses were coupled to a high numerical aperture (NA) microscope objective, NA = 0.95–1.4, and localized to cellular and subcellular sites. Beam dwell times ranged from milliseconds to seconds and pulse energies delivered to the sample were 0.03 to several nanojoules per pulse (nJ/pulse). Model systems that have been used in fs laser pulse mediated nanosurgery include human metaphase chromosomes,4 Chinese hamster and canine kidney epithelial cells,1,2 plant chloroplasts,5 mitochondria in endothelial and HeLa cells,6,7 yeast microtubules,8 the actin cytoskeleton in fixed 3T3 fibroblast and bovine endothelial cells,6,9 hamster ovary cells,10,17 Caenorhabditis elegans,11,12 Drosophila melanogaster,16 Sprague-Dawley rats and Danio rerio (zebrafish).13 Using these biological systems, intrachromosomal dissections,4 membrane surgery,1 cell isolation,1 cytoskeletal and microtubule ablation,6,8,9 knockdown of plastids,5 laser axotomy of neurons,11 intravascular disruption of microvessels,13 cellular delivery of exogenous DNA, carbohydrates and quantum dots2,3,17 and the surgical ablation of Drosophila16 and zebrafish embryos1,15 have been demonstrated. In this paper, we present a review of current developments in fs laser mediated nanosurgery of cells and embryos with emphasis on the fs laser as a tool able to induce ablation with high spatial resolution and with minimal transfer of thermal and mechanical stresses to the material investigated.

LASER INTERACTION WITH BIOLOGICAL MATERIALS

Features that distinguish fs laser pulses from longer pulse durations (i.e., nanosecond pulses) include the ability to localize cellular disruption to a sub-micron resolution, the low threshold energy needed to elicit ablation and the lower conversion of energy into shockwaves and cavitation bubbles, which are adverse side effects known to increase the spatial extent of cellular damage.18–22 When fs laser pulses are focused to a high peak intensity of 1011–1013 W/cm2, optical breakdown occurs, resulting in the ablation of...
the biological material. The mechanism by which the material is ablated depends on the strength of the peak intensity, leading to quasi-ionized electrons (the electrons are not completely ionized from the atom, rather they occupy a higher energy state within the conduction band of the material) produced via multiphoton absorption or tunneling ionization. The Keldysh parameter \( \gamma \) determines the extent to which multiphoton absorption or tunneling ionization governs the ablation process. In Eq. (1), \( \omega, e, c, \epsilon_0, m, \Delta, I \) represent the frequency of light, electron charge, speed of light, permittivity of free space, electron-hole reduced mass, bandgap energy, and peak intensity of the light pulse, respectively. When \( \gamma < 1 \), tunneling ionization dominates the laser-matter interaction process, while for \( \gamma > 1 \) multiphoton ionization dominates. As to a very good approximation we can consider biological tissue as water, Sacchi suggested that water should be modeled with a bandgap energy of 6.5 eV. The value of 6.5 eV arises from work conducted by Boyle et al. which examined the photolysis of liquid water. If we consider laser light produced from a Ti:Sapphire laser oscillator with an emission spectrum centered at 800 nm (1.55 eV) and a pulse duration of 100 fs, then for \( \gamma > 1 \), the biological tissue must simultaneously absorb five photons to excite a valence electron to the conduction band. This conduction electron represents the 'seed electron' that undergoes free carrier linear absorption by nonresonantly absorbing laser photons through inverse bremsstrahlung. As the electron energy increases, a condition is reached where the electron undergoes impact ionization, defined as 1.5\( \Delta \) where \( \Delta \) is the effective ionization potential given by:

\[
\Delta = \frac{2}{\pi} \sqrt{1 + \gamma^2} E \left( \frac{\pi}{2}, k \right) \tag{2}
\]

where \( E \left( \frac{\pi}{2}, k \right) \) represents the elliptical integral of the second kind with \( k = (1 + \gamma^2)^{-1/2} \). At 1.5\( \Delta \) the electron impact ionizes a valence electron, resulting in two electrons in the conduction band (seed electron and ionized valence electron). Through linear absorption of laser photons, these two electrons can participate in impact ionization, causing a rise in the density of conduction band electrons. This cascade effect is properly termed avalanche ionization, where the ionized electron density quickly rises to a critical value where optical breakdown occurs. At optical breakdown, the plasma frequency and the critical electron density equals the laser frequency and the critical electron density becomes:

\[
N_{\text{crit}} = \frac{\omega^2 m_e \epsilon_0}{c^2} \tag{3}
\]

where \( m_e \) is defined as the electron mass. At a wavelength of 800 nm, \( N_{\text{crit}} = 10^{21} \text{ cm}^{-3} \), beyond which the plasma becomes highly reflective and absorbing to laser light.

As a consequence of using fs laser pulses for ablation, seed electrons can be generated with an intensity value lower than the threshold intensity for optical breakdown. With nanosecond laser pulses, no seed electrons are created by multiphoton ionization for intensities below the threshold for optical breakdown. Therefore, the requirement that the intensity must equal the threshold for optical breakdown to produce seed electrons indicates an increase in the deposition of laser energy. However, this increased energy is funneled into shockwaves and cavitation bubbles, leading to a larger spatial disruption of the material. In fact, Vogel et al. showed that the conversion of energy into cavitation bubbles for fs laser pulses was 6.8% versus 12.7% for nanosecond pulses. As a result, less energy is required to elicit ablation of the material, which reduces the amount of transient stresses such as shockwaves and cavitation bubble formation imparted to the sample.

In fs laser-tissue interaction the mechanism of ablation between high and low repetition rate laser oscillators (i.e., 80 MHz vs. 1 kHz) is different. For instance, in 80 MHz ablation the pulse energy is below the threshold energy for optical breakdown with each pulse producing a low density plasma. Ablation of the biological material occurs through the interaction of multiple pulses through free electron induced chemical decomposition of the material via bond-breaking. In contrast, with low repetition rates such as 1 kHz, the pulse energy for ablation is near or above the breakdown threshold energy. Larger plasma densities are created in comparison to 80 MHz with the formation of minute cavitation bubbles. It has been suggested that the cavitation bubbles are responsible for the dissection of the biological material.

Using fs laser pulses, highly spatially localized ablation is achievable through the nonlinear multiphoton ionization process (\( \gamma > 1 \)). When a laser beam is focused by a high NA objective, it is the electron density profile and not the irradiance profile that governs the spatial extent of ablation. (The NA of the microscope objective lens can alter the ablation profile. Using low NA objectives (i.e., NA < 0.9) plasmas can be generated ahead of the focus. Such a plasma...
can effectively shield successive laser photons from reaching the focus and induce plasma defocusing.\textsuperscript{19,27} Spatially asymmetric plasmas are created with low NA objectives, with the observance of a high plasma density (before the geometrical focus) surrounded by a lower density region.\textsuperscript{27} In contrast, for NA $\geq 0.9$, smaller symmetric plasmas are formed.\textsuperscript{27} The diffraction limited laser spot size has transverse, $d_{\text{trans}}$, and longitudinal, $z$, dimensions\textsuperscript{28}

$$d_{\text{trans}} = \frac{1.22 \lambda}{\text{NA}} \quad \text{and} \quad z \approx 2 \left(\frac{\pi w_0^2}{\lambda}\right)$$

where, for example, NA = 1.3, $\lambda = 800$ nm and $w_0 = 375$ nm are the NA, wavelength of light and radius of the beam waist, respectively. The irradiance profiles along the transverse and longitudinal direction are 750 and 1104 nm, respectively. As the simultaneous absorption of five photons is required to produce a small symmetric plasma,\textsuperscript{27} the ablation dimensions are effectively reduced by $\sqrt{5}$,\textsuperscript{19} yielding 335 and 494 nm for the transverse and longitudinal dimensions, respectively. (The transverse and longitudinal dimensions of the ablation profile represent theoretical estimates. The formation of cavitation bubbles, particularly with high repetition rate laser oscillators (i.e., MHz), can increase these values.) Therefore, the ablation of biological tissue can be localized to a high spatial resolution, allowing key structures within biological material to be removed or altered without affecting adjacent cellular sites. This unique property has made the application of fs laser pulses a novel tool for the nondestructive study of biological materials.

**Cellular and Subcellular Nanosurgery**

In a study by Konig et al.,\textsuperscript{4} the authors reported the nanodissection of fixed air-dried human metaphase chromosomes using fs laser pulses (170-fs, 800 nm, 80 MHz). Intrachromosomal dissections were made by 500–2500 consecutive single line scans across the chromosome using an average laser power of 100 mW (1.25 nJ/pulse).\textsuperscript{4} Dissection depths were analyzed using scanning force microscopy and revealed a full width at half maximum cut size of 170 ± 10 nm for 500 consecutive scans.\textsuperscript{4} A reduction in the number of laser line scans to 250 produced smaller cut sizes on the order of 85 ± 10 nm.\textsuperscript{4} It was also found that the cut sizes increased with an increasing number of consecutive scans, from 200 to 400 nm for 1000 to 2500 scans. In addition to line scans, the authors performed stationary ablation of the chromosomes with an average laser power of 15 mW (0.19 nJ/pulse) and varying beam dwell times.\textsuperscript{4}

In the work conducted in our lab, Kohli et al.\textsuperscript{1} demonstrated the surgical dissection of biological material using fs laser pulses. With a pulse energy of 5 nJ/pulse (sub-10 fs, 800 nm, 80 MHz) several dissection cuts were made in the plasma membrane of live Madin-Darby Canine Kidney (MDCK) cells.\textsuperscript{1} Figure 1 shows membrane surgery on the mammalian cell, where the arrows represent the ablated extracellular matrix. Post-laser surgery, the cell maintained normal morphology without evidence of membrane re-orientation, cell collapse or bleb formation, Figure 1. It was hypothesized that the absence of cell dissociation after laser surgery was likely as a result of coalescence of the dissected upper and lower plasma membrane.\textsuperscript{1} Further work by this group used fs laser pulses as a novel tool for single cell isolation. Figure 2 depicts nanosurgical isolation of a live Chinese hamster fibroblast cell. Two fibroblast cells are shown initially tethered together by focal adhesions. As shown in Figure 2(b–d), by scanning the cells along the dissection interface relative to the laser.

![FIGURE 1 Membrane surgery on a live MDCK cell. (a) Illustrates a cell of 12 μm in length where three ~ 800 nm incisions have been made. (b) When the sample is traversed along its long axis an additional incision is made, with (c), two extra sub-micron surgical incisions. The arrows in (a) indicate the ablated extracellular matrix secreted by the cell. Unlike fibroblasts, MDCK cells are devoid of focal adhesions, and cell-substrate bonds anchor the cell to the substrate. With precise sample movement, the isolation of single MDCK cells can be achieved when the laser traces the exterior contour of the cell membrane. Laser parameters: pulse duration sub-10 fs; excitation wavelength 800 nm; oscillator repetition rate 80 MHz; pulse energy for surgery 5 nJ/pulse; 0.95 NA 100× air microscope objective. (Reprinted, with permission, from Ref. 1. Copyright 2005 Wiley Periodicals, Inc.).](image-url)
FIGURE 2 | Live video observation of nanosurgical isolation of live fibroblast cells. (a) The arrows depict two fibroblast cells (V79-4), with a tethered width of $\sim 1 \mu m$. The dashed line represents the dissection interface the sample traverses relative to the fs laser spot. (b) The application of focused laser pulses ($10^{13}$ W/cm$^2$/pulse), indicated by the arrow, nanosurgically ablates the focal adhesions adjoining the two fibroblast cells. (c) The surgery precisely isolates and detaches the cell, indicated by the dotted box. This is achieved without morphologically compromising the cell. (d) An in-focus image, depicted in the dotted box, shows a live isolated folded fibroblast cell. Laser parameters: pulse duration sub-10 fs; excitation wavelength 800 nm; oscillator repetition rate 80 MHz; pulse energy for surgery 5 nJ/pulse; 0.95 NA $100 \times$ air microscope objective. (Reprinted, with permission, from Ref. 1. Copyright 2005 Wiley Periodicals, Inc.).

spot, removal of the focal adhesion resulted in the isolation of a single fibroblast cell from its neighbor.\(^1\)

Shen et al.\(^6\) ablated fluorescently labeled actin cytoskeleton in fixed 3T3 fibroblast cells using a pulse energy ranging from 1.5 to 3 nJ/pulse (100-fs, 1 kHz). By translating the cells relative to the laser pulse, nanometer scale channels were made in the cytoskeleton. The diameter of the ablated channels was found to decrease as the pulse energy was lowered, with a threshold for actin ablation of 1.5 nJ/pulse.\(^6\) Confirmation that the cytoskeleton was ablated and not photobleached was obtained by restaining the cells after laser irradiation.

Heisterkamp et al.\(^9\) also dissected fluorescently labeled actin in both fixed and live bovine capillary endothelial cells using a pulse energy ranging from 1.8 to 4.4 nJ/pulse (100-fs, 1 kHz). Similar to the observations of Shen et al., the width of the cuts was found to decrease as the pulse energy was reduced, from 600 to 240 nm for 4.4 and 2.2 nJ/pulse, respectively.\(^9\) Figure 3 shows the dissection of a single green fluorescent protein (GFP) tagged microtubule in a live cell irradiated with 1000 pulses at a pulse energy of 1.5 nJ/pulse. It was found that within 2 s, the microtubule retracted because of depolymerization\(^9\) (Figure 3(b)) (arrows). In addition to the ablation of actin, the authors also dissected the nucleus in a fixed endothelial cell. Transmission electron microscopy analysis revealed that the nucleus could be ablated with a pulse energy as low as 1.8 nJ/pulse.\(^9\)

Recently, Sacconi et al.\(^8\) demonstrated nanosurgery (100-fs, 80 MHz) of GFP-labeled microtubules in fission yeast cells. Individual mitotic spindles in anaphase B were irradiated with an average laser power of 4 mW (0.05 nJ/pulse) for 150 ms.\(^6\)
Following nanosurgery, the spindles were bent and broken into segments. In a similar experiment, the authors determined the optimal average laser power for nanosurgery of cytoplasmic microtubules in interphase cells. For an average laser power below 2 mW (0.03 nJ/pulse), it was shown that the shape and length of the microtubule remained unchanged after nanosurgery. However, above 2 mW, disassociation of the microtubules was observed, with the frequency of breakage increasing with higher average laser powers (4–8 mW). The optimal average laser power for microtubule disassociation was found to be 4 mW, yielding a 75% disassociation efficiency and 100% cell survival.

Konig et al. demonstrated the nanodissection of chromosomes in the nucleus of live Chinese hamster ovary cells, using a pulse energy of 0.4 nJ/pulse and an exposure time of 500 μs. At this pulse energy, chromosomes could be ablated without disruption to the nuclear envelope. However, at 0.63 nJ/pulse, dissection of the chromosomes was accompanied by damage to the nuclear envelope and the outer cell membrane.

In a study by Tirlapur and Konig, the authors used fs laser pulses (170-fs, 720 nm, 80 MHz) for the nanodissection of plant cell walls and the partial and complete removal of chloroplasts in Elodea densa. Using an average laser power ranging from 30 to 50 mW (0.38–0.63 nJ/pulse), lesions with a width of < 400 nm were made in the plant cell wall. Figure 4 depicts transmission and autofluorescence images of the chloroplast in E. densa before and after removal of this organelle. Figure 4(a, b) shows several chloroplasts in the epidermal cell of the plant (arrows), where the lightning symbol identifies the chloroplast chosen for removal. Using an average laser power of 30 mW and a beam dwell time of 13 ms, portions of the targeted chloroplast were removed, (Figure 4(c, d)), without compromising the functionality or integrity of adjacent chloroplasts. To verify that adjacent plastids remained functional, phase-contrast transmission microscopy was used to examine the cytoplasmic movement of the organelles in the cortical region. Normal cytoplasmic movement was observed in all nonirradiated chloroplasts. To address whether subcellular removal of the plastids altered cell viability, an examination of the presence of propidium iodide (PI)
in the cytoplasm of the irradiated cells was performed. Using transmission and two-photon fluorescence, no accumulation of PI was observed in the targeted cells, indicating that the cells remained viable.5 Shen et al.6 targeted a single fluorescently labeled mitochondrion in bovine adrenal capillary endothelial cells with fs laser pulses (100-fs, 1 kHz). The purpose of the study was to elucidate the connective properties of mitochondria to determine whether this organelle forms a continuous network or represents an independent structural unit. After stationary irradiation of the mitochondrion with a few hundred pulses at an energy of 2 nJ/pulse, surgical removal of the mitochondrion from the endothelial cell was accomplished without affecting neighboring mitochondria6 (Figure 5). Since only the targeted mitochondrion was structurally damaged and removed, (Figure 5(b,c) arrow), the authors claimed that the absence of adjacent mitochondrial damage provided direct evidence that this organelle exists as an independent unit.6 Watanabe et al.7 removed a mitochondrion in a human carcinoma cell line, HeLa, using a pulse energy ranging from 2 to 7 nJ/pulse (150-fs, 1 kHz) and a beam dwell time of 250 ms. At 7 nJ/pulse, the removal of the mitochondrion was accompanied by plasma membrane disruption indicated by cellular PI uptake.7 However, membrane disruption was not observed following mitochondrial ablation for pulse energies between 2 and 4 nJ/pulse, indicating that the cells remained viable after laser irradiation. The authors used confocal imaging to confirm that the
targeted mitochondrion was ablated, and that its absence (as detected by fluorescence microscopy) was not because of its diffusion out of the focal plane (by cytoplasmic streaming). Similar to the observations of Shen et al., Watanabe reported that neighboring mitochondria remained intact.

In a study by Tirlapur and Konig, the authors employed fs laser pulses (800 nm, 80 MHz) to introduce DNA into Chinese hamster ovarian (CHO) cells and rat-kangaroo kidney epithelial cells (PtK2). With an average laser power ranging from 50 to 100 mW (0.625–1.25 nJ/pulse), the cell membrane was disrupted in the presence of DNA plasmid vector pEGFP-N1 encoding enhanced green fluorescent protein (GFP). Disruption of the cell membrane after 16 ms of irradiation resulted in the introduction of DNA. Expression of the DNA construct was verified by two-photon fluorescence imaging.

In similar work to that of Tirlapur and Konig, Stevenson et al. transfected CHO cells with fs laser pulses (120-fs, 800 nm, 80 MHz) using a pulse energy and beam dwell time ranging from 50 to 225 mW and 10 to 250 ms, respectively. Contrary to the claim of 100% transfection efficiency by Tirlapur and Konig, Stevenson measured an average transfection rate of 50 ± 10% in 4000 laser-treated CHO cells. The nonfluorescent dye, trypan blue, was used to confirm cell membrane viability.

In our lab, fs laser pulses were used to disrupt the cell plasma membrane for the purpose of introducing foreign substances into the cytoplasm of live MDCK cells. Kohli et al. showed that when fs laser pulses were localized to the cell membrane, transient pores could be formed, exposing the extracellular space to the intracellular environment. Using a pulse energy of 3 nJ/pulse (sub-10 fs, 800 nm, 80 MHz) cryoprotective disaccharides were cytoplasmically
FIGURE 10 | Multiphoton ablation allows quantified modulation of specific morphogenetic movements (a) and (b), control; (c) and (d), middorsal ablation, (e) and (f), postdorsal ablation). (a) Development of an intact sGMCA embryo. Green represents images recorded at the equator. Red represents images recorded \( \approx 20 \mu m \) under the surface. (c) Development of a sGMCA embryo after a \( 100 \times 40 \mu m \) middorsal ablation, resulting in disrupted lateral cell movements and no cephalic furrow formation (gray arrowheads). (e) Development of a sGMCA embryo after \( 100 \times 40 \mu m \) postdorsal ablation resulting in disrupted lateral cell movements only. (b),(d), and (f) Corresponding velocimetric analysis for the same embryos at stage 7. Each experiment was reproduced on five different embryos and gave similar results. Scale bar: 100 \( \mu m \). Black scale arrow, 5 \( \mu m/min \). Laser parameters: pulse duration 130-fs; excitation wavelength 830 nm; oscillator repetition rate 76 MHz; pulse energy for embryo manipulation 0.6–4 nJ/pulse; 0.9 NA water immersion microscope objective. (Reprinted, with permission, from Ref. 16. Copyright 2005 The National Academy of Sciences of the USA).

introduced through laser-induced transient pores for biopreservation applications. When MDCK cells were suspended in 1.0 M cryoprotective sucrose, the cells were found to swell to a new equilibrium volume following transient pore formation as a result of an intracellular accumulation of sucrose and water (Figure 6(a, b)). The authors used volumetric analyses to determine the longevity of the transient pore created in the cell membrane. Figure 6(c) depicts the kinetics of the cell following permeabilization. Since the volumetric change was found to plateau within 200 ms (Figure 6(c)) it was hypothesized that this time corresponded to the lifetime of the laser-induced transient pore. The transient lifetime of the pore in varying molar concentrations was also determined by the authors. A survival analysis was performed using a membrane integrity assay consisting of ethidium bromide and Syto 13. In addition, transport equations were used to estimate the delivered intracellular concentration as a function of the extracellular osmolarity.

In a recent study by Yanik et al., the authors used fs laser pulses to perform laser axotomy of D-motor neurons in L4 larval-stage C. elegans. Severing of the D-neurons induced muscle contractions preventing backward locomotion. Figure 7 depicts time lapse images of the laser axotomy, where individual neurons were cut at the mid-body position using 100 laser pulses at a pulse energy of 40 nJ/pulse (200-fs, 1 kHz). The authors observed that the severed neurons retracted following axotomy (Figure 7(c, d)). Analysis of neuron regeneration revealed that 54% of the laser-treated neurons (52 axons in 11 worms) re-grew within 12–24 h (Figure 7(e)). A test of the motor neuron function showed that backward locomotion resumed within 24 h, with a functionality approaching that of wild type C. elegans.

Chung et al. used fs laser pulses to study the role of AFD neurons in C. elegans. Using a pulse energy of 3 nJ/pulse (100-fs, 800 nm, 1 kHz), individual dendrites within a bundle of amphid dendrites were severed. Severing of the dendrites was accomplished without visible damage to adjacent dendrites. Similar to the observations made by Yanik et al., the ablated dendrites were found to retract following ablation, with a retraction distance of 5 \( \mu m \). To determine whether the dendrites re-grew after laser dissection, the authors severed fluorescently labeled PHA and PHB sensory neurons and monitored neuron growth for 24 h. In over 50 C. elegans, none of the sensory neurons repaired, indicating that the cuts were permanent.

Nishimura et al. used fs laser pulses to photodisrupt microvessels in the parenchyma of rat brains using a range of average laser powers from 0.03 to 0.5 \( \mu J/pulse \) (100-fs, 1 kHz).
FIGURE 11 | Middorsal ablation modulates morphogenetic movements at the anterior pole, which are correlated with twist expression. (a)–(f) Sequence of development at the anterior pole of control and photoablated sGMCA embryos, showing the disrupted movements of SP cells after middorsal ablation. Approximate time after the onset of gastrulation is indicated in minutes (inverted contrast images). Black scale arrow: 2 μm/min. Laser parameters: pulse duration 130-fs; excitation wavelength 830 nm; oscillator repetition rate 76 MHz; pulse energy for embryo manipulation 0.6–4 nJ/pulse; 0.9 NA water immersion microscope objective. (Reprinted, with permission, from Ref. 16. Copyright 2005 The National Academy of Sciences of the USA).

FIGURE 12 | (a) When sub-10 fs laser pulses were focused through the chorion, laser-induced transient pores were created at the blastomere–yolk interface or in individual blastomeres of zebrafish embryos. Transient pores were formed only at the focus, leaving the chorion layer undamaged. The pores were used to introduce foreign material into the embryonic cells. Three-dimensional movement of the laser focal spot allowed for precise targeting of any location on or within the embryo. (b) An early 8-cell stage embryo was targeted for pore formation at the blastomere–yolk interface (arrow). (c) A sub-micron (~800 nm) transient pore was created at the interface dividing the blastomeres (B) and yolk (Y) (arrow). The sub-micron pore is obscured by a laser-generated cavitation bubble. An energy of 3 nJ/pulse at a gated pulse train of 200–300 ms was used to form the pore. (d) Depicts the developing embryo at 64/128-cell stage 45–60 min post-fs laser poration. Scale bar for (b), (d) and (c) represents 200 μm and 5 μm, respectively. Laser parameters: pulse duration sub-10 fs; excitation wavelength 800 nm; oscillator repetition rate 80 MHz; pulse energy for embryo manipulation 3 nJ/pulse; beam dwell time 200–300 ms; 1.0 NA 60× water immersion microscope objective. (Reprinted, with permission, from Ref. 3. Copyright 2007 Wiley Periodicals, Inc.).

depicts three different vascular lesions that were produced using varying pulse energies and pulse densities. These included laser induced hemorrhaging, extravasation and intravascular clot formation13 (Figure 8). At relatively high laser pulse energies above the threshold for extravasation of fluorescently labeled blood plasma (0.03 μJ), hemorrhage of the blood plasma and red blood cells from the targeted vessel was observed.13 Lowering the pulse energy resulted in more controlled vascular lesions. However, both extravasation of intact vessels with continued blood flow and clot formation resulting in complete vessel obstruction were observed.13 The authors also measured the changes in adjacent and downstream blood flow in the obstructed vessel following laser-induced clot formation.

Embryo Nanosurgery

While the application of fs laser pulses has been extensively used in the nanosurgery of simple cells, the study of complex multicompartmental biological systems such as embryos remains a challenge. The ability to noninvasively manipulate the intracellular environment of individual embryonic cells has
important implications for future developments in medical and developmental biology.

In a recent study by Supatto et al., the authors used fs laser pulses to induce morphogenetic movements in *Drosophila* embryos. The authors demonstrated that laser nanosurgery (130-fs, 830 nm, 76 MHz) below the vitelline membrane could be achieved within the developing embryo without disturbing cytoskeletal dynamics adjacent to the ablated area. A series of dissection line cuts, 100 × 40 µm, were made 5–15 µm beneath the vitelline membrane with varying pulse energies and pulse number densities (number of incident fs laser pulses per area). The dissections were characterized by observing endogenous fluorescence emission using two-photon excited fluorescence. For pulse densities and pulse energies below 10^5 µm^-2 and 4 nJ/pulse, respectively, no endogenous fluorescence emission was observed. However, with increasing pulse density, fluorescence emission was observed along the dissection cut with microexplosions in the perinuclear region of the cytoplasm. With a pulse density approaching 10^6 µm^-2, large cavitation bubbles in excess of 5–6 µm in diameter were observed.

Further work examined the *in vivo* modulation of cellularization front invagination (CFI) in embryos ablated by fs laser pulses. Figure 9 depicts the rate of CFI in control and laser ablated *Drosophila* embryos. As shown in Figure 9(a), an increase in the rate of CFI was observed for the early fast phase one min after laser ablation, relative to the controls. Fifteen minutes after ablation, no difference in the CFI rate for the fast phase was observed. Figure 9(b). Despite the increase in CFI for the early fast phase, the authors reported that kymograph analysis showed that cellularization completed in cells adjacent to the laser ablated area. In addition to monitoring changes in the cellularization rate, the *in vivo* morphogenetic movements in embryos targeted at dorsal ablation sites were quantified. Figure 10 depicts the morphogenetic movements and velocimetric analysis of the ablated and control embryos. In Figure 10(a, b), both cephalic furrow formation and lateral cell motions were clearly observed in control embryos (arrows). However, middorsal dissection, (Figure 10(c, d)), resulted in no cephalic furrow formation and the disruption of lateral cell movements. Ablation of the postdorsal region was found to affect the lateral cell movements only, (Figure 10(e, f)), with furrow formation occurring normally. The authors speculated that the mechanism responsible for the modulation likely arose from the disruption of the motor region associated with the ablated area. Further investigations examined cell movement and *twist* expression after laser ablation. When embryos were targeted at the middorsal site, the stomodeal primordium (SP) cell motions were affected (Figure 11). In control embryos, expansion and compression of the SP cells were readily observed (Figure 11(a–c)) however, this movement was suppressed by middorsal ablation and the loss of furrow closure (Figure 11(d–f)). While ventral cells at the anterior pole in control embryos were found to have forward movement, SP cells exhibited more backward directed motion in ablated embryos (Figure 11(d–f)). At ablation sites other than middorsal, no significant changes in the *twist* expression were observed.

Recently, in our lab Kohli et al. used fs laser pulses (sub-10 fs, 800 nm, 80 MHz) to introduce exogenous material into early stage cells of live developing embryos. The animal model system chosen was the zebrafish (*Danio rerio*), an aquatic vertebrate organism that is genetically and developmentally closer to humans than the common invertebrate *Drosophila melanogaster*. Presently, zebrafish are used in the study of genetics, drug monitoring, human disease, cardiac function and blood disorders. Figure 12(a) depicts the method used for targeting individual embryonic cells of the developing zebrafish. In both chorionated and dechorionated embryos, the authors focused fs laser pulses with a pulse energy ranging from 0.56 to 2.7 nJ/pulse to a location near the blastomere-yolk (B-Y) interface for transient pore formation (Figure 12(a)).
The authors first addressed whether the applied laser pulses were deleterious to the development of the embryo. Fs nanosurgery was performed at the B–Y interface (Figure 12(b, c) arrows), in early cleavage to early blastula (2-cell to 128-cell) stage embryos using a pulse energy of 2.7 nJ/pulse and a beam dwell time of 200–500 ms. Figure 12(d) shows normal development of a laser treated 8-cell stage embryo, which has developed to 128-cell stage 45–60 min post-laser surgery.3 Other targeted embryos were found to develop normally as compared to control embryos. To determine if laser surgery at the B–Y interface or on individual blastomere cells lead to the formation of a transient pore, the authors suspended early cleavage to early blastula (2-cell to 128-cell) stage dechorionated embryos in a fluorescent reporter molecule, fluorescein isothiocyanate (FITC), and examined fluorescence uptake in the embryonic cells. Figure 13 depicts FITC fluorescence in the blastomere cells of a 16-cell stage embryo, confirming transient pore formation and exogenous material delivery. In 39 targeted embryos, a FITC loading efficiency of 87% was reported.3 It was conjectured that the distribution of the fluorescent probe to adjacent blastomeres likely occurred through blastomere bridges or gap junctions, depending on the developmental stage.38–40

Figure 12(a) depicts the chorion, a proteinaceous membrane surrounding the developing embryo, which provides protection from the environment. To show that the applied laser pulses could still be focused for pore formation at the B–Y interface, the authors focused fs laser pulses beyond the structure of the chorion as shown in Figure 12(a). Early cleavage to early blastula (2-cell to 128-cell) stage chorionated embryos were suspended in the presence of FITC, and the fluorescent probe was allowed diffuse into the perivitelline space (FITC was previously shown to be impermeable to the blastomeres). Targeting the B–Y interface with a pulse energy of 2.7 nJ/pulse and a beam dwell time of 200–300 ms, the authors found that they could introduce perivitelline FITC into the embryonic cells without compromising the structure of the chorion.3 This is evident in Figure 14(c, f, i), where after proteolytic digestion of the chorion (to remove the interfering fluorescent signal from the perivitelline region), fluorescence was observed in the individual blastomere cells. In a total of 27 laser-treated embryos, a FITC loading efficiency of 78% was found.3

Exogenous material delivery was not limited to FITC, as the authors also demonstrated the delivery of conjugated quantum dots and plasmid DNA. Quantum dots and DNA are important materials that have
potential uses for cell fate mapping and the development of stable transgenic fish lines. Using a pulse energy of 1.5–2 nJ/pulse, streptavidin-conjugated quantum dots (targeted near the B–Y interface) were introduced into 2-cell stage dechorionated embryos\(^3\) (Figure 15(a, b)). Quantum dot fluorescence was observed in the early embryonic cells (Figure 15a) while in later cell stages up to germ ring (Figure 15b) the quantum dots were visibly dispersed throughout the blastomeres.\(^3\) To determine if the applied laser pulses constituted a valid alternative method for DNA delivery, the authors laser transfected early to mid cleavage (2-cell to 8/16-cell) stage dechorionated embryos in the presence of a circular plasmid, sCMV-EGFP, with a pulse energy of 0.56 nJ/pulse and a beam dwell time ranging from 200 to 500 ms.\(^3\) Expression of the plasmid construct was observed in a 24 h post-fertilization (hpf) larva (Figure 15(c, e)) with the expression seen along the yolk-extension, floor plate, somites and tail cells of the larva.\(^3\) In over 45 chorionated and dechorionated laser treated embryos, survival approached 90%, with embryo morphology and behavior similar to the control sample.\(^3\)

Kohli and Elezzabi\(^{15}\) further examined the development of zebrafish embryos after laser surgery with the fs laser (sub-10 fs, 800 nm, 80 MHz). Using a pulse energy of 0.56 nJ/pulse and a beam dwell time of 100 ms, individual chorionated blastomere cells were surgically ablated at the early 2-cell stage in over 40 embryos.\(^{15}\) Each blastomere cell was ablated at three different locations with a total laser exposure time of 300 ms per targeted site. The authors reared the embryos to 2 and 7 days post-fertilization (dpf) and used light microscopy (LM) and scanning electron microscopy (SEM) to determine if the applied laser pulses induced morphological changes in the development of the embryos. Under LM, the body plans of control and laser-manipulated embryos were inspected with emphasis on the development of the body axis.\(^{15}\) Short-term survival (before 2 dpf), as determined by the above analysis, revealed a survival percentage of 93%.\(^{15}\) Viable larvae showed no differences in developmental or hatching rates as compared to the controls. SEM imaging showed key developmental structures including the caudal fin, dorsal fin, yolk sac extension, yolk sac and the olfactory pit to be morphologically similar in laser-manipulated and control larvae.\(^{15}\) As the laser-treated larvae aged, the pectoral fin buds lifted away from the yolk sac and developed into mature pectoral fins along the lateral extent of the zebrafish body. This morphological development was consistent with control larvae.\(^{15}\) The authors concluded that no short-term effects of the laser on the development were observed.

While no short-term effects were observed, Kohli commented that the laser’s effect on embryonic
development may not become apparent until later developmental stages. Using SEM, the authors examined control and laser-manipulated larvae reared to 7 dpf. Developmental structures inspected included the protruding mouth, olfactory pit, pectoral fin, eye, otic capsule, otic vesicle, ventral fin, notochord, posterior forebrain, and dorsal midbrain. No differences in the placement or patterning of these structures were observed between the samples. Figure 16(a, b) depict mosaics of a laser-manipulated and a control larva with the developmental structures indicated as mentioned above. High magnification images revealed that the olfactory pit in the control and laser-manipulated larvae was surrounded by epidermal cells, with the pit rims covered by long kinocilia. In the lumen of the ear cristae was found on the lateral wall with kinocilia projecting from the sensory epithelial, as seen in Figure 16 (c, d). The authors found no differences in neuromast patterning, with projecting kinocilia that were distributed along the lateral line of the zebrafish body. In control larvae, neuromasts were found anterior to the olfactory pit, at the outer rim of the otic capsule, anterior to the diencephalon and adjacent to both sides of the optic tectum (dorsal midbrain) and diencephalons (posterior anterior-forebrain). The neuromast patterning in laser-manipulated larvae was found to be identical to that seen in the controls. It was concluded that no long-term developmental effects could be observed, thereby making the application of fs laser pulses an important noninvasive tool for the study of live embryos. Further work is being conducted by the authors to determine if any physiological responses are induced following fs laser nanosurgery.

CONCLUSION

The noninvasive nature of fs laser pulses and their ability to target subcellular sites with high spatial resolution are the major features that have made these ultrafast lasers an attractive tool for the study of live cells and embryos. This review article has explored developments in cell and embryo nanosurgery; each reported study identified unique applications to biology. These include the knockdown of subcellular organelles, the opto-injection of exogenous materials, and functional analyses of laser-induced morphogenetic and morphological changes in embryonic development. However, despite these advances, the full potential of fs laser pulses has yet to be realized. Like the laser itself, the fs laser is a tool being developed without having fully elucidated all of its potential uses. In addition, the application of fs laser pulses is in some ways developing as a technique ‘in search of a biological problem’. It is through continued research that we will uncover novel applications that will undoubtedly benefit many biological disciplines. We envision that in the near future fs laser pulses will be used in the study of cell fate mapping to identify how individual cells contribute to the overall embryonic development of organisms. It will be possible to cryopreserve embryos with low solute permeabilities by delivering impermeable and permeable cryoprotective agents. The generation of genetically modified
organisms will be possible as a result of the interference of delivered exogenous nucleic acids, as will be the potential development of stable transgenic cell lines. The collaboration of physicists, engineers, and cell and developmental biologists will enable the pursuit of such applications, with the fs laser providing a new prospective for understanding essential biological systems.

REFERENCES


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Pharmacokinetics of nanomaterials: an overview of carbon nanotubes, fullerenes and quantum dots

Jim E. Riviere*

A full understanding of the pharmacokinetic parameters describing nanomaterial disposition in the body would greatly facilitate development of a firm foundation upon which risk assessment could be based. This review focuses on the disposition of carbon based fullerenes and nanotubes, as well as quantum dots (QD) after parenteral administration to primarily rodents. The common theme across all particle types is that a major determinant of nanomaterial disposition is the degree of interaction with the reticuloendothelial (RE) cell system. Small water-soluble particles evading this system may be excreted by the kidney. Larger particles and those with the proper surface charge may get targeted to RE cells in the liver, spleen and other organs. Most nanomaterial kinetics are characterized by relatively short blood half-lives reflecting tissue extraction and not by clearance from the body. In fact, another common attribute to nanomaterial kinetics is retention of particles in the body. Finally, unlike many small organic drugs, nanomaterials may preferentially be trafficked in the body via the lymphatic system that has obvious immunological implications.

The technological and biomedical advancements inherent to the application of nanomaterials are becoming increasingly evident. In the field of medical applications, a thorough understanding of their pharmacokinetic properties is crucial for their safe and efficacious application. From the perspective of characterizing potential adverse effects, these data are also required for quantitative risk assessments to define the toxicology of the material. Pharmacokinetics is defined as the science of quantifying the rate and extent of the absorption, distribution, metabolism and elimination (ADME) of chemicals and drugs in the body using mathematical modeling approaches. The aim of pharmacokinetics is to relate drug dose or chemical exposure to biological effect. Pharmacokinetics has developed distinct sets of models and parameters which have been useful to describe and predict drug and chemical disposition, and also have specific meanings and interpretations from the perspective of the regulatory agencies assigned to either approve drugs or conduct occupational or environmental risk assessments of potentially adverse materials.

Most pharmacokinetic models and approaches have been defined and applied to the disposition of small organic drugs, chemicals or metals. The physiochemical processes driving disposition of small molecules in the body are related to diffusion and transport or metabolism (biotransformation) by enzymatic processes. Most of the models are defined by transport through the blood. Are these processes and pathways also relevant to the disposition of larger nanomaterials, some with unique physical properties? Pharmacokinetic analyses have been applied to proteins that in some cases are of dimensions equivalent to the manufactured nanomaterials. Viruses and lipid particles circulating in the body also are of similar sizes. However, not known are the effects on physiologic disposition of the unique quantum physical properties that characterize manufactured nanoscaled materials of 1–100nm, which confer on them unique
Physical attributes (fluorescence, electrical conductivity, resistance to biological degradation etc.).

Physiological processes interacting with similarly sized compounds include many cellular recognition, opsonisation, adhesion, and uptake processes including phagocytosis, as well as lymphatic transport, that are not usually relevant to the disposition of most small organic molecules. A central question of nanopharmacokinetics is whether these processes, which would be expected to apply to small organic molecules and biodegradable nanomaterials, also apply to the manufactured particles made from inert and degradation resistant novel materials such as fullerenes, carbon nanotubes, QD or metallic particles? What nanomaterial properties and characteristics best correlate to their disposition defined by the pharmacokinetic parameters of bioavailability, clearance, volume of distribution, or half-life? Do known nanomaterial issues of aggregation, binding, and particles administered with varying sizes modify interpretation of pharmacokinetic studies?

The focus of this manuscript is to review the literature on the pharmacokinetics of nanomaterials conducted to date and assess whether any overarching findings can be defined. Studies which would be characterized as ‘classic’ for drugs or chemicals have not been conducted for most of the manufactured nanomaterials. Therefore, an assessment will be made of the basic principles of experimental design which should be followed if robust and precise pharmacokinetic parameters are to be obtained for these materials. This review is restricted to carbon based nanomaterials and QD since they have been relatively well studied. Focus is also on systemic pharmacokinetics after parenteral administration. Inhalational, dermal and oral routes of administration have not been included.

OVERVIEW OF PHYSIOLOGICAL DISPOSITION

The goal of pharmacokinetic and absorption, distribution, metabolism and elimination studies is to assess the fraction of a dose of a chemical, drug, or nanomaterial administered to an animal that is absorbed into the systemic circulation and subsequently distributed to tissues or is excreted from the body. Figure 1 depicts the stages involved in ADME processes as a function of route of administration, as well as subsequent elimination. Pharmacokinetic models quantitate the rate and extent of a compound’s sojourn through the body determined by these physiological pathways by mathematically analyzing blood (or plasma) concentrations over time. From such data, a number of descriptive parameters can be determined, including:

- **Volume of Distribution (Vd):** Proportion of drug distributed in the body used to relate administered dose to observed blood concentrations. Vd = Concentration / dose.
- **Clearance (Cl):** Efficiency of removal of a compound from the blood. This can be determined for the whole body (ClB) or for specific organs such as the kidney and liver.
- **Half-life (T_{1/2}):** Time it takes for 50% of a process (e.g., absorption, elimination) to be completed. This often calculated parameter assumes first-order, linear behavior. T_{1/2} = [0.693 Vd] / Cl.
- **Mean Residence Time (MRT):** A parameter similar to T_{1/2} denoting the average time a compound remains in the body.
- **Bioavailability (F):** The fraction of a dose absorbed into the body and available for systemic distribution. This is calculated from blood as the ratio of the area under the curve (AUC) in blood seen after a specific route divided by that seen after intravenous administration. Alternatively, other methods of assessing total body burden may be used in lieu of measuring blood concentrations.

The parameters above are often determined using specific mathematical models that account for more complex patterns of distribution or elimination from the body (e.g., multidistribution compartments resulting in multiple Vds; non-linear pathways of elimination, stochastic modeling approaches). Other modeling approaches go into more realistic detail [e.g., physiological-based pharmacokinetic—(PBPK) models] but require more elaborate and data intensive experimental designs. A PBPK model describes the disposition of a chemical based on a mathematical model that mirrors the physiological structure of the body, with compartments linked by tissue blood flow. Such models easily incorporate in vitro data and define target doses of materials. Pharmacokinetic texts should be consulted for more detail on model construction and data analysis.1-3

Most pharmacokinetic studies are conducted from the perspective of nanomaterial behavior in the systemic circulation assessed by analysis of plasma or blood concentrations. It is generally assumed that a compound must reach the systemic circulation in
order to be distributed to tissues and be available for excretion by the liver or kidney. When such an approach is applied to drugs, a decay in blood concentrations over time usually translates into excretion of the compound from the body via the urine, feces or in the inhaled breath. Under these circumstances, $T_{1/2}$ relates the length of time a drug is available in the body. If extensive tissue distribution occurs, as is seen for many lipophilic contaminants such as chlorinated hydrocarbons, decay from blood may be very slow as is seen above by the dependency of $T_{1/2}$ on $V_d$. Elimination from the body may be inefficient (low $Cl$) further increasing $T_{1/2}$. In fact, elimination may only occur by metabolism of the chemical to a more hydrophilic, and thus easily excretable chemical form. However, for nanomaterials, decay in blood concentrations may be related to the compound movement into tissues where further excretion does not occur [e.g., trapped in reticuloendothelial (RE) system, bound to tissue proteins, postdistributional aggregation]. In these cases, blood half-life may paradoxically be relatively short despite the prolonged body persistence. Nanomaterials may also be transported in the body via the lymphatic system, a phenomenon which complicates their pharmacokinetic analysis based on blood sampling and also exposes lymphoid tissue to higher concentrations than would be seen secondary to distribution from blood. These idiosyncrasies of nanomaterials compared to most drugs or small molecule xenobiotics require caution in using classic interpretations of the meaning of basic ADME parameters.

REVIEW OF THE LITERATURE

There have been a number of recent studies that have attempted to define basic disposition and pharmacokinetic parameters for a number of nanomaterials including fullerenes, carbon nanotubes and QD. A review of these studies presents a reasonable perspective on the nature of research conducted to date and a relatively consistent picture of basic concepts that seem to apply to nanomaterial ADME. All of these studies have been conducted in laboratory animal species, primarily rodents. Human data are not available.

Carbon-Based Nanomaterials—Fullerenes ($C_{60}$) and Nanotubes

An interesting literature has developed on the disposition of carbon nanomaterials in laboratory animals. Much of this work has resulted from attempts to derivatize fullerenes or carbon nanomaterials to serve as vectors for delivery of specific therapeutic ligands (e.g., antiviral compounds, anticancer drugs). This complicates data interpretation since nanomaterial controls often do not exist due to solubility issues, making it difficult to separate nanomaterial effects from that of the attached ligand. There are limited studies that independently alter size, surface charge or solubility to assess their effect on ADME parameters. A number of different vehicles are often used to dose different particles, making vehicle effects difficult to identify. Finally, the nanomaterial is often monitored using further molecular modifications to facilitate assaying or tracking (e.g., fluorescent or

FIGURE 1 | Stages involved in absorption, distribution, metabolism and elimination (ADME).
radioactive label) which may further alter ADME properties.

Rajagopalan et al.⁴ conducted a pharmacokinetic study in Sprague-Dawley rats using 15 mg/kg MSAD-C₆₀, a water-soluble C₆₀ derivative with antiviral properties. Terminal blood T₁/₂ was approximately 7 h, MRT 11 h, and the Vd was 2 L/kg indicating extensive distribution. There was no evidence of urinary excretion and C₆₀ was 99% bound to protein in plasma. There was great variability in disposition, not reflected in the T₁/₂ data, with 2/5 rats having two-fold differences in Cl and Vd parameters. The authors attributed this to the extensive protein binding. This observation underlines the weakness in using T₁/₂ alone as the sole pharmacokinetic descriptor, since T₁/₂ is physiologically confounded by opposite changes in Vd and Cl (recall above that T₁/₂ ≈ Vd/Cl).

Yamago et al.⁵ studied a ¹⁴C labeled (trimethylenemethane (TMM) derived) lipophilic yet water-soluble C₆₀ after IV and oral administration (dosed in ethanol/PEG/albumin vehicle) to mice and Fischer rats. In both species, oral absorption was minimal. After IV administration, only 5% of the compound was excreted from the body, all by the fecal route. Most radiolabel was retained in the liver after 30 h, primarily in Kupffer and perisinusoidal fat cells, not hepatocytes. Some C₆₀ derivatives were also located in the spleen, kidney and importantly the brain. From 30 to 160 h, label in organs slowly decreased without observable excretion from the body. However, redistribution to skeletal muscle and hair was observed.

Qiingnuan et al.⁶ reported that tissue distribution of ⁹⁹mTc labeled C₆₀(OH)₅ in mice and rabbits after IV dosing occurred primarily to the kidney, bone, spleen and liver with slow elimination from the body occurring after 48 h, except for bone which accumulated label. T₁/₂ in blood was 17 h in mice. Cagle et al.⁷ studied the biodistribution of endohedral metallofullerenes (¹⁶⁶HoC₈₂(OH)₅) in mice and reported relatively rapid clearance from blood over a few hours, bone accumulation, and liver localization with slow elimination. As with the C₆₀(OH)₅ studies above, total body Cl was low with only 20% of intact compound being excreted by 5 days, a retention time much longer than when control metal chelates alone were administered. After 5 days, blood concentrations did not appreciably decay. In contrast to other work, these C₆₀(OH)₅ were excreted in urine, which may be a function of increased water solubility compared to more lipophilic derivatives.

Bullard-Dillard et al.⁸ using ¹⁴C labeled particles dosed to Sprague-Dawley rats showed longer persistence in the circulation of water-soluble ammonium salt derivative C₆₀ compared to very rapid clearance of C₆₀. Both primarily targeted the liver. For watersoluble C₆₀ derivatives, Cl was low and material was seen in spleen, lung and muscle, as well as the liver. Significantly, there was no radioactivity detected in urine and feces collected every 24 h for 5 days. Similarly, Qiang et al.⁹ studied 0.5 mg/kg intravenous C₆₀(OH)₂₄ disposition in mice and demonstrated organ accumulation, as well as only 50% excretion after 3 days, with over 50% excreted in feces and only 4% in urine. Bone showed a pattern of continued accumulation. In tumor-bearing mice, tumor/muscle and tumor/blood averaged 2–6 fold depending on the tumor type. Studies of nanoparticle size distributions in aqueous versus protein media showed different patterns indicating particle agglomeration in biological environments, a phenomenon which could impact the pattern of tissue kinetics seen.

Some workers have investigated the biodistribution of carbon nanotubes in laboratory animals. Most of these have used indirect measures of concentration (infrared),¹⁰ positron emission tomography,¹¹ or particles functionalized with specific tracers.¹² Methods using such modifications rely on radiolabel or fluorescent tags to both remain attached to the nanomaterial throughout its sojourn through the body, as well as not to impart any different physiochemical properties that would alter ADME. The lack of a sensitive analytical assay for these materials has hindered this work. Studies have also been done in small numbers of laboratory animals often with insufficient time points for proper pharmacokinetic analyses. Nanotube length has not been rigorously controlled, nor in some cases even determined, in the studies reported to date. Nevertheless, some interesting patterns emerge.

Singh et al.¹² studied various functionalized single walled nanotubes (SWNT), as well as multiwalled nanotubes (MWNT) administered intravenously to BALB mice. This work demonstrated urinary excretion and accumulation in muscle, skin and kidney for neutral and positively charged SWNT, as well as MWNT. In contrast to other studies, extensive body accumulation was not seen and clear urinary excretion was evident. Guo et al.¹³ using labeled MWNT dosed intraperitoneally to mice showed a blood T₁/₂ of 5.5 h. Material was retained in the stomach and haircoat and was excreted primarily in the feces. Cherukuri¹⁴ studying rabbits showed SWNT (1 × 300 nm) dispersed in pluronic F108 preferentially accumulated in liver after 24 h. IR spectra suggested that the SWNT dissociated from the pluronic dosing media and interacted with proteins, making the SWNT actually a study in nanotube-protein disposition. This is consistent with the findings of Dutta et al.¹⁴ who demonstrated
that protein adsorption onto SWNT altered biological interactions, although binding to albumin could be prevented by pretreatment with Pluronic F127 surfactant. Liu et al.\(^\text{11}\) studied phospholipid-coated SWNT in mice using PET. Tissue distribution (liver, spleen) and blood \(T_{1/2}\) (0.5–2 h) were dependent upon phospholipid substituents. Significant body burden persisted after a 24 h sacrifice. Finally, Deng et al.\(^\text{15}\) studied the disposition of taurine functionalized MWNT after intravenous dosing to mice and also observed distribution primarily to liver, lung, spleen and heart, but not brain, stomach or bone. As reported for many materials, liver deposition was primarily to Kupffer cells. These studies establish a pattern of distribution of larger material to tissues that compose the RE system in the liver as Kupffer cells, spleen, lymph nodes and bone marrow. As will be seen, this theme is also played out with other types of nanomaterials.

**Quantum Dots**

There are systemic disposition studies reported using inherently fluorescent QD derivatized for medical imaging. As seen with the carbon nanomaterials, most studies do not rigorously determine particle concentrations using analytical techniques as is required in pharmacokinetic studies. However, general patterns of particle distribution can often be assessed. These studies suggest that QDs, tagged with homing peptides, can be targeted to specific tissues (e.g., lung, vessels) after intravenous administration of \(\approx 10\) mg/kg to mice. Similar to carbon materials reviewed above, they accumulate in liver and spleen.\(^\text{16}\) Coating QD with polyethylene glycol allows particles to escape detection by RE tissues (liver, spleen, lymph nodes). Imaging studies in mice clearly show that QD surface coatings alter their disposition and pharmacokinetic properties.\(^\text{17}\) Plasma \(T_{1/2}\) was less than 12 min for amphiphilic poly (acrylic), short chain (750 Da) methoxy-PEG or long chain (3400 Da) carboxy-PEG QD, but over an hour for long-chain (5000 Da) methoxy-PEG QD. These coatings determined the pattern of *in vivo* tissue localization, with retention of some QDs occurring up to 4 months.

Fischer et al.\(^\text{18}\) studied the pharmacokinetics of CdSe/ZnS QD after intravenous administration to Sprague–Dawley rats, either coated with bovine serum albumin [(BSA)-QD; hydrodynamic radius of 80 nm] or bound to mercaptoundecanoic acid crosslinked to lysine (LM-QD; hydrodynamic radius of 2.5 nm). Blood clearance of BSA-QD was 1.23 compared to 0.59 mL/min-kg for LM-QD. BSA-QD \(T_{1/2}\) was 39 min versus 58 min for LM-QD. The \(V_d\) for both was approximately 65 mL/kg. By 90 min, the liver had accumulated 40 and 99% of LM-QD and BSA-QD, respectively. Electron microscopy located QD primarily to Kupfer cells within the liver. No QD of either form were detected in urine or feces for up to 10 days. This study nicely illustrates the uncoupling of blood decay to elimination from the body or tissue distribution as represented by \(V_d\) estimates, as these pharmacokinetic parameters do not reflect the nature of tissue distribution, nor are sensitive to irreversible tissue binding of substances such as nanomaterials.

Several very recent studies using QD further extend these observations. Schipper et al.\(^\text{19}\) studied QD with various coatings after intravenous administration to mice. As seen in other studies, liver was a primary target of uptake, with smaller amounts going to spleen, bone and lung. Within the ranges of sizes studied (12—21 nm), size had no influence on biodistribution. There was no evidence of clearance from the body through 36 h. Yang et al.\(^\text{20}\) demonstrated a complete lack of excretion after 28 days for QD 705 after IV injection to mice. Although plasma \(T_{1/2}\) was short (18.5 h), there was continued redistribution from body sites to liver and kidney over 28 days. As seen with the carbon-based material, decay in blood is not necessarily associated with clearance from the body.

As can be appreciated from this research, most investigators have failed to detect excretion from systemically dosed QD. Choi et al.\(^\text{21}\) reported in a series of studies that QD with zwitterionic or neutral organic coatings prevented QD adsorption to serum proteins that kept the QD hydrodynamic radius less than 5.5 nm. Under this size, QD could be excreted by the kidney. Larger sizes of QD were not excreted from the body. This phenomenon followed what would be expected based on size cutoffs for renal filtration of various peptides and proteins. Globular proteins with diameters of 5–6 nm are regularly excreted, while larger proteins are not. This work is also consistent with 5 nm colloidal gold nanoparticle studies by Balogh et al.\(^\text{22}\) whereby positive charged particles were excreted by the kidney, whereas negative charged or neutral small particles and 22 nm particles of all surface charges were targeted to the liver, spleen or lung presumed secondary to opsinisation and RE cell removal.

In order to begin exploring the biodistribution kinetics of nanomaterials, our laboratory infused three concentrations of PEG-coated or COOH-coated QD621 into an isolated perfused porcine skin flap preparation and modeled arterial-venous (AV) extraction quantified by fluorescent intensity and validated using inductive coupled plasma emission
spectroscopy for cadmium.\textsuperscript{23} Perfusion media was a Krebs Ringer albumin containing buffered solution. Data was analyzed based on a pharmacokinetic model previously optimized for platinum chemotherapeutic compound distribution in infused skin, a compound class also marked by irreversible tissue binding.\textsuperscript{24} COOH-QD 621 (negative charge) uptake into skin was 2–3 fold greater than for PEG-QD621 (neutral). This is consistent with increased tissue distribution of negative charged QD discussed above. However, the data for both QDs were also marked by a statistically significant periodicity (period \(\approx 90\) min) in vascular uptake, a phenomenon never previously seen with infused drugs (cisplatin, carboplatin, lidocaine or testosterone). This finding appears unique for nanomaterials and suggests that the tissue distribution of QD may be different than other organic molecules. In fact, such periodicity in QD tissue extraction from blood is consistent with the tissue redistribution seen for QD in mice,\textsuperscript{20} as well as an erratic pattern of early QD deposition in skin reported by Ballou et al.\textsuperscript{17} The charge selectivity for tissue distribution seen in the perfused skin study is important as RE cells are not present in this isolated organ model.

OTHER NANOMATERIALS

It is beyond the scope of this review to discuss biodistribution studies conducted using nanoparticles composed of other materials, as the above studies using carbon and QD are representative. However, a few studies are worth noting. Kreyling and co-workers have conducted a number of elegant biodistribution studies using defined sizes of \(^{192}\)Ir radio-labeled iridium nanoparticles after inhalational and parenteral administration.\textsuperscript{23,26} These studies suggest a size-dependent pattern of pulmonary deposition after inhalation. However, their work also clearly demonstrate that a small amount of inhaled iridium (<1\%) is translocated to other organs, including the liver, spleen, heart and brain. Similar to other materials discussed above, liver concentrations continued to increase and minimal excretion was seen after parenteral dosing. Smaller particles (15 vs 80 nm) had significantly increased tissue translocation after inhalational exposure.

Many studies have been conducted using pharmaceutical ‘nanoformulations’. The materials are often polymers of drug or carrier substances previously used in parenteral drug administration but now formulated as nano-scale material (particles, liposomes, nanocapsules, micelles, dendrimers and nanoplexes).\textsuperscript{27,28} The bulk of the applications in this area are formulations (e.g., Nanoedge and NanoCrystal platforms for increasing oral delivery) where the extreme surface area to mass ratios of nanomaterial result in refined control of drug delivery, or nanof ormulation surfaces improve solubility or permeability in biological systems. In other cases, nano-sized drugs appear to have enhanced and/or targeted tissue biodistribution and have surface properties designed to reduce RE cell uptake. Some nano-based pharmaceutics are already on the US market, examples being Doxil—doxorubicin HCL liposome injection and Abraxane—paclitaxel protein-bound particles for injectable suspension. Manufactured materials such as 60 nm bioconjugated quantum rods have been developed for receptor-mediated transport across the blood brain barrier.\textsuperscript{29} The fundamental difference between most of these pharmaceutical materials compared to the manufactured materials discussed earlier is that pharmaceutics are specifically constructed of materials that are biodegradable, and thus not-persistent in the body. Because of this property, excretion ultimately occurs and standard pharmacokinetic approaches have been used to describe their disposition. In contrast, the manufactured nanomaterials are not biodegradable and thus once within certain tissue, may accumulate. Finally, the unique surface chemistry of these ‘hardened’ materials may further alter disposition or bioactivity.

LYMPHATIC TRANSPORT

Although most ADME and pharmacokinetic studies focus on the blood circulatory system, animals and humans also have another system which traffics cells and large lipophilic molecules and proteins throughout the body, the lymphatic system, a component of the RE system. The lymphatics have been extensively studied relative to their role in absorption of particulates and protein therapeutics for molecules with molecular weights greater than 16 KD\textsubscript{a}.\textsuperscript{30–32} After absorption in local lymphatic vessels, a compound moves to regional lymph nodes and ultimately re-enters the systemic circulation via the thoracic duct. An important toxicological implication of this pathway is that all such transported material has the potential for interaction with the immune system resident in regional lymph nodes. Nanomaterials, being relatively large and depending on surface modifications, are ideal candidates for lymphatic transport. In support of this hypothesis, studies have shown that after subcutaneous QD
injection, some nanomaterials end up in draining lymph nodes.\textsuperscript{33} Solid lipid nanoparticles designed for magnetic resonance imaging have been shown to enter lymph after duodenal administration to rats.\textsuperscript{34} In other imaging studies conducted in pigs, intradermal injection of 400 pMoles of fluorescent QD targeted sentinel lymph nodes,\textsuperscript{35} a finding relevant to dermal absorption of even minute fractions of topically applied or orally dosed nanomaterials. In fact, it has been suggested that nanocapsules, ultrafine oily droplet-coated polymeric drug substances, may be one of the most promising candidates for lymphatic targeting.\textsuperscript{36} Similarly, QD injected into porcine lung parenchyma was used to map lymphatic drainage and visualize lymph nodes during surgery.\textsuperscript{37} This preferential uptake of some nanomaterials by lymph coupled with the tendency of some materials to also interact with the RE system makes this aspect of nanomaterial deposition different than most small organic drugs.

**CONCLUSIONS**

There are a few conclusions that can be drawn from the available literature on nanomaterial biodistribution and kinetics based on study of carbon based materials and QDs. First, most nanomaterials tend to accumulate in the liver, potentially because of RE cell trapping. However, particles also distribute to other tissues, including the kidney, depending on the surface characteristics and size. The effect of size across different nanomaterials has only begun to be evaluated. There is some consensus beginning to develop that possibly particles with hydrodynamic radii less than 5–6 nm may be eliminated from the kidney. However, if they are larger and have specific surface characteristics (e.g., negative charge), they may interact with the RE system or become protein bound and not be excreted in the kidney.

Secondly, all classes of particles also have extensive tissue retention, a property of potential toxicological significance since tissue accumulation and persistence in the body may occur.\textsuperscript{38} This happens in the face of relatively short blood T\textsubscript{1/2} since these particles are effectively being ‘cleared’ into tissue depots rather than excreted from the body into the urine or feces as most drugs are. Note that as tissue deposition occurs, Vd may also increase resulting in prolongation of T\textsubscript{1/2}. What is the driving force for tissue redistribution between tissues observed in some studies?

A third issue relates to how preferential transport by the lymphatic system affects interpretation of classic pharmacokinetic parameters. What is the relationship between persistence of nanomaterial absorbed into the lymphatic system relative to redistribution back to the central blood circulation?

A fourth issue is the exact state the nanomaterial exists once deposited in a tissue. Do surface coatings persist after translocation from the blood? The association of a carbon nanotube with a surfactant such as sodium dodecyl sulfate (SDS) is pH dependent as is the actual surface area of a nanotube covered by the compound.\textsuperscript{39} For example, what is the nature of a nanomaterial ‘sequestered’ in a lysosome characterized by a very acidic pH? Subtle differences in the physiological milieu of the nanotube (e.g., local ion concentration) would also be expected to alter subsequent particle disposition as well as toxicity.

Finally, studies are difficult to directly compare (different species, doses, vehicles, different approaches to functionalizations, lack of common characterization techniques) both within and across nanomaterials, making interpretation problematic at best and definitely not adequate to begin risk assessment analyses for manufactured materials. Longer-term studies with complete particle characterization before dosing and after tissue deposition are required. Are nanomaterials deposited into tissues stable over long time frames? Studies in nonrodent species with body mass and thus physiological time-clocks closer to that of humans are required. Finally, there is a need for more classic pharmacokinetic studies to be conducted so that physicochemical parameters across nanomaterials can be correlated to the parameters of disposition.

**REFERENCES**


**RELATED ONLINE ARTICLES**

Toxicology of nanomaterials.
Human health implications of nanomaterial exposure.
Characterization of nanomaterials for toxicity assessment.
Catalyst-functionalized nanomaterials

Yi Lu* and Juewen Liu1

With rapid development in both nanotechnology and biotechnology, it is now possible to combine these two exciting fields to modulate the physical properties of nanomaterials with the molecular recognition and catalytic functional properties of biomolecules. Such research efforts have resulted in a larger number of sensors that can detect a broad range of analytes ranging from metal ions, small molecules, and nucleic acids down to proteins. These sensors will find important applications in nanomedicine. In this article, the design of sensors with four classes of nanomaterials (metallic, semiconductor, magnetic, and carbon nanotube nanoparticles) is reviewed. Metallic nanoparticles possess distance-dependent optical properties and are useful for designing colorimetric sensors. Semiconductor nanoparticles or quantum dots (QDs) appear to be superior alternatives to traditional organic fluorophores in many aspects, such as broad excitation range, narrow emission peaks, and high photo stability. QD sensors based on either energy transfer or charge transfer are summarized. Furthermore, magnetic nanoparticles are shown to be useful as smart magnetic resonance imaging (MRI) contrast agents. Finally, some carbon nanotubes show near-IR emission properties, and thus, are potentially useful for in vivo sensing. Sensors based on either tuning the emission intensity or wavelength are discussed.


With the recent focus of research on nanoparticle synthesis, functionalization, characterization, and application, many physical and chemical properties of these materials have been extensively explored, such as optical, magnetic, electronic, thermal, and catalytic properties.1,2 The ability to modulate these properties in response to external chemical environment allows us to use nanomaterials as sensor components.1–6 By definition, nanoparticles are in the scale of 1–100 nanometers, which is comparable to many important biological macromolecules, such as proteins and nucleic acids. It is known that these biopolymers possess highly specific molecular recognition abilities. For example, a protein enzyme catalyzes the turnover of only certain substrates, while a piece of single-stranded DNA can bind its complementary strand specifically. Therefore, conjugation of these biopolymers to nanoparticles may transduce specific molecular recognition and catalytic properties of proteins and nucleic acids into the change of physical properties of nanoparticles, and one major application of these catalyst-functionalized nanomaterials is sensing. Because of their small sizes, these sensors could be used as probes inside a cell. Currently, since the toxicity of nanomaterials has not been fully established, only in vitro applications of these sensors, such as dipstick tests of serum samples, are reviewed here, which may serve as a basis for their future biomedical applications in the human body.

Many biopolymers can be used as molecular recognition elements in sensor design, such as antibodies, enzymes, DNA, and RNA. This chapter focuses on molecules with catalytic properties including protein and nucleic acid enzymes. For sensing applications, sensitivity could be increased with catalytic turnovers of enzymes. In the enzyme world, protein has long been the only player. This situation was changed in the early 1980s with the discovery of catalytic RNAs or ribozymes.7,8 In 1994, the first catalytic DNA (also known as deoxyribozyme or DNAzyme) molecule was isolated.9-15 Compared to RNA, DNA has much higher stability and it is also relatively cost-effective to chemically synthesize DNA.
Many DNAzyme-based sensors have been developed recently. Biopolymer-based sensors with other properties, such as ligand binding, will also be discussed. This chapter will be divided into several sections and each section focuses on a particular class of nanomaterials, including metallic, semiconductor, magnetic, and carbon nanotube nanoparticles.

**CATALYST-FUNCTIONALIZED METALLIC NANOPARTICLES AND THEIR APPLICATIONS IN COLORIMETRIC SENSING**

**Metallic Nanoparticles as Color-Reporting Agents**

Noble metallic nanoparticles such as gold and platinum nanoparticles possess a number of unique properties including size and distance-dependent optical properties, electric conductivity, and catalytic properties. Gold nanoparticles (AuNPs) measuring from several nanometers to below 100 nm, for example, are red in the dispersed state. The color gradually changes to purple or blue when AuNPs are aggregated due to surface plasmon coupling. In addition to this distance-dependent optical property, AuNPs have extinction coefficients three to five orders of magnitude higher than traditional organic chromophores. Therefore, highly sensitive sensors can be constructed with minimal consumption of materials. The chemistry of bioconjugation to gold surfaces has been well established. Proteins can be covalently linked to AuNPs through the thiol group and DNA can be attached through covalent linkage to AuNPs. The secondary structure of the DNAzyme is shown in Figure 1(a). It contains an enzyme strand (in green) and a substrate strand (in black). The substrate has a single RNA linkage (rA) that serves as the cleavage site. In the presence of Pb2+, the enzyme strand cleaves the substrate into two pieces (Figure 1(b)). To incorporate AuNP binding functions, the substrate strand was extended on both ends with the extended fragments being complementary to the DNA attached to AuNPs (Figure 1(c)). In each assembled AuNP aggregate, there are hundreds to thousands of AuNPs. For the clarity of the figure, only two particles are drawn to show the linkage between AuNPs. Addition of Pb2+-induced cleavage of the substrate and disassembled the AuNPs, accompanied by a blue-to-red color change. Because the DNAzyme is selective for Pb2+, addition of other metal ions did not cause cleavage or color change. As a result, this DNAzyme-linked nanostructure is useful for colorimetric detection of lead ions. Alternatively, dispersed AuNPs were mixed with the DNAzyme. Addition of Pb2+ cleaved the substrate, and therefore, inhibited the assembly of AuNPs. Both approaches have been demonstrated and a representative thin layer chromatography (TLC) plate spotted with the sensor solution reacted with different metal ions is shown in Figure 1(d). Increasing Pb2+ concentration resulted in a color progression from bluish purple to red, and all other competing ions showed only background color, suggesting the high selectivity of the DNAzyme for Pb2+ has been maintained. Under optimized conditions, the sensor can change color in ~5 min in the presence of Pb2+ with a detection limit around 100 nM. This method can be generally applied to other DNAzyme-functionalized AuNPs in response to different chemical or biological stimuli. By replacing the lead DNAzyme with a uranium-specific DNAzyme, colorimetric sensors for uranium has also been obtained. To improve the sensitivity even further, a label-free DNAzyme-AuNP system was designed to take advantage of the salt-induced aggregation of AuNPs for colorimetric sensing, reducing the detection limit of Pb2+ to 3 nM.

**Heavy Metal Detection with DNAzyme-Functionalized AuNPs**

The use of DNA-functionalized AuNPs for sensing applications was first reported by Mirkin and coworkers. It was demonstrated that these AuNPs can self-assemble in the presence of a complementary DNA with a vivid red-to-blue color transition. With appropriate signal amplification methods, the sensitivity of such nanoparticle-based nucleic acid sensors rivals that of polymerase chain reaction (PCR).

Introducing catalysts that require certain cofactors for their activities allows design of colorimetric sensors for analytes beyond nucleic acids. We first employed a Pb2+-dependent DNAzyme to assemble AuNPs. The secondary structure of the DNAzyme is shown in Figure 1(a). It contains an enzyme strand (in green) and a substrate strand (in black). The substrate has a single RNA linkage (rA) that serves as the cleavage site. In the presence of Pb2+, the enzyme strand cleaves the substrate into two pieces (Figure 1(b)). To incorporate AuNP binding functions, the substrate strand was extended on both ends with the extended fragments being complementary to the DNA attached to AuNPs (Figure 1(c)). In each assembled AuNP aggregate, there are hundreds to thousands of AuNPs. For the clarity of the figure, only two particles are drawn to show the linkage between AuNPs. Addition of Pb2+-induced cleavage of the substrate and disassembled the AuNPs, accompanied by a blue-to-red color change. Because the DNAzyme is selective for Pb2+, addition of other metal ions did not cause cleavage or color change. As a result, this DNAzyme-linked nanostructure is useful for colorimetric detection of lead ions. Alternatively, dispersed AuNPs were mixed with the DNAzyme. Addition of Pb2+ cleaved the substrate, and therefore, inhibited the assembly of AuNPs. Both approaches have been demonstrated and a representative thin layer chromatography (TLC) plate spotted with the sensor solution reacted with different metal ions is shown in Figure 1(d). Increasing Pb2+ concentration resulted in a color progression from bluish purple to red, and all other competing ions showed only background color, suggesting the high selectivity of the DNAzyme for Pb2+ has been maintained. Under optimized conditions, the sensor can change color in ~5 min in the presence of Pb2+ with a detection limit around 100 nM. This method can be generally applied to other DNAzyme-functionalized AuNPs in response to different chemical or biological stimuli. By replacing the lead DNAzyme with a uranium-specific DNAzyme, colorimetric sensors for uranium has also been obtained. To improve the sensitivity even further, a label-free DNAzyme-AuNP system was designed to take advantage of the salt-induced aggregation of AuNPs for colorimetric sensing, reducing the detection limit of Pb2+ to 3 nM.

**Biosensors with Tunable Detection Ranges**

The DNAzyme/AuNP-based colorimetric sensors have a unique and useful feature of a tunable detection range. This is important because the safety level (or maximum contamination levels, MCL) for certain toxic chemicals such as many heavy metal ions are different, depending on the media to be tested. For example, the MCL for lead in water is 75 nM while the MCL for lead in blood is 500 nM. A sensor with tunable dynamic ranges can apparently match different MCL requirements of various applications. As shown in Figure 2(a), there is a GT wobble base
pair close to the cleavage site in the Pb$^{2+}$-specific DNAzyme. Changing the T base in the enzyme strand to a C base completely abolishes the enzyme activity$^{31}$ (Figure 2(b)). Although inactive, the mutated DNAzyme can still assemble AuNPs, and the extinction spectra of DNAzyme-assembled AuNPs in the presence or absence of Pb$^{2+}$ are shown in Figure 2(c) and (d) for the native and mutated DNAzymes, respectively. The red curves are the spectra of AuNPs in the presence of Pb$^{2+}$, while the blue curves are those in the absence of Pb$^{2+}$. A significant increase in the 522 nm plasmon peak and a drop in the 700 nm region were observed for the native DNAzyme in the presence of Pb$^{2+}$ (Figure 2(c)), while no change was observed for the mutant (Figure 2(d)). The extinction ratio at these two wavelengths were used for quantifying the color of the system, with a high ratio associated with dispersed AuNPs of red color and a low ratio with aggregated particles of blue color. By using all native DNAzyme, a detection range from 0.1 to 2 µM was obtained (solid squares, Figure 2(e)). When 5% of native DNAzyme were used along with 95% mutant, the detection range shifted to 10–200 µM (open squares, Figure 2(e)). Recently, a new method of using pH to adjust the detection range is also reported.$^{29}$

**FIGURE 1** | Colorimetric sensing with functional nucleic acids and gold nanoparticles (AuNPs). (a) The secondary structure of a Pb$^{2+}$-specific DNAzyme. (b) Schematic presentation of cleavage of the substrate by the enzyme in the presence of Pb$^{2+}$. (c) In the presence of Pb$^{2+}$, the DNAzyme-assembled AuNPs are dispersed with a blue-to-red color transition. (d) A TLC plate with the sensor solutions spotted. The sensor shows a red color only in the presence of Pb$^{2+}$. (e) Schematics of the adenosine aptamer binding to its target. (f) Schematic presentation that the aptamer can be either a random coil or in a complexed structure upon binding to its target. (g) The adenosine aptamer-linked AuNPs change color from blue to red in the presence of adenosine through a structure-switching process. (h) Color of the sensor in the presence of different nucleosides. (i) An adenosine-dependent aptazyme constructed on the basis of the Pb$^{2+}$-specific DNAzyme and the adenosine aptamer. The aptazyme is active only in the presence of adenosine. (j) Color of the aptazyme-based adenosine sensor spotted on a TLC plate.

**FIGURE 2** | The secondary structure of the native (a) and mutated (b) DNAzyme. The position of mutation is shown in blue. The extinction spectra of DNAzyme-assembled gold nanoparticles (AuNPs) in the presence (red) or absence (blue) of Pb$^{2+}$ for the native DNAzyme (c) and the mutated DNAzyme (d). (e) Pb$^{2+}$-dependent color change of AuNPs with 100% native DNAzyme and with only 5% native DNAzyme and 95% mutated DNAzyme.
Small Organic Molecule Detection with Aptamer-Functionalized AuNPs
In addition to catalytic functions, DNA has also been shown to be a useful ligand to bind many molecules of choice with high affinity and specificity, and these binding DNAs are known as DNA aptamers.32,33 Similar to antibodies, aptamers can be selected to essentially any chemical or biological species of choice, ranging from metal ions, small organic molecules, and proteins, to whole cells.34–36 Similar to DNAzymes, the binding properties of aptamers have also been employed to control the assembly state of AuNPs for sensing applications. Shown in Figure 1(e) is an aptamer for adenosine,37 which is a piece of short single-stranded DNA. This DNA aptamer can adopt either random coil structures or a folded structure in solution. The equilibrium between these two states can be shifted by addition of adenosine, because adenosine can associate with the aptamer to induce its folding.38–41 As a result, the color of the solution changed from blue/purple to red. As shown in Figure 1(h), only adenosine induced a red color while other ribonucleosides all gave purple colors. The rate of color change was very fast and it took only seconds to observe the color transition to red in the presence of target molecules. This method is also generally applicable to constructing colorimetric sensors for other chemical targets, such as cocaine, potassium ions and their combinations.42,44 Aptamers have also been directly functionalized onto AuNPs. Some macromolecules, such as thrombin and platelet-derived growth factor, possess multiple aptamer-binding sites. Therefore, these protein molecules can crosslink aptamer-functionalized AuNPs to form aggregates and change the color of the systems to blue.45,46 Recently, the use of AuNP stability differences in high salt conditions to probe aptamer binding has also been demonstrated. In these systems, AuNPs aggregated due to colloidal instability to salt instead of DNA crosslinking.47,48

Aptazyme-Based Detections
A combination of DNAzymes and aptamers results in an interesting allosteric molecule, referred to as allosteric DNAzymes or aptazymes.49,50 For example, by inserting the adenosine aptamer into one of the substrate binding arms of the Pb2+-dependent DNAzyme, an adenosine-dependent aptazyme was designed (Figure 1(i)). Binding of adenosine strengthened formation of the Pb2+ binding site in the DNAzyme and allowed cleavage, whereas in the absence of adenosine, Pb2+ binding was disrupted. Based on this aptazyme and similar methods developed for colorimetric detection of Pb2+, a colorimetric assay for adenosine was demonstrated.51

Dipstick Tests with Aptamer-Functionalized AuNPs
Even though AuNP-based colorimetric sensors can eliminate the use of analytical instruments for detections, there still is room for further improving their user friendliness. For example, a sensor in the format of a pH paper or a pregnancy test strip can make it more readily accepted by users in medical diagnostics. One of the most useful methods to convert antibody-based assays to user friendly test kits is the lateral flow technology. The application of this technology in nucleic acid-based detections, however, is rarely reported.53,54 We explored the feasibility of using lateral flow devices and developed aptamer-based sensors that could be used as simple dipsticks.55

The lateral flow device contained four overlapping pads placed on a backing (Figure 3(a)). The four pads were from top to bottom: absorption pad, membrane, glass fiber conjugation pad, and wicking pad. Aptamer-linked AuNPs were spotted on the conjugation pad while streptavidin was applied on the membrane as a thin line (Figure 3(a), left strip). To be captured by streptavidin, some AuNPs were labeled with biotinylated DNA (black stars in Figure 3(a)). When the wicking pad of the device was dipped into a solution, the solution moved up along the device and rehydrated the AuNPs. In the absence of adenosine, the rehydrated AuNP aggregates migrated to the bottom of the membrane where they stopped because of their large size (Figure 3(a), middle strip). In the presence of adenosine, the AuNPs were disassembled due to binding of the aptamer to adenosine. The dispersed AuNPs then migrated along the membrane and were captured by streptavidin to form a red line (Figure 3(a), right strip).

To carry out detection, the adenosine-sensitive devices were dipped into buffers containing various nucleoside species at different concentrations (Figure 3(b)). No red band was observed in the absence of adenosine. With increasing adenosine concentrations, intensified red bands were observed, and the detection limit was ~ 20 μM. No red bands were observed with 1 mM cytidine or uridine, suggesting that the high
selectivity of the aptamer was maintained. Similarly, cocaine-sensitive strips were also prepared and the possibility of using such devices to detect analytes in human blood serum was tested. As can be observed from Figure 3(c), a distinct red line was observed when the serum contained 0.2 mM cocaine, and the color intensity increased with increasing cocaine concentration; while adenosine failed to produce a red line. These results demonstrate that the device is compatible with biological samples, making its application in medical diagnosis possible.

Nanoparticles as Enzyme Carriers

In the examples illustrated above, the role of AuNPs was mainly as color-reporting groups. In other cases, metallic nanoparticles were also used as enzyme carriers for signal amplification. Shown in Figure 4(a) is the proposed secondary structure of a DNAzyme that can bind hemin (denoted as the diamond shape). This aptamer/hemin complex is a DNAzyme that can catalyze conversion of luminal. The target DNA acted as a linker between this piece of DNA extension and a DNA immobilized on a solid support (Figure 4(b)). In the presence of the target DNA, chemiluminescence could be detected on the solid support. In this case, AuNPs acted as not only as a support for DNAzyme immobilization, but also as a means of signal amplification because each AuNP could bind ~96 DNA molecules. Compared to directly immobilized DNAzyme, the AuNP/DNAzyme system gave at least a 10-fold increase in sensitivity.

Nanoparticles as Catalysts for Signal Generation and Amplification

Noble metal nanoparticles, such as gold and platinum, have high catalytic activities for many chemical
transformations. Coupled with biopolymers, this property has also been utilized for sensor design.\textsuperscript{50} Thrombin is an important serine protease in the blood coagulation cascade. The secondary structure of a thrombin DNA aptamer is shown in Figure 4(c). The authors indicated that there are two aptamer-binding sites in each thrombin molecule. Therefore, by attaching one thrombin aptamer on a solid support and another on a Pt nanoparticle, the presence of thrombin can link the nanoparticle to the solid surface (Figure 4(d)). The catalysis of H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O was used as a way to electrochemically signaling the presence of thrombin.\textsuperscript{61}

Nanoparticles as Wiring for Electron Relay
AuNPs also possess excellent electric conducting properties. Because their sizes can be made to be comparable to protein and nucleic acids, these nanoparticles can be used as a nanowire to conduct electrons from the enzyme-active site to bulk electrodes, and thus facilitate the detection of enzymatic reactions by electrochemical methods. Willner and coworkers immobilized 1.4 nm AuNPs on an Au electrode with a dithiol linkage.\textsuperscript{59} N-aminoethyl flavin adenine dinucleotide (FAD) was attached to the AuNPs through thiol linkages. FAD is the cofactor for glucose oxidase (GOx). Apo-GOx was prepared and reconstituted on the immobilized FAD, which allowed electrocatalytic oxidation of glucose. Higher concentration of glucose produced stronger currents, which enabled sensitive glucose detection. This method has also been applied to reconstitute glucose dehydrogenase.

Detection Based on Fluorescence Resonance Energy Transfer
Fluorescence resonance energy transfer (FRET) is a widely used technique in biology and biophysics.\textsuperscript{64} A FRET system contains a donor and an acceptor. The donor should be fluorescent and its fluorescence spectrum should have some overlaps with the absorption spectrum of the acceptor. The acceptor can be either fluorescent or nonfluorescent. In the latter case, it is usually called a quencher. When the donor and acceptor are close to each other (usually within 10 nm), energy can transfer from the excited donor to the acceptor. FRET efficiency depends on the distance between the two fluorophores, and a shorter distance gives stronger energy transfer. Most FRET experiments were performed with organic fluorophores. Recently, nanoparticles (including QDs) have been used both as FRET donors and acceptors for sensing applications.

Medintz et al. functionalized 530 nm QDs (emission peaks at 530 nm) with Cy3-labeled maltose binding proteins (MBP)\textsuperscript{65} (Figure 5(a)). Cy3.5-conjugated β-cyclodextrin specifically binds to the pocket of MBP. Therefore, there are two FRET pairs in this system. For the QD/Cy3 pair, QD is the donor, and for the Cy3/Cy3.5 pair, Cy3 is the donor. Exciting the 530 nm QD led to the energy relay to Cy3 and then to Cy3.5. In the presence of maltose, the Cy3.5 labeled β-cyclodextrin was displaced, which decreased the FRET from Cy3 to Cy3.5, and increased the Cy3 emission. This system is therefore useful for maltose detection.

In addition to separating the donor and acceptor by the ligand displacement reaction as mentioned above, the two can also be separated by cleavage reactions. Proteases are protein enzymes that can cleave proteins and peptides. Assays for protease activities are important for understanding a number of bioprocesses and diseases including cancer and infectious diseases. Medintz et al. prepared several quencher-labeled peptide sequences and attached these peptides to QDs (Figure 5(b)). These peptides were substrates for various proteases including caspase-1, thrombin, collagenase, and chymotrypsin. Initially the QD emission was quenched. In the presence of the proteases, the peptide was cleaved, releasing the quencher and unmasking the QD emission.\textsuperscript{66} In a slightly earlier report, Chang et al. employed AuNPs as quenchers, which were linked to QDs by a substrate peptide for collagenase. Similarly, enhanced emission was observed in the presence of the protease.\textsuperscript{67} This method is not limited to protease assays. Rao and coworkers attached a Cy3-labeled substrate for β-lactamase to QDs, and the QD emission was
Ellington and coworkers prepared thrombin aptamer-functionalized QDs. Quenchers were brought close to the QD surface by a small piece DNA that was complementary to a fraction of the aptamer sequence (Figure 5(c)). In the presence of thrombin, the aptamer switched its structure and bound thrombin, releasing the quencher-labeled DNA to produce enhanced emission. One of the main advantages of QDs is that under the same excitation light, emissions of different wavelengths can be generated in the same solution, which is useful for information encoding. We recently encoded adenosine and cocaine aptamer-linked AuNPs with QDs that emitted at 525 and 585 nm, respectively. This system was capable of detecting both analytes in one pot.

Detection Based on Charge Transfer

In addition to energy transfer, charge transfer has also been applied to modulate the emission properties of QDs. Benson and coworkers immobilized MBP on the QD surface (Figure 6(a)). A ruthenium compound was used as an electron donor. In the absence of maltose, the ruthenium compound was close to the QD surface and the charge transfer efficiency was high, inhibiting the QD emission. However, in the presence of maltose, the MBP underwent a conformation change and the ruthenium compound was separated from the QD surface, leading to increased emission. A similar strategy has also been used to couple the...
same ruthenium compound to an intestinal fatty-acid-binding protein. Upon palmitate binding, the environment around the ruthenium compound became more hydrophobic, which enhanced electron transfer and led to decreased emission. Strano and coworkers found that thrombin aptamer-functionalized PbS QDs showed thrombin-dependent emission decrease (Figure 6(b)), while other proteins such as bovine serum albumin (BSA), streptavidin, proteinase K, or lysozyme did not have such an effect. They attributed this drop in emission to the charge transfer from the surrounding water proton, and thus, are useful as contrast agents in magnetic resonance imaging (MRI).

We have prepared adenosine aptamer-functionalized iron oxide nanoparticles similar to those described in Figure 1(g), except that AuNPs were replaced by iron oxide nanoparticles. In the presence of adenosine, aggregated nanoparticles disassembled into individual ones. In a second example, thrombin aptamer-functionalized magnetic nanoparticles were assembled upon addition of thrombin to form aggregated structures, taking advantage of multiple aptamer-binding sites in each thrombin molecule. In both cases, altered water proton relaxation (T2) was observed, suggesting potential applications of these functionalized magnetic nanoparticles as smart magnetic contrast agents.

**CATALYST-FUNCTIONALIZED NANOTUBES AND THEIR APPLICATIONS IN NEAR-IR FLUORESCENT SENSING**

Carbon nanotubes have recently been found to be useful materials for sensing applications because of their near-IR fluorescence property. Near-IR sensors are very useful in molecular diagnostics and nanomedicine. Similar to QDs, nanotubes are also highly photostable. However, it is relatively difficult to functionalize nanotubes for bioconjugation while still maintaining its useful optical property. Other than optical detection, nanotubes can also be incorporated into microelectronic devices such as field effect transistors (FETs). Attaching biopolymers to these nanotubes allows other modes of detection.

**Sensing with Change of Emission Intensity**

Strano and coworkers reported that electroactive species such as K3Fe(CN)6 could irreversibly adsorb on the surface of single-walled carbon nanotubes and act as a quencher for nanotube emission with a quenching efficiency up to 83.3%. K3Fe(CN)6, on the other hand, quenched the emission only by 27.4% under identical conditions. With a dialysis method, glucose oxidase was immobilized on the nanotube surface through van der Waals interactions. This enzyme converted β-d-glucose into gluconic acid and hydrogen peroxide. The latter could react with K3Fe(CN)6 to produce K2Fe(CN)6, thus reducing quenching (Figure 7(a)). With this method, a detection limit of 34.7 µM glucose was achieved. Detection of glucose in blood under physiological conditions was also demonstrated. Other glucose oxidase immobilization methods, such as through a DNA interlayer, were also reported to be successful. With the unique IR-emitting property of carbon nanotubes, the sensors may be useful for in vivo applications.

**Sensing with Shift of Emission Wavelength**

In addition to emission intensity-based sensing, modulation of the chemical environment of the nanotubes to generate shifted emission wavelengths has also been shown to be a useful way of making sensors. Strano and coworkers demonstrated that DNA hybridization on the surface of solution-suspended single-walled carbon nanotubes induced shifted emission wavelength through a band gap fluorescence modulation. Unmodified 24-mer DNA was immobilized on nanotube surface with a dialysis method. Upon hybridization to its complementary strand, the nanotube emission wavelength showed a blue shift with an energy of ~2 meV, while noncomplementary DNA did not show such a shift. The system had a detection limit of 6 nM. The time...
required for the detection was relatively long, and it took ~13 h to reach the steady state. The same group has also demonstrated other means of shifting the nanotube emission wavelengths. For example, native double-stranded DNA are right-handed double helix, or known as the B-form DNA. Certain DNA sequences under certain conditions can switch conformation to a left-handed Z-form (Figure 7(b)). When such a B-Z transition takes place on the surface of a single-walled carbon nanotube, the emission wavelength of the nanotubes will also change. Hg²⁺ was found to be particularly effective in inducing such B-Z transitions and a sensor for Hg²⁺ detection was designed based on this observation. Again, because of the near-IR emission property of nanotubes, Hg²⁺ could be detected even in whole rooster blood and black dye solutions.

**CONCLUSION**

In this review, we have summarized some recent developments in the design of catalyst-functionalized nanomaterials and their applications in sensing relevant to nanomedicine. Nanoparticles and other nanomaterials possess useful physical properties that can be modulated by changing their chemical environment. Biocatalysts and biopolymers, on the other hand, possess highly specific target recognition properties. Conjugation of these biomolecules to nanomaterials can therefore provide a useful means of making biosensors for biomedical applications. Sensors based on the modulation of optical properties of metallic nanoparticles, semiconductor nanoparticles, magnetic nanoparticles, and carbon nanotubes have all been realized. The changes in the optical properties were either induced by different assembly states, or by changes in local chemical environment. Most of the examples are focused on in vitro applications of the sensors. Future works are likely to move towards in vivo detection. To achieve this goal, however, the toxicity of these nanomaterials has to be investigated to make sure they are safe for medical applications. Future research efforts will also focus on demonstration of sensors for clinically relevant targets under physiological conditions.

**NOTES**

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Nanoparticle-based biologic mimetics

David E. Cliffel,∗ Brian N. Turner1 and Brian J. Huffman1

Centered on solid chemistry foundations, biology and materials science have reached a crossroad where bottom-up designs of new biologically important nanomaterials are a reality. The topics discussed here present the interdisciplinary field of creating biological mimics. Specifically, this discussion focuses on mimics that are developed using various types of metal nanoparticles (particularly gold) through facile synthetic methods. These methods conjugate biologically relevant molecules, e.g., small molecules, peptides, proteins, and carbohydrates, in conformationally favorable orientations on the particle surface. These new products provide stable, safe, and effective substitutes for working with potentially hazardous biologicals for applications such as drug targeting, immunological studies, biosensor development, and biocatalysis. Many standard bioanalytical techniques can be used to characterize and validate the efficacy of these new materials, including quartz crystal microbalance (QCM), surface plasmon resonance (SPR), and enzyme-linked immunosorbent assay (ELISA). Metal nanoparticle–based biomimetics continue to be developed as potential replacements for the native biomolecule in applications of immunoassays and catalysis.

THE IMPORTANCE OF BIOMIMICS

The accurate mimicking of biologically important materials in a benign form is critical for the development of drug carriers, sensors, and catalysts. The use of whole or modified pathogens presents many challenges to researchers in terms of personal safety, facility requirements, and overall time and cost. Additionally, while the inactivated or killed form of a given pathogen can be used, there are always risks such as conformational changes or losses during inactivation, or a specimen that remains partially active. These challenges require the development of a surrogate that circumvents the need for active biological systems. Biomimetic nanoparticles offer an easy way to present the active part of a biomolecule with better stability and without the harmful payload. Additionally, nanoparticles provide a way to modify a surface with multiple functional groups because of their high surface area. All these attributes have led to nanoparticles becoming a diverse platform for biomimicking.

Since the development of water-soluble, ligand-capped nanoparticles almost 15 years ago,1 the use of nanoparticles in biological systems has increased dramatically. This is due, in part, to the fact that they can be chemically modified to mimic an antigen or biological marker of interest. Unlike growing cell cultures or working with live animals, which is time consuming and expensive, nanoparticle synthesis is relatively straightforward and can be carried out on a larger scale. The chemistry to conjugate functional ligands and macromolecules to nanoparticles has been well developed (especially place exchange2 and amide linkage3) and can be adapted to fit a myriad of systems, for example, antigen/antibody interaction, via different synthetic routes. Nanoparticles offer a method whereby a surface can be multifunctionalized to create a broad spectrum of functionality, whether presenting multiple epitopes of the same antigen or two different reactive species from a catalyst. This review will discuss the creation, modification, characterization, and uses of nanoparticle-based biological mimics, and the tools that can be used to validate their biological activity.

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NANOPARTICLE SYNTHESIS AND FUNCTIONALIZATION

The scientific study of colloidal metal particles dates back to Faraday in the mid-19th century. The synthesis and characterization, notably by electron microscope, of water 'soluble' gold colloids as small as 18 nm was completed by Turkevich and coworkers in 1951. Schiffrin and Brust, 43 years later, reported metal particles stabilized by alkanethiols. Murray and coworkers termed these 'monolayer-protected clusters' (MPCs) and defined them as differing from metal colloids because they can be repeatedly dried as well as isolated from and redissolved in common solvents without decomposing or aggregating. MPCs are synthesized using a bottom-up approach, suggesting that a wide variety of nanomaterials is possible from a small number of building blocks.

Nanoparticles are created with a variety of core types and capping ligands to create water- or organic-soluble products with desired functions. Both metallic and nonmetallic starting materials are used in the creation of nanoparticles, such as MPCs, organic polymers, virus-like particles (VLPs), protein particles, colloidal particles, and semiconductor quantum dots. Thiol-capped MPCs have received more focus because of their ease of creation, water and air stability, electrochemical and optical properties, and their ability to be surface-functionalized by the addition of biologically relevant ligands, such as peptide sequences of epitopes. Gold MPCs can range in size from 1 to 10 nm, containing approximately 55–1000 gold atoms with molecular weights between 10 and 200 kDa.

We acknowledge that a broad spectrum of nanometer-sized materials is present in the literature as previously mentioned. However, the focus of this review is stable, water-soluble gold-core MPCs and their targeted use in biological mimetics.

Synthetic Routes

Water solubility of MPCs is best accomplished by using a thiolated, polar protecting ligand in a modified Brust reaction as seen in Figure 1. In the Brust reaction, tetrachlorauric acid is reduced from Au to Au in the presence of the thiol capping ligand, yielding a colorless gold-thiol solution. This is either composed of a gold-thiol polymer or tetramer. Following the initial reduction, the gold is further reduced to Au in the presence of sodium borohydride (NaBH), yielding a black to dark brown solution. Other potent reducing agents, such as lithium aluminum hydride (LiAlH) or lithium triethylborohydride, have been used to reduce different metal cores such as palladium and platinum.

Key examples of thiolate ligands that have been used to produce water-soluble and long-term (months) air- and water-stable clusters are tiopronin, glutathione, 4-mercaptobenzoic acid, 1-thio-β-D-glucose, and N,N,N-trimethyl(mercaptoundecyl)ammonium (TMA) as depicted in Figure 2.

Functionalization of Monolayer-Protected Clusters

Transformation of water-soluble MPCs into biological mimics has been accomplished using a variety of synthetic functionalization strategies. However, the most widely used, straightforward method is the thiol place-exchange reaction seen in Figure 3.

Solution-Phase Place Exchange

In the place-exchange reaction, an incoming ligand, such as a thiol-containing biomolecule, replaces one...
of the original capping ligands in a 1:1 ratio. Place exchange on nanoparticles was first described by Murray and coworkers who used alkanethiolate clusters with \( \omega \)-functionalized thiols in toluene.\(^2\) This reaction has since been expanded to aqueous solutions and can also be carried out in aqueous buffer solutions.\(^10\) Multiple research groups have studied the dynamics by which place exchange occurs in ligand solutions. According to Murray’s work, the rate of ligand exchange depends upon both the concentration of incoming and exiting ligands, implying an associative (S\(_n\)2-like) mechanism.\(^33\) Lennox and coworkers, on the other hand, report that the reaction is zero-order with respect to the incoming ligand.\(^34\) Zerbetto’s lab found that the associative mechanism is accurate, but that the newly introduced ligand interacts with multiple existing ligands on the cluster.\(^35\) These interactions cause the kinetics to change as the reaction proceeds. Nevertheless, while the exact mechanism for place-exchange reactions may be complicated, the utility of place-exchange for functionalizing MPCs results from its simplicity.

Reaction rates also play an important role in the place-exchange dynamics. The reaction rate increases with smaller-sized entering ligands and shorter chain length of the protecting ligand.\(^33\) Consequently, it is thermodynamically favorable to place-exchange a large biomolecule, such as a peptide or protein fragment, with a small protecting ligand such as tiopronin. Further, it is important to consider that subtle differences in the structure of the incoming ligand, such as branching, can have a significant effect on both the rate of place exchange and the stability of the monolayer.\(^36\) Additionally, it should be noted that the reaction proceeds more favorably at different sites on the core: vertex sites > edge sites > near-edge sites > terrace sites,\(^33\) as depicted in Figure 4.

The variations in reactivity due to thermodynamics and kinetics originate from the differences in electron density\(^37\) and steric accessibility\(^38\) of these sites. This unique property of nanoparticles leads to some degree of predictability, and therefore control, in where the place-exchanged functional groups will anchor on the core. The rate of exchange is also increased by oxidative electronic charging of the core by electrochemical means\(^39\) or in the presence of dioxygen.\(^40\) The extent of reaction can be enhanced by increasing the incoming ligand concentration, but it should be noted that the extent of exchange rarely approaches 100\%, owing to the difficulty of exchange at terrace sites.\(^33\)

It is also important to realize that the rate of ligand place exchange on MPCs becomes slower as the particles age, probably because of a slow rearrangement of the ligands on the surface.\(^41\) Unfortunately, no kinetic or mechanistic study of place exchange has considered the new findings about the presence of gold-thiolate tetramer rings on the surface of MPCs as reported by the Cliffel group using mass spectrometry\(^28\) and Häkkinen and coworkers in a theoretical paper.\(^42\) Most recently, Kornberg et al. determined the specific crystal structure of a \( p \)-mercaptobenzoic acid MPC.\(^33\) The X-ray structure showed surface bridging interactions between gold atoms and the thiol groups of the protecting ligands. Also, the structure contains conformational features specific to the phenyl ligands, for example, phenyl stacking, T stacking, and sulfur–phenyl interactions. All the recent findings show nuances in surface structure that could help to better explain the complexities of the place-exchange mechanism.

### Solid-Phase Place Exchange

As an alternative to the solution-phase place exchange discussed above, Huo and coworkers have studied solid-phase place-exchange reactions. In their original report of this reaction, they employed a polystyrene Wang resin with acetyl-protected 6-mercaptophexanoic acid attached via an ester bond.\(^44\) The thiol groups were deprotected and allowed to undergo place exchange with butanethiolate-protected gold nanoparticles, followed by washing away of unexchanged product and cleaving of the exchanged particles. Their results showed that they could place-exchange one ligand on to a particle surface. This was proven using coupling chemistry to make dimer nanoparticle complexes, rather than trimers or larger aggregates that would result from multiple exchanged ligands.
The same group has compared this solid-phase approach to the solution-phase approach and found the solid phase approach to be advantageous in terms of controlling the number of ligands attached per cluster and preserving the order of ligands on the surface. Recently, the same group has reported a solid-phase approach using a noncovalent interaction of the incoming ligand with silica gel, which is depicted in Figure 5. This strategy employs milder reaction conditions, thereby making it amenable to a wider class of molecules, such as large biologically relevant functional groups.

Adapting Biological Functional Groups to Place Exchange

Macromolecules often contain thiols, for example, cysteine residues in proteins or adenosyl phosphorothioate residues in DNA oligonucleotides. These groups make biomolecules readily amenable to the place-exchange reaction. Strategies to introduce thiol groups into macromolecules include, but are certainly not limited to, the use of Traut’s reagent (2-iminothiolane) in the case of proteins, the inclusion of terminal cysteine residues during the synthesis of peptides, and the conversion of phosphates to phosphorothioates using 3H-1,2-benzodithiole-3-one 1,1-dioxide. It is also possible to introduce ligands into the MPC monolayer which undergo electrostatic interactions with biomolecules, for example, the use of biotin–streptavidin interaction or biotin–anti-biotin interaction. All these routes provide straightforward methods to ready a group for place exchange and create functional nanoparticles.

Direct Monolayer Functionalization

There are other strategies to functionalize MPCs. An important class of these strategies, which is gaining popularity, is the use of simple organic reactions on ligands already bound to the MPC. Examples include triazole cycloaddition to a bromine functionality, direct functionalization of a hydroxyl group, amide coupling, and ester coupling. All these methods enable post-exchange reaction chemistry to occur, allowing a surface to be modified in a controlled fashion.

Characterization Methods for Monolayer-Protected Clusters

A thorough characterization of MPCs and functionalized MPCs is critical before applying them in biological uses. Determination of core size is easily accomplished via transmission electron microscopy (TEM). Core sizes have also been determined by mass spectrometric methods. Thermogravimetric analysis (TGA) provides an easy method to determine the ratio of organic ligand to inorganic core material. Combining the core size and organic : inorganic ratio data yields an approximate average molecular formula for homofunctionalized MPCs. Nuclear magnetic resonance (NMR) spectroscopy is useful for determining the structure and composition of the protecting monolayer. Protecting ligands have broadened peaks in both 1H and 13C spectra due to spin–spin relaxational (T2) broadening, dispersity in binding sites, and dipolar broadening due to packing density gradients.

Once functionalized as a biomolecular mimic, it is critical to characterize MPCs for two attributes. The first is the quantity of biologically relevant functional groups attached per cluster, generally reported as an average of all the clusters in a sample. The second factor is the secondary structure of the biomimics post conjugation. 1H NMR is a simple way to semiquantitatively determine the number of antigen peptides per cluster via integration of known protecting ligand peaks versus new broadened biomolecule peaks. The accuracy of this method can be enhanced through the use of I2-induced MPC decomposition (termed the ‘death reaction’), which leads to sharper peaks with less overlap.

Secondary structure determination has proven to be more challenging. Drobny and coworkers describe the use of novel solid-state NMR techniques to investigate the secondary structure of peptides immobilized on gold MPCs via amide coupling. For their experiments, they used cross-polarization magic angle spinning (CPMAS) and double-quantum dipolar
They showed that a peptide maintained a helical structure upon conjugation, but with a slight change in backbone torsion angle. Mandal and Kraatz recently described similar characterizations of peptides place-exchanged onto MPCs using Fourier transform infrared spectroscopy (FT-IR) and Fourier transform reflection absorption spectroscopy (FT-RAIRS). Using amide I bands, they observed that the secondary structure of a leucine-rich peptide bound to gold transitions from $\alpha$-helical to $\beta$-sheet with greater surface curvature. Results showed that free peptides, 2-D self-assembled monolayers (SAMs) on gold, and peptides on 20-nm gold MPCs showed $\alpha$-helical structure because of less surface curvature. However, 10-nm and particularly 5-nm gold MPCs showed increasing amounts of $\beta$-sheet conformation due to the increased surface curvature. Understanding the nature of primary and secondary structure becomes critical as functionalized nanoparticles are used for practical applications.

ANTIGENIC VALIDATION USING IMMUNOASSAYS

One of the most effective ways to validate functionalized nanoparticles' ability to mimic a biological antigen is to look for recognition from a specific antibody. Since antibodies are generally targeted for one epitope of interest, they allow for specificity and serve as the keystone in many bioanalytical techniques. This section will quickly highlight some selected analytical tools used to detect antigen mimics in a sensitive and specific fashion.

Enzyme-Linked Immunosorbent Assay

ELISA describes a family of techniques used for the validation of antigen–antibody interaction through the detection of antigen or antibody. This technique was first described by Engvall and Perlmann in 1971. ELISA generally involves the adsorption of an antigen onto a plastic substrate, followed by recognition with a primary antibody. Then, detectable secondary antibody, specific to the primary, is incubated. Secondary antibodies use tags: for example, horseradish peroxidase or alkaline phosphatase that give a detectable signal upon activation with a specific substrate.

Quartz Crystal Microbalance

A powerful tool used by our lab and others to detect antigens against specific antibodies is the quartz crystal microbalance (QCM). This technique is based on a piezoelectric oscillator that changes frequency with the addition of a mass load, specifically, the antigen. This frequency shift is then converted to a mass load, so the instrument acts as a highly sensitive mass balance. Depicted in Figure 6 is a cartoon representation of a QCM biosensor, with a generic antibody–antigen system. The detection limit of QCM technology is continuously increasing as higher-frequency crystals are developed, reaching easily to the nanogram level and down to hundreds of picograms. A convenient reason to use QCM for measuring biomimic binding is the built-in amplification of using nanoparticles. Since QCM is essentially a mass detection method, the large molecular weight of the gold nanoparticle improves the sensitivity for biomimic studies.

Surface Plasmon Resonance

Another popular tool for bioanalytical measurements is the optical technique, surface plasmon resonance (SPR). This technique detects the refractive change of an incident laser source, which then translates into the on and off rates of the antigens. SPR utilizes commercially available gold surfaces with prefabricated substrates. These prefabricated substrates allow easier surface functionalization to create the biosensor. With subnanogram detection limits, SPR is another powerful tool for bioassays.
NANOPARTICLE-BASED MIMETICS

Protein-Functionalized Nanoparticles

Protein A–Coated Nanoparticles

Nanoparticles are capable of being functionalized with whole proteins, while still undergoing the same biomolecular recognition events as the free proteins. Recently, Rosenzweig and Thanh demonstrated the viability of biomolecular recognition of whole protein–coated gold nanoparticles in the development of an aggregation-based assay. They were able to detect anti-protein A in serum by aggregating protein A–coated gold nanoparticles and observing an absorbance change at 620 nm.

Antibody Fragment–Conjugated Nanoparticles

Kornberg and coworkers described single-chain Fv (scFv) antibody fragments conjugated to glutathione gold MPCs. The scFvs were rigidly coupled and exhibited specificity in binding to the antigen protein. By eliminating the flexible regions present in the whole antibody, rigidity was accomplished. Conjugation was accomplished by attaching a cysteine-terminated C-terminal affinity tag (FLAG) to the scFv. To assist the place exchange of the glutathione with scFv, they used oxidative charging of the metal core. Using cryo-electron microscopy, they were able to verify the antibody activity by observing the attachment of four Au71–scFv–glutathione units to single tetrameric influenza N9 neuraminidase units. In both these cases, the nanoparticle was used to aid in the detection of antibody–antigen binding, without actually using it as a biomimetic building block.

Peptide-Functionalized Gold Nanoparticles

Peptide-Functionalized Particles for Biological Recognition

Nanoparticles can be surface-functionalized by particle assembly and stabilization with a peptide or by place-exchange with the ligand after particle assembly. The first example of biologically relevant particles is the synthesis of nanoparticles with a protecting peptide from the histidine-rich protein II (HRP-II) of Plasmodium falciparum. Using standard fmoc procedures, Wright and coworkers recreated this peptide from the histidine-rich protein II and used it as a stabilizing ligand on different metal core particles: ZnS, Au, Ag, TiO2, and AgS. The biological significance comes from the recognition of the particle by a monoclonal antibody specific for P. falciparum. They were able to detect the peptide-encapsulated particles as they would the whole protein. This antibody–nanoparticle recognition shows that their particle mimics the native epitope.

Recently, Clifel and coworkers developed several MPCs that mimic antigens of interest. The first was a glutathione (GSH)-passivated gold cluster (GSH-MPC) that was then detected with a polyclonal anti-GSH antibody. The antibody very specifically recognized the GSH-MPC versus a standard tiopronin-passivated nanoparticle, even though both surface ligands only differ by about one amino acid, seen in Figure 7. While glutathione is not a traditional antigen, it serves as a proof of concept that an MPC can be functionalized with a surface peptide, and then specifically recognized via its antibody.

Another MPC this group synthesized contains an epitope from the hemagglutinin (HA) protein of influenza. termed an HA-MPC. The 10-amino acid peptide was again synthesized with standard fmoc procedures with a terminating cysteine residue to promote place-exchange chemistry. This peptide was selected because it is a neutralizing site for influenza and there was a commercially available monoclonal antibody specific for this epitope on HA. Also, this experiment compared 2-D SAMs to 3-D nanoparticles as depicted in Figure 8. It was shown that the HA-MPC was more efficient in presenting the peptide to the antibody, resulting in a higher ratio of antibody to peptide binding when compared to the 2-D surface.

Another novel feature of epitope presenting MPCs is that they can be size-separated. Using a specific sized particle, the peptide is forced into adopting a conformation closer to the native structure. Previous work by Murray and coworkers had shown that ligands are dynamically attached to the surface and will therefore migrate across the MPC to find the most stable conformation possible. Clifel’s research group applied this concept to their work on the protective antigen (PA) of Bacillus anthracis. The PA protein is one of three precursors of the anthrax toxin. PA was selected because it precedes the

![Figure 7](https://example.com/figure7.png)

**Figure 7** Comparison of the glutathione (a) and tiopronin (b) ligands used to functionalize MPCs. Tiopronin is a truncated form of the 3-amino acid glutathione, with overlap shown in red. (Reprinted, with permission, from Ref. 62. Copyright 2005 American Chemical Society.)
other two proteins (edema factor and lethal factor) in their transport for infection, which makes it an ideal target for neutralizing antibodies. Specifically, the C-terminus and two loops of the PA protein were identified as cell-receptor sites, making them the best candidates for their work.

Again, tiopronin MPCs were used and place-exchanged with the relevant peptide for the regions on PA. Since some of the PA epitopes selected were loop regions, the peptide was designed so that it could mimic its native conformation by putting cysteine residues on both the N- and C-termini. This allowed bidentate attachment across the nanoparticle surface to reconstruct the natural loop. Shown in Figure 9 is the stepwise process in the creation of the conformational mimic.

For comparison, a second cluster was created that only had a cysteine on the C-terminus for monodentate attachment. This creates two types of clusters, both with the proper primary structure, but only one with the secondary structure closer to the native conformation, as illustrated in Figure 10.

A QCM-based antibody–antigen binding study revealed that the loop-presenting cluster was more strongly recognized than the linear epitope cluster. More specifically, the loop epitope had a higher affinity constant ($K_a$) for this particular antibody than the linear epitope, especially at physiological saline concentrations. This data shows that the bidentate structure was better recognized and bound more tightly. This suggests that the commercial antibody may have a conformational paratope. The QCM was used to detect the antibody binding to the MPC, which was electrostatically held to the sensor.

Peptides are not only limited to use as the functional group. Naik and coworkers used peptides in a novel and sophisticated way. Multifunctional peptides were used as the reducing agent, gold-protecting ligand, and presenting epitope. Peptide A3 was selected from a phage peptide display library and found to both bind to gold and reduce it. Flg,
a peptide commonly used in tagging proteins with a biomolecular recognition domain, was also found to reduce gold. They were able to produce Flg-A3 and A3-Flg gold nanoparticles in a one-pot synthesis with good monodispersity and were capable of binding to anti-Flg IgG on glass slides.

As an extension of peptide-epitope-protected gold MPCs, a collaboration of the Cliffel and Wright research groups synthesized tiopronin MPCs functionalized with either the flag epitope (flag-MPC), HA epitope (HA-MPC), flag and HA epitope (flag/HA-MPC), or no epitope.75 The peptide epitopes were attached to the cluster via a cysteine-terminated polyethylene glycol (PEG) hexamer using place exchange. The PEG linker provides enhanced accessibility by moving the epitope away from the particle’s surface. QCM immunosensors, as previously described, using either anti-flag or anti-HA IgG were used to evaluate the immunological activity of the mimics synthesized. They were able to detect the HA-MPC and HA/flag-MPC using the anti-HA immunosensor, and the flag-MPC and HA/flag-MPC using the anti-flag immunosensor. Neither one detected the tiopronin MPCs without peptide epitopes. In all these trials, biological recognition serves as a quick means to validate peptide nanoparticles and determine binding constants.

**Peptide-Functionalized Gold Nanoparticles for Biological Targeting**

Biomimetic nanoparticles have shown promise as a tool for targeted cell entry. Targeted entry is complex, but the small size of gold nanoparticles and the functionality available from synthetic peptides make this delicate task a possibility. Inspired by viruses, Tkachenko and coworkers conjugated peptides to bovine serum albumin (BSA) via an ester linker, and then conjugated the BSA to gold nanoparticles.76 The four peptides they used were from viral cell entry/targeting proteins, and they were able to achieve targeted entry of the gold nanoparticles into the nucleus of HepG2 cells. Furthermore, it should be noted that the cells were still viable after entry of the gold nanoparticles.76

**Cell-Binding Studies of Functionalized Nanoparticles**

Gold nanoparticles, as previously mentioned, can be functionalized with many different ligands. Results from Rotello’s group show that the charge of the capping ligand can affect how the particle binds to cell surfaces.77 Positively charged ligands, such as TMA, cause an attraction between the particle and the negatively charged cell wall. The increased binding leads to higher toxicity and cell lysis. Conversely, the same negatively charged cell wall has little attraction to a carboxylate nanoparticle, leading to less cell lysis. Cell walls with no overall charge, however, lysed slightly more with negatively charged particles. These findings present interesting considerations when conducting studies at the cellular level.

Further work by Schmid and coworkers has shown that very small gold nanoparticles (Au53 cores, 1.4 nm) can actually bind to DNA in the cell. This is due to gold’s preference for the negatively charged backbone of DNA, which partially removes the protecting ligand group.78 Au53 clusters entrenched in the DNA grooves are depicted in Figure 11. Cell entry and specific targeting can serve as tools to either mark cells for imaging or cause controlled cell death.

**Peptide-Functionalized Gold Nanoparticles for Biological Catalysis**

The first report of using multifunctionalized gold MPCs for catalysis was by Frigeri and coworkers earlier this decade.79 Using N-methylimidazole-functionalized gold nanoparticles, they were able to catalyze the hydrolysis of an activated ester. Scrimin and coworkers have created water-soluble gold MPCs place-exchanged with histidine-phenylalanine dipeptides that are capable of mimicking hydrolytic enzymes.80 These two examples represent steps toward gold nanoparticle-based enzyme mimics that inspired Scrimin and coworkers to term them ‘nanozymes’. More recently, Morse and coworkers were able to use gold nanoparticles to mimic the catalytic activity of an enzyme in the sponge *Tethya aurantia* responsible for producing silica needles by simply conjugating organic molecules to the protecting monolayer of gold nanoparticles.81 The catalytic site of the aforementioned enzyme in *T. aurantia* uses a nucleophilic -OH group interacting with a hydrogen-bonding imidazole group to accomplish hydrolysis of...
Hydroxy- and imidazole-functionalized nanoparticles working together to catalyze silica formation. The ligands used to functionalize the particles are shown in (a), while the interaction between particles in (b) and (c). Part (d) shows the stepwise synthetic route of the ligands. (Reprinted, with permission, Wiley Periodicals, Inc.)

a silicon alkoxide precursor and subsequent polycondensation to silica. Hydroxy-terminated nanoparticles were afforded simply by using 11-mercaptoundecanol as the protecting ligand in a Brust synthesis. The imidazole-terminated nanoparticles were obtained by using amide coupling of an imidazole functionality to 11-mercaptoundecanoic-protected gold nanoparticles. This idea is illustrated in Figure 12.

Carbohydrate-Functionalized Nanoparticles
Many important processes in biology rely on carbohydrate–protein interactions, and it may become convenient to functionalize gold nanoparticles with carbohydrates instead of proteins or peptides. This was first accomplished by Penades and coworkers when they used carbohydrate-functionalized gold nanoparticles to mimic glycocalyx, the sticky film found on the outside of many different cells. As a further example of non-protein-related gold nanoparticle biomimetics, Chen and coworkers observed high affinity and specificity for binding of carbohydrate-encapsulated nanoparticles to concanavalin A. Carbohydrates were attached to the gold core using a thiol linker in a place-exchange reaction. Interaction with concanavalin A was monitored using SPR.

CONCLUSION
The exciting field of nanoparticle-based biological mimetics is rapidly developing. The results will continue to grow as new discoveries are made in the secondary structure of proteins, opening the door for even more material to incorporate into an expanding toolbox. Also, new techniques are being created to better characterize the interface between biological recognition and nanoscale structures.

The field has brought out many interesting results concerning the synthesis and conjugation of nanoparticle-based mimetics that can compete with their native analogs in functional complexity. The future of this field will be focused on creating more sophisticated nanoparticle-based biomimetics, centered on biological recognition, catalysis, and targeting. Metal nanoparticle–based drug targets may work well under ideal conditions for analytical merit and in vitro studies, but their effect in complex
biological systems is only beginning to unravel. The field of biological mimetics will continue to thrive, with nanoparticles playing a critical role in research, diagnostics, and therapeutics.

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**RELATED ONLINE ARTICLES**

Metal nanoparticles for DNA and antigen detection. Using nanoparticles to push the limits of detection.
Informatics approaches for identifying biologic relationships in time-series data

Brett A. McKinney

A vital goal of the genomic era is to identify biologic relationships between genes and gene products and to understand how these relationships influence phenotypes. Time course data contain a vast amount of causal and mechanistic information about complex systems, but experimental and informatics challenges must be overcome to produce and extract this information from biologic systems. Mathematical modeling and bioinformatics methods are being developed in anticipation of experiments involving the coordinated measurement of cellular and molecular quantities at various spatial and temporal scales. Experimental methods that probe at the nanoscale will facilitate the exploration of biologic systems at the single-cell and single-molecule level, but will also introduce special challenges for mathematical modeling because events at nanoscale concentrations are subject to the influence of intrinsic noise. This review addresses the progress, challenges, and frontiers in the field of time-series informatics. The ultimate goal of time-series informatics is to move beyond descriptive relationships and toward predictive models of emergent, or systemic, behaviors of biologic systems as a whole.

In recent years, high-throughput, genome-wide experiments have led to the vigorous development of new bioinformatics tools and algorithms that identify genes and gene products associated with phenotypic variables and that model causal interactions in gene networks. These large-scale experiments have created vast amounts of data, but most are limited to a single snapshot in time, allowing only a coarse approximation of the underlying dynamic system. Because of the lack of time-rich biologic data, most bioinformatics efforts have focused on a static viewpoint of biology. For example, statistical methods to discriminate between phenotypic classes from microarray data are reaching maturity. However, technologies such as kinetic reverse transcription polymerase chain reaction\(^1,2\) will soon allow for the coordinated measurement of dense time-series involving concentrations and expression levels of biologically active molecules. Bioinformatics approaches are under development in anticipation of the availability of more time-enriched data sets. This article reviews the promising developments and challenges in the area of time-series bioinformatics. These challenges include model structure inference and parameter estimation, the identification of models that predict phenotypic outcomes, and understanding the mechanisms of noise regulation in biologic systems.

Nanobased approaches are better suited than conventional methods to probe biologic systems at scales relevant to molecular mechanisms, in particular at the single-cell level.\(^3,4\) Processes at this scale involve statistical fluctuations that may be large, and biologic systems have necessarily evolved to function in the presence of such noise. In fact, there is evidence that biologic networks exploit noise, but if not properly controlled, fluctuations may lead to inappropriate systemic behavior of the network and possibly to an increased susceptibility to disease. Modeling time series at the nanoscale, where noise effects become more pronounced, is particularly challenging but may hold important keys to understanding the etiology of...
phenotypes that have eluded standard statistical analysis.

GRAPH-BASED NETWORKS

In this article, a model-free network is defined as a graph whose nodes represent genes and gene products and whose edges represent physical or functional interactions. The structure, or topology, of a graph provides insight into the organizing principles of cellular systems as well as into the functional motifs and interactions between genes and gene products. Statistical clustering is a potentially useful way to infer graph edges by identifying the patterns of correlation between profiles in time series, but inferring network connections by correlation or other metrics may belie the underlying molecular interactions that are implicit in the time-series profiles. For example, consider the simple hypothetical time series shown in Figure 1. Profiles for quantities y and z are the most correlated and correlation-based clustering would predict a close connection between y and z, with x more distantly related. In fact, y and z co-inhibit x; however, clustering cannot capture all of the complexity of the actual model shown in Figure 2. Specifically, there is directionality and information flow to this negative feedback loop with x activating y, y activating z, z suppressing x, and each gene product degrading in proportion to its own concentration.

Clustering has limited the ability to identify the directional interactions displayed in Figure 2, but may be useful for organizing information as input for more mechanistic algorithms discussed later in this review. For clustering time series, it is important to recognize that the time-series profile of each biomarker is not a collection of independent and identically distributed (iid) observations. To properly cluster time series, the dynamics should be taken into account explicitly, as for example in Ref. 6, where the authors use a linear approximation of the dynamics in the form of an autoregressive (AR) model to guide the clustering of gene expression profiles. This method should be more reliable than metric-based clustering, but AR has the disadvantage of being linear and univariate.

Graph theoretic methods facilitate the visualization of biologic relationships, but the lack of an underlying model hinders graphical methods from making experimentally verifiable predictions from system perturbations. The nodes of a realistic biologic network should represent time-varying activity and edges should represent flux through the network. At the other extreme of formalism complexity, nonlinear differential equations, which will be discussed in the following section, have been used to derive detailed models of gene networks, but the computational complexity of numerical integration and parameter estimation currently limits the size of the network that can be analyzed. The rest of the review will focus on mechanistic and data-driven modeling approaches.

MODEL-BASED NETWORKS

A Bayesian network (BN) combines probability theory and graph theory by constructing directed acyclic graphs (DAGs) that represent the dependencies between variables through probabilistic models. BNs provide a probabilistic framework for network graphs and have been used for gene network analysis of static gene expression data. However, BNs are designed for time-independent data, and their basis in acyclic graphs prevents them from handling feedback loops, which are necessary to model real biologic networks.
In contrast to graph-based networks, which are qualitative, static representations of cellular processes and pathways, dynamic model-based networks are governed by an underlying model that allows them to generate experimentally testable output. Numerous formalisms have been used to model kinetic data, but this article will focus on more quantitative formalisms based on differential equations. For a broad overview of model formalisms, see Ref. 8.

A more reductionist approach to understanding cellular networks would be to model the noncovalent bonding and enzymatic reactions of each macromolecule. However, this would not be feasible even if precise quantitative biologic data were available; thus, in practice mathematical approximations to the physical system are used. The simplest ordinary differential equation (ODE) that may describe biologic time-series panel data is a linear system:

$$\frac{dy_i}{dt} = \sum_{j=1}^{n} A_{ij} y_j, \quad i = 1, \ldots, n \quad (1)$$

where $y$ is a vector of functions describing the time variation of each molecule $i$ and the constant matrix $A$ summarizes the coupling strengths between molecules $i$ and $j$. The matrix $A$ lends itself to visualization as a graphical network, but fails to accurately model the nonlinear dynamics of a real biologic system. Equation (1) can be expressed naturally as a dynamic Bayesian network (DBN), which is a generalization of BNs to time-series data. DBN is a promising inference algorithm that has been applied to the analysis of gene networks from time series.9–12 A DBN can also be formulated as a Kalman filter (KF),13 a widely used tool in engineering for tracking and estimation. Using a linear system of ODEs like Eq. (1), the KF has been used to estimate DBN parameters for gene network inference.14 The KF is a Bayesian method in the sense that it provides a way to incorporate prior information to update the current state of the system. The KF can be extended to nonlinear models by replacing the matrix in Eq. (1) with a vector of nonlinear functions as in Eq. (2) below, but the model becomes more general than a network, and the system might be better described as a dynamic Bayesian model or generalized DBN (GDBN). The unscented Kalman filter (UKF) is an accurate and computationally efficient method for estimating parameters of nonlinear dynamic systems.15,16 It has been used to model in vivo protein time-series with noise and nonlinearities17,18 and has the ability to model unobserved state components, yet computational limitations still must be overcome for high-dimensional systems.

Numerous classes of ODE systems have been proposed from mathematical biology to model nonlinearity in biologic systems. For example, the operon model19,20 with nonlinear Hill functions was used to simulate the profiles shown in Figure 1. The general form of a nonlinear differential equation is

$$\frac{dy_i}{dt} = f_i[y(t), \lambda, \varepsilon], \quad i = 1, \ldots, n \quad (2)$$

where the model $f$ is a vector of nonlinear functions of vector $y$ with model parameter vector $\lambda$, and noise vector $\varepsilon$. Popular nonlinear ODEs for modeling biologic systems include generalized mass action (GMA) and synergistic-systems (S-systems).21–24 The S-system preserves some of the interpretability of a purely graph-based approach while having the ability to model realistic nonlinearities. In addition, the S-system has a bounded number of parameters, making it more efficient for analyzing cellular and molecular networks than GMA. The canonical form of the S-system without noise is the following power-law system of nonlinear differential equations

$$Y_i = \alpha_i \prod_{k=1}^{N} Y_k^{g_{ik}} - \beta_i \prod_{k=1}^{N} Y_k^{h_{ik}}, \quad i = 1, \ldots, n.$$

Each equation for the time rate of change of biochemical $Y_i$ is composed of a term for net production from metabolic biomolecules that contribute to the increase of $Y_i$ with rate $\alpha_i$ and a term representing net degradation of $Y_i$ from catabolic biomolecules with rate $\beta_i$. The $Y$s may represent a molecule, cell, protein, or other gene product within the system. Kinetic order parameters $g_{ik}$ and $h_{ik}$ on the real number line, represent the regulatory influence of $Y_k$ on $Y_i$. In principle, the S-system reduces structure identification to parameter estimation; however, in practice, the number of parameters is too large for nonlinear systems-identification algorithms. A promising approach to reduce the computational expense of parameter estimation is to decouple the ODE system into independent algebraic equations.25 Parameters estimated in this way may be used as initial guesses to speed up other, more computationally intensive, estimators.

It is rare that the biologic mechanism of a given process is completely described; thus, one of the goals of bioinformatics is to develop data-driven algorithms to automate the identification of the model structure and parameters from time series. The enormity of the search space of possible model structures calls for heuristic search methods such as evolutionary algorithms.17,18,26,27 When learning the structure of
a model, it is often necessary to include parsimony constraints in the objective function. A typical choice for objective function involves some variant of least squares deviation of the model prediction from the time-series panel data. It is often useful to divide the terms in the least squares sum by the corresponding data value to prevent variables with extreme values from dominating the objective function. To penalize high-connectivity models that over-fit the data, one may add a parsimony or complexity term that is usually a function of the number of parameters in the model.30

**SAMPLING FREQUENCY**

In classical model inference, the model structure is fixed and a parameter is unidentifiable if it cannot be estimated from the data, no matter how large the sampling frequency is. The least squares definition of identifiability is often used because it takes measurement error into account.29 Identifiability is more difficult to assess for dynamic network inference from biologic time series because the model structure is often nonlinear and/or unknown. For an experiment, a more practical quantity is the minimum sampling frequency—or the number of time points sampled for the duration of the experiment—needed to unequivocally identify a model. If the experimental system is insufficiently sampled, the system is underdetermined, meaning multiple models may fit the data. The problem is analogous to a sample size calculation to achieve a desired statistical power in a clinical trial involving multiple regression.30 If the structure of the model is fixed, one can minimize the reduced chi-square statistic (i.e., the maximum likelihood parameter estimation) \( \chi^2/\nu \), where \( \nu \) is the number of degrees of freedom, and then the level of significance can be estimated in terms of the incomplete gamma function.31 Of course, biologic model identification typically involves the identification of the model structure as well as its parameters. It is an open research question as to how to rigorously calculate the minimum sampling frequency for biologic network identification; however, the minimum number of measurements will depend on the measurement error, the variation in the profile curvatures, the number of biomarkers in the network, and the sparseness of the connectivity of the network. For a sparse network of Boolean functions with \( K \) regulatory inputs per gene, the minimum number of sampling points needed to identify a network of \( N \) biomarkers was shown to be of order \( 2^K (K + \log(N)) \).22 This value for \( M \) was derived under ideal conditions but represents a reasonable lower bound for modeling with a more complex continuous formalism such as nonlinear differential equations.

To overcome low-frequency sampling, one could use interpolation, random effects regression, or smoothing; however, these methods could be problematic for systems with high levels of noise that cause the system to deviate from smooth profiles. A recursive approach using the UKF has been successful for parameter estimation of dynamic biologic models.17,18 Figure 3 shows the results of this parameter estimation approach for data (filled circles) simulated based on the hypothetical model shown in Figure 2, disturbed by a large measurement noise and sparsely sampled (only five time points). The recursive steps are depicted as multiple lines for each variable shown in Figure 3 at the end of each UKF pass through the time series. In the absence of prior information on the parameters, all parameters are initialized to zero, resulting in an initial system with constant solutions. The predicted parameters at the end of each pass through the time series are used as input for the next recursion step. The UKF is insensitive to the initial choice of parameters for this model and converges to the correct parameters after 11 recursive steps. Recursion can help overcome sparsely sampled systems but also leads to increased computation time. Fewer loops through the time series and improved performance may be achieved by using high-quality initial guesses33 or more qualitative guesses based on known pathway connectivity.

For proper experimental design, simulations should be performed to determine the necessary

![Figure 3](image-url) **FIGURE 3** Recursive parameter estimation with unscented Kalman filter for extremely sparse, noisy data (filled circles) simulated with model shown in Figure 2. In each panel, each overlaid predicted time curve corresponds to the recursive steps through the time series. Parameters converge after 11 steps.
sampling frequency to reduce the false-positive model rate. A true-positive detection of a dynamic model is not an all or nothing prospect; one can correctly identify parts of the model and misidentify others. For stochastic system-identification algorithms, rather than taking the top-scoring system as the final model, a useful strategy to detect false-positive model components might be to inspect the set of top-scoring models to identify consensus model components and components that show more inter-model variation, and then run the algorithm again, this time focusing on the uncertain model components, which are more likely to be false. The best way to reduce false-positive models that all reasonably describe the available experimental data is to make computational predictions to design a new experiment that can discriminate between the hypothetical models. The time-series sampling frequency need not be uniform. In a more rapidly varying domain, it is advantageous to use a higher sampling frequency in order to capture detailed features of the profile. Another practical challenge from an experimental standpoint is anticipating when such a rapid variation will occur for a given biomarker. For example, transcription occurs on the scale of hours while metabolic reactions occur on the scale of minutes. Knowledge of such scales as well as time delays can aid experimental and algorithm design.

**SUPERVISED MODELING**

The next frontier in time-series informatics is to identify global properties and predict global states of dynamic network models. How does the perturbation of individual inputs of a noisy dynamic network affect properties of the network as a whole? Such systemic properties might be disease susceptibility or drug/vaccine response. It is not currently clear how to predict such a property directly from a dynamic network, but in other areas of bioinformatics, involving time-independent data, supervised statistical learning and data-mining algorithms have been used to predict the state of a phenotypic variable from multiple input variables. A similar approach has been used with knowledge-driven dynamic models to simulate time-series output, which is used to train a decision tree to predict the state of a selected output variable from perturbations in initial concentrations. By itself, this approach does not predict a global phenotype of an individual; however, coupled with other bioinformatics to identify gene products associated with the phenotype, the final decision leaves could predict some phenotypically relevant functional of the simulated gene product outputs. Among other things, a potential application of this technique might be the rational design of preventative and therapeutic interventions. The complexity of biologic networks poses many challenges to model-driven therapeutic design strategies due to interconnected clusters in transcription networks and the evolutionary evidence of network rewiring. The robustness of gene networks to noise (Ref. 39 and next section) may also make them robust to external manipulation, or may give rise to adverse side effects. Thus, a multivariate strategy is necessary to design combination therapies, which may be the best treatment strategy for many diseases.

Introduced here is an integrative, supervised strategy for vaccine improvement using aspects of the dynamic model simulation method described in Ref. 37. In machine learning, a problem is supervised if there is an outcome/class variable, such as a phenotype, which typically is used for classification. For rational vaccine development or improvement of existing vaccines, the goal is to maximize immunogenicity while minimizing reactogenicity. Step 1 of the proposed strategy would involve high-throughput screening to identify target cytokines associated with adverse events (e.g., Ref. 41) and a parallel analysis of antibody titers to identify target cytokines associated with protective immunity. Assuming that a dynamic model exists—either knowledge or data driven—Step 2 involves the generation of a large artificial data set with random initial perturbations of cytokines and other signaling and regulatory molecules of the model as the independent variables, and the response variable is a functional involving the target-molecule (found in Step 1) expression levels at the initial and final time point. The functional acts as the outcome variable for the set of perturbations. An example functional that measures the ratio of immunogenic to reactogenic expression change from the initial to final time points for a given combination of initial perturbations \( p \) is of the form

\[
F_p = \frac{\sum_{\text{immunogenic}} [y_p^i(t_{\text{final}}) - y_p^i(t_{\text{initial}})]}{\sum_{\text{reactogenic}} [y_p^i(t_{\text{final}}) - y_p^i(t_{\text{initial}})]} \tag{3}
\]

The next step is to find a combination of molecular perturbations that maximizes Eq. (3). Hence, Step 3 uses clustering or other methods to discretize \( F_p \) across the random Step 2 simulations into ‘high’, ‘medium’, and ‘low’ states, and then a decision tree is trained with the goal of identifying multivariate tree paths from the root node to ‘high’ output leaves. This strategy could generate hypotheses for improving protective immunity while reducing adverse events, and identify
network perturbations that lead to unstable behavior of the system. The success of this strategy is contingent upon an accurate model of the immune regulatory system and the availability of time-series data to tune such a model. An additional challenge to realizing the rational design of therapeutics is the possibly naive assumption that vaccine immune response kinetics can be modeled by the same model structure or kinetic parameters for all individuals; that is, the models may show genetic heterogeneity. A related goal will be to identify dynamic network motifs or modules associated with a given phenotype and to target these motifs rationally to achieve the desired outcome.

**PHENOTYPIC EFFECT OF NOISE AT NANOSCALE**

When using the differential equation formalism to predict network outputs from perturbed inputs, it is commonly assumed that the concentration of each molecule or expression of each gene product varies smoothly. In reality, the expression of each molecule depends on the number and state of other molecules, which are subject to random fluctuations. These external fluctuations, or extrinsic noise, cause the number of molecules to change abruptly from time point to time point. Furthermore, isogenic, identically prepared populations of cells may vary in expression level for a particular gene due to the order of the cascade of microscopic events leading to that gene’s expression. The conditions for the dominant effect of intrinsic noise in gene regulatory networks have been created in multicolor fluorescence experiments and aspects have been modeled with detailed simulations.

For the measurement of a molecule across M cells, the variance of the measurements is of order \( \sigma_i^2 / M \), where \( \sigma_i^2 \) is the variance of a single measurement. Thus, if the measurements are averaged over a large number of cells, then one expects low noise effects and smooth time-series profiles. At the other extreme, abrupt changes in profiles may be magnified at nanoscale concentrations, where there is low copy number or low concentration. Kalman filters using the differential equation formalism can handle limited noise effects, but when statistical fluctuations of concentrations become very large, a purely stochastic formalism may be more suitable. The Gillespie algorithm has become a popular method to directly simulate the stochastic mechanisms of a dynamic system. Under typical experimental conditions, it is sufficient to model with deterministic differential equations and is preferred for larger systems due to the computational cost of stochastic simulation. However, for sparsely sampled time series it may be difficult to estimate the noise strength, making it difficult to determine whether a time-series profile is merely random.

Biologic networks have evolved to function in the presence of noise and in most cases they behave in such a way as to reduce the effect of noise; however, in certain situations noise may be magnified to create heterogeneity in cell populations or to allow cells to adapt to a fluctuating environment. Thus, cells and networks may exploit noise, but it is conceivable that this flexibility, if not properly controlled, may also lead to adverse systemic behavior such as disease. A possible model for the pathogenesis of some diseases that have eluded the reductionist approach to the prediction of disease susceptibility directly from the genome may be the failure of a network motif to properly regulate the intrinsic noise. Obviously environmental factors also contribute to disease, but in certain cases a disease phenotype may be a rare, emergent property of a stochastic network caused by the network’s lifetime exposure to noise. For example, a stochastic mechanism has been hypothesized for haploinsufficiency diseases in which one allele in diploid cells is insufficient to assure normal function. In this model, the decrease in gene dose to one allele leads to an increased susceptibility to stochastic interruptions in gene expression. This interruption may lead to the increased probability of a drop in gene expression below a critical threshold and consequently to an increase in lifetime disease susceptibility. This effect of increased noise in haploinsufficiency may also be found in tumor suppressors. Another source of noise may arise from epigenetic factors, such as DNA methylation, which can modify transcriptional activity stochastically. The way gene and cellular networks deal with noise and its potential role in the etiology of disease phenotypes, particularly late onset, is far from understood and represents an opportunity and challenge for time-series bioinformatics.

**CONCLUSION**

Biologic time series contains considerably more causal information than gene expression or protein abundance measured at a single time point; thus, the development of high-throughput technologies to gather data with high temporal information content is eagerly anticipated. However, the identification of mathematical models for these dense data poses many practical and fundamental challenges. A challenge beyond the scope of this article is the role of spatial effects as they could be important in situations such as modeling protein activity, which depends on the...
protein’s location within the cell. This adds another level of complexity to the computational challenges discussed above, and tools such as partial differential equations will be needed to model networks when such spatiotemporal data become more readily available. Another challenge to reaching a systems-level understanding of organisms will be to integrate other data types—genomic, proteomic, structural, environmental, clinical, and phenotypic—into time-series modeling.

A dynamic model is just that: a phenomenological model of the true underlying system. However, an accurate model can reveal insight into biologic relationships and may act as an in silico experimental tool to generate testable hypotheses. Possibly the most ambitious time-series bioinformatics research frontier is to predict global/systemic properties, such as disease susceptibility, of a biologic system from dynamic network inputs. The reductionist approach has been very successful at identifying susceptibility genes for many phenotypes, but many common multifactorial phenotypes that have eluded this reductionist strategy may be an emergent property of the entire system, as opposed to a property that is possessed by any isolated part of the system. In future predictive dynamic network models, the phenotype may be an emergent property of the model or perhaps may be modeled as a hidden variable that describes the state of the whole system. Such a model may need to include genomic, proteomic, environmental, and epigenetic factors as well as the lifetime effect of intrinsic noise expressed in regulatory networks. Such a global model of genetic and cellular networks may also lead to improved preventative and therapeutic interventions by indicating ways to modulate multiple targets and simultaneously reduce adverse side effects.

NOTES
The java software used in this paper for recursive parameter estimation of generalized dynamic Bayesian networks with the unscented Kalman filter is available from the author upon request.

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REFERENCES


**RELATED ONLINE ARTICLES**

Noise in biological circuits.
Radioactive liposomes
William Thomas Phillips, Beth Ann Goins and Ande Bao

Many methods of labeling liposomes with both diagnostic and therapeutic radionuclides have been developed since the initial discovery of liposomes 40 years ago. Diagnostic radiolabels can be used to track nanometer-sized liposomes in the body in a quantitative fashion. This article reviews the basic methods of single photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging and labeling of liposomes with single photon and dual photon positron emission radionuclides. Examples of the use of these diagnostic imaging agents will be shown. The ability to track the uptake of liposomes in humans and research animals on a whole body basis is providing researchers with an excellent tool for developing liposome-based drug delivery agents. The attachment of therapeutic radionuclides to liposomes also has great promise in cancer therapy. Recent developments in the use of liposomes carrying therapeutic radionuclides for cancer therapy will also be reviewed. Many of the radiolabeling and tracking technologies developed for nanosized liposomes will also be useful for the imaging and tracking of other nanoparticles.

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Liposomes are spontaneously forming lipid bilayers that enclose an aqueous space. Very soon after their initial discovery more than 40 years ago, they were recognized as promising drug carriers for diagnostic and therapeutic agents. Composed of naturally occurring components, liposomes have a myriad of possible compositions and modifications, making them extremely flexible drug carriers. Liposomes range in size from 50 nm to several micrometers, with the most stable and useful size being in the lipid nanoparticle size range of 90–250 nm. In particular, several liposome formulations in the size range of 90–100 nm are clinically approved for the delivery of antifungal, antiparasitic, and anticancer agents. Incremental, continuous progress in the field of liposome technology has greatly increased the viability of liposomes as clinically useful drug carriers. These achievements include the development of (1) methods to produce homogeneously sized liposomes in the nanometer size range, (2) methods for large-scale liposome manufacture, (3) methods to stably encapsulate sufficient quantities of therapeutic agents within the liposomes, (4) methods to prolong the circulation time of liposomes in the blood, (5) methods to actively target liposomes in vivo by surface modification with targeted ligands, and (6) effective methods of non-invasively tracking and quantifying the distribution of liposomes in the body. This article will review the uses of radioactive liposomes for in vivo applications. Basic methods of labeling liposomes with radionuclides suitable for detection using nuclear imaging cameras placed outside the body and in vivo imaging methods with these radioactive liposomes will be described. Examples of practical imaging applications of radioactive liposomes for research and potential clinical application will also be reviewed. Finally, liposomes labeled with therapeutic radionuclides will be discussed.

SCINTIGRAPHIC IMAGING
One of the most effective methods of tracking and quantitatively determining the distribution of liposomes in the body is through the scintigraphic imaging of radiolabeled liposomes. Scintigraphic imaging, also known as gamma photon imaging, is a common clinically used technology that involves the detection of gamma photons emitted from radioactive molecules. Clinical imaging of injected radioactive molecules or molecular composites is widely used to detect the in vivo behavior of radionuclides and indirectly the in vivo behavior of these molecules, or...
liposomes, and other particle molecular composites in the body that cannot be detected by more anatomically based imaging methods such as standard X-ray and X-ray computed tomography (CT) imaging. Technological advances over the last 30 years have resulted in great improvements in the resolution and sensitivity of the cameras used for scintigraphic imaging. Over this same period, a variety of methods have been developed for the labeling of liposomes with various radionuclides.

TWO TYPES OF SCINTIGRAPHIC IMAGING

There are two types of gamma photon imaging that detect photons ($\gamma$-rays) resulting from the radioactive decay of radionuclides. These are based on either single photon image detection or dual-annihilation-photon image detection. Single photon imaging with a gamma camera can either be planar projection imaging, or tomographic three-dimensional imaging known as single photon emission computed tomography (SPECT) imaging. A second type of imaging known as positron emission tomography (PET) imaging is based on the detection of two annihilation photons that are simultaneously emitted at approximately 180° from each other. These annihilation photons are generated from the annihilation reaction between an emitted positron and a shell electron. PET imaging is always acquired for display of tomographic images, and planar projection imaging is not generally feasible.

Single Photon Imaging

For diagnostic applications with single photon imaging, the liposomes are labeled with a radionuclide that emits $\gamma$-rays with energies ranging from 100 to 200 keV (Table 1). These energies are high enough to penetrate a human body while low enough to be easily collimated by lead and be detected with a scintillation crystal. In single photon gamma camera imaging, 100–200 keV photons are emitted from liposomal radionuclides that have been previously administered into the body and allowed sufficient time to distribute in particular locations. In general, owing to their particulate nature, radiolabeled liposomes localize by physiologic processes responsible for clearing particles from the body; however, their blood clearance profile can be modified by adjusting particle sizes or attaching certain molecules, such as polyethylene glycol (PEG). Liposomes can also be molecularly targeted to specifically localize in a disease process or organ of interest. The emitted photon travels through body tissue and exits the body whereupon it is detected by a crystal inside the gamma camera. A lead collimator in front of the crystal allows only the photons from predictable projections to traverse through the collimator and thus provides spatial localization of the single photon emission. Following successful passage through the collimator, the photon interacts with the crystal detector and generates a scintillation that is detected by photon amplifiers, and the position of the scintillation on the crystal is determined.

Positron Emission Tomography Imaging

In PET imaging, a proton-rich radionuclide decays, resulting in emission of a positron (positively charged electron). When this positron reaches almost zero kinetic energy, it immediately reacts with a shell electron, resulting in the transition of these two electrons into the emission of two annihilation photons that are emitted at approximately 180° from each other. These dual photon emissions can be localized along a line by the simultaneous detection of the two 180° emitted photons by crystal scintillation detectors that are part of a ring of detectors surrounding the body. Multiple emissions can be iteratively localized in the body, providing a three-dimensional tomographic image of the distribution of the positron radionuclide. Collimators are not required for this type of imaging, although fairly thick crystals are required to detect the high-energy 511 keV photons that are emitted from all positron emission radioisotopes. The most commonly used radionuclides for PET imaging have relatively short half-life, such as oxygen-15 (2 min), carbon-11 (20 min), and fluorine-18 (110 min) (Table 1). PET radionuclides with longer half-lives, such as copper-64 (64Cu) (12.7 h) and iodine-124 (124I) (4.2 days), may be particularly promising for tracking the distribution of long-circulating nanoparticles such as liposomes.

METHODS OF LABELING LIPOSOMES WITH RADIOACTIVE MOLECULES

Commonly used radionuclides for the radiolabeling of liposomes for imaging studies with a gamma camera are technetium-99m ($^{99m}$Tc), indium-111 ($^{111}$In), and gallium-67 ($^{67}$Ga) (Table 1). These radionuclides are all widely available from local clinical radiopharmacies. $^{99m}$Tc has the most ideal properties of the readily available single photon emission diagnostic imaging radionuclides due to its optimal imaging characteristics which include an ideal photon energy of 140 keV and the fact that
Radioactive liposomes

it is relatively inexpensive because it can be eluted daily from a commercially available molybdenum-99 (69Mo)/99mTc generator. Because 67Ga, 111In, and 123I are cyclotron products, these agents are more expensive and not always available in every nuclear medicine department.

Several detailed reviews have been recently written describing the methodology for radiolabeling liposomes (Table 2). When designing a liposome labeling method, important factors to consider are ease of preparation, and in vitro and in vivo stability. Ideally, liposomes should be labeled just prior to the initiation of experiments by using premanufactured stock liposomes. This situation is ideal because the radionuclides used in clinical imaging and research studies are fairly short-lived (on the order of hours to days) (Table 1). This type of postmanufacture labeling is known as ‘afterloading’ or ‘remote labeling’ in which the preformed liposomes are labeled just prior to the start of the experiment. Also, after radiolabeling the radioactive liposomes should be stable, with a high percentage of the radionuclide remaining with the liposomes. Any significant release of the radionuclide from the liposomes during or after injection will obviously lead to complications in interpreting biodistribution results.

Table 2 summarizes several different methods that have been reported for labeling liposomes with both single photon and dual photon emission (PET) radioactive molecules. A relatively simple method of labeling liposomes is to incubate the premanufactured liposomes with a lipophilic radiolabel. This results in association of the label within the lipid bilayer. This approach generally yields very unstable radiolabeling and is therefore not preferable. In contrast, two afterloading approaches have proven to yield radiolabeled liposomes that have a high efficiency and good radiochemical stability. With these approaches, the radionuclide is either (1) trapped in the enclosed aqueous phase of liposomes, or (2) bound to a lipid-conjugated chelator incorporated in the lipid bilayer of preformed liposomes.

One type of afterloading method that has been developed for labeling liposomes uses radionuclide chelators attached to the liposomal surface. One method that has been found to be stable for the labeling of liposomes with 111In uses the metal chelator diethylenetriamine pentacetic acid (DTPA) conjugated to phosphatidylethanolamine (PE) lipid. A detailed description of the preparation and application of radiolabeled DTPA–PE liposomes has been previously published. DTPA–PE is not very effective for labeling liposomes with 99mTc. For this purpose, a new chelation method based on the technetium chelator, N-hydroxysuccinimidyl hydrazino nicotinate hydrochloride (HYNIC), has been developed by Laverman et al. With this method, the HYNIC ligand is conjugated to the amino group of distearoyl phosphatidylethanolamine (DSPE) and subsequently incorporated into the lipid bilayer during the liposome preparation. Just prior to the imaging study, the liposomes are incubated with 99mTc. This HYNIC labeling methodology has been investigated as a method to label liposomes for detection of infection. It has also been used to investigate the relatively short blood

<table>
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<th>Radionuclide (abbreviation)</th>
<th>Half-life</th>
<th>Type of Photon</th>
<th>Method of Production</th>
<th>Photons (keV), (Abundance (%))</th>
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<td>Gallium-67 (67Ga)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>300 (17)</td>
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<tr>
<td>Technetium-99m (99mTc)</td>
<td>6.01 h</td>
<td>Single photon</td>
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<td>Cyclotron</td>
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<td>245 (94)</td>
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<td>Iodine-123 (123I)</td>
<td>13.2 h</td>
<td>Single photon</td>
<td>Cyclotron</td>
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<td>20 min</td>
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circulation times that occur when very low doses of lipids are used, as well as the greatly shortened circulation time of PEG-coated liposomes when they are reinjected at frequent intervals.\(^{32,53}\)

In another type of afterloading method, a radionuclide is coordinated with a lipophilic chelator and then mixed with an aliquot of liposomes encapsulating a second molecule. Once the lipophilic chelator carries the radionuclide across the lipid bilayer, the second molecule interacts with the radionuclide complex and traps the radionuclide within the interior of the liposome. Several methods have been developed that use this second afterloading approach to label liposomes. This approach to labeling preformed liposomes has an important advantage in that it does not leave the radiolabel on the surface of the liposomes where it might interfere with specific targeting molecules or where it would have the opportunity to interact with natural metal-binding molecules in the body.

This approach has been used with \(^{111}\)In, \(^{99m}\)Tc, and \(^{67}\)Ga.\(^{16,30,43,54–56}\) One method using the radionuclide \(^{111}\)In has been widely adopted for liposome imaging studies.\(^{57–59}\) With this method, a clinically available \(^{111}\)In complex, \(^{111}\)In-oxine, is used for liposome labeling. The \(^{111}\)In-oxine is incubated with premanufactured liposomes encapsulating DTPA.\(^{60}\) \(^{111}\)In-oxine migrates into the aqueous interior of a liposome where it is trapped by trans-chelation onto DTPA. These \(^{111}\)In-labeled liposomes are stable and can be used for long-term tracking of liposomes because of the long half-life of \(^{111}\)In (68 h).

A method for labeling liposomes with \(^{99m}\)Tc uses the lipophilic complex \(^{99m}\)Tc-hexamethylpropyleneamine oxime (\(^{99m}\)Tc-HMPAO).\(^{16}\) With this method, lipophilic \(^{99m}\)Tc-HMPAO enters the liposome where it interacts with pre-encapsulated glutathione and converts to the hydrophilic form, and thus is trapped in the liposome. This method has been successfully used by many investigators.\(^{16,61–69}\)

Another similar afterloading method for radiolabeling liposomes with \(^{99m}\)Tc uses \(^{99m}\)Tc-N,N-Diethyl-ethylene diamine (\(^{99m}\)Tc-BMEDA).\(^{43}\) This method has been shown to have good in vitro and in vivo stability with a variety of preformed liposome formulations. Compared with \(^{99m}\)Tc-HMPAO, an additional advantage of the BMEDA-labeling method is that it is effective...
for labeling liposomes with β-particle-emitting therapeutic radionuclides, rhenium-186 (186Re) or rhenium-188 (188Re). A second advantage of this BMEDA method is that it can be used to directly label commercially available liposome formulations such as liposomal doxorubicin (Doxil) via sharing the pH gradient mechanism for loading drugs into the liposomes. BMEDA for research purposes is available from a commercial vendor (ABX, Radeburg, Germany).

A new method has recently been described to label preformed liposomes with radiohalogenated agents such as iodine-123 (123I) and iodine-124 (124I). With this method, a Bolton–Hunter reagent, in the form of an activated ester, crosses the liposome membrane and reacts with arginine that has been previously encapsulated in the liposome. Use of 123I may have some advantages because it is a single photon emitter with a longer half-life of 13.2 h, as compared to 6 h for 99mTc. This radionuclide is readily available from the local radiopharmacy in many locations, although it is fairly expensive because of the fact that it must be produced by a cyclotron. This same method could also be used to label liposomes with 124I, which is a long-lived positron emitter with a half-life of 4.2 days. The labeling of liposomes with this long-lived PET radionuclide may have useful research applications. However, the possibility of a high patient dose and its challenge in radiation protection may need to be considered with 124I owing to its relatively high β-particle energy and a high-energy γ-photon emission (1.69 MeV γ-ray, 10.4%).

A new method has recently been described for labeling liposomes with the PET radionuclide, fluorine-18 (18F). In this method, 18F is incorporated into the dipalmitoylglycerol lipid molecule by nucleophilic substitution of the p-toluenesulfonyl moiety. This procedure has a decay-corrected yield of 43%. Following rapid production of the 18F-lipid, long-circulating liposomes are labeled by adding the 18F-lipid during a rapid manufacturing procedure, and then used for PET imaging. Although this procedure, in which the liposome is labeled as part of the manufacturing process, is less ideal than the afterloading method of labeling preformed liposomes, the PET image produced by a very high-resolution state-of-the-art microPET camera is of excellent quality. This high-quality image demonstrates the future potential for PET imaging of radioactive liposomes, which will provide improved anatomical localization of labeled liposomes and other nanoparticles labeled with PET imaging radionuclides. A disadvantage with the use of 18F is that it has a short half-life of 109 min so that it would be useful for tracking liposomes only for a short time period of no more than 8 h; however, this short time of imaging may still prove useful for certain applications.

SPECIFIC USES OF SCINTIGRAPHIC IMAGING FOR LIPOSOme DRUG DEVELOPMENT

Scintigraphic imaging of liposomes has been successfully used in the last 15 years for studies of drug delivery and as potential diagnostic imaging agents. Several comprehensive reviews have been written describing in detail these diagnostic uses of radioactive liposomes. In the following section some specific examples of the use of radioactive liposomes will be provided with emphasis on research carried out in the last 3 years.

Noninvasive Determination of Drug Concentrations

The tracking of radiolabeled liposomes by scintigraphic imaging can be used as a method to noninvasively and quantitatively determine how much of a drug is taken up by the targeted site. A validation of this approach has recently been shown by Kleiter et al. In this research, liposomes were labeled with 99mTc using the HMPAO method. The purpose of the study was to determine whether noninvasive imaging could be used to predict the amount of doxorubicin that accumulated in a rat fibrosarcoma tumor following the administration of long-circulating PEG liposomes containing doxorubicin (Doxil) without the need to biopsy the tumor. Scintigraphic images were obtained at 18 h following injection of the 99mTc-labeled liposomes, and the tumors were removed for radioactivity counting and for measurement of the doxorubicin concentration. The results of this study demonstrated that there was a significant positive correlation between the intratumoral radioactivity and the amount of doxorubicin in the tumor. This provides good support for the use of this labeling and imaging methodology to noninvasively track the distribution of drugs delivered by liposome carriers. This report was also interesting in that these studies were performed on rats that had received local hyperthermia directed at the tumor. The images clearly demonstrated the increased uptake in the hyperthermia-treated tumor as compared with the tumors that were not treated with hyperthermia. Hyperthermia increased the uptake of the radio-label associated with the liposomes by approximately fourfold, and it increased the uptake of measured doxorubicin concentrations in the tumor by a slightly smaller increment that ranged from 2.6- to 3-fold.
Infection and Inflammation Targeting of Liposomes

Liposomes have been shown to accumulate heavily at sites of inflammation and infection by passive targeting. Microscopic studies have shown that the liposomes accumulate by enhanced extravasation and become mainly localized in macrophages and to a lesser extent in endothelial cells in the region of the infection/inflammation. Liposomes labeled with 99mTc have been effective tools in quantitating and localizing the site of uptake in the infection. Planar whole rat images shown in Figure 1 clearly demonstrate the uptake of liposomes in induced colitis in a rat. At 24 h, rats with colitis had 13.5% of the injected dose of liposomes located in the inflamed colon as compared to 0.1% in the colon of control normal animals. These planar images demonstrate the capability of scintigraphic imaging for noninvasively quantitating the percentage of injected dose that accumulates in a targeted region of the body. Liposomes continue to be very promising carriers for delivery of drugs to inflamed regions of the body, although, to date, no clinical products have specifically taken advantage of the inflammatory targeting of liposomes.

Liposomes for Treating Arthritis

Liposome imaging has been used in the preclinical development of liposomes containing prednisolone for the therapy of rheumatoid arthritis in an animal model. Long-circulating PEG-coated liposomes containing prednisolone as well as DTPA were labeled with 111In-oxine using the method described previously. These liposomes were found to accumulate in the inflamed joints by imaging. The increased uptake was very obvious on the images, and the 111In labeling of the liposomes was used to determine the clearance from the blood and the biodistribution in the tissues. The inflamed hind paws had sevenfold increased activity in comparison with the hind paws of normal rats. A single dose of liposome-encapsulated prednisolone resulted in complete remission of the inflammatory response in 1 week.

A similar use of imaging has also been recently reported for liposomes that carry superoxide dismutase (SOD) on their surface. These liposomes were used to treat experimental arthritis. The use of liposomes as a carrier for SOD greatly improved the pharmacokinetic behavior of SOD, allowing this powerful antioxidant to reach the site of inflammation in the joint. A comparison of the therapeutic efficiency was made between liposomes that had no SOD on their surface versus liposomes in which the SOD was carried on their surface. Imaging using the 111In-oxine/DTPA method demonstrated that even though both liposome formulations had similar accumulations in the inflamed joints, the liposomes with the SOD on their surface were significantly more effective for the treatment of arthritis. The investigators have named this type of liposome, an ‘enzymosome’.

Bone Marrow–Targeted Liposomes

A recent paper has reported that liposomes with a special surface modification have very high uptake in bone marrow. With this special surface modification, rabbits injected with these liposomes, also referred to as vesicles, had a very high uptake in the bone marrow as revealed by imaging with the 99mTc-HMPAO method as demonstrated in Figure 2. The special modification to the liposome surface was the addition of a negative charge to the liposome surface by adding a nonphospholipid anionic amphiphile, L-glutamic acid, N-(3-carboxy-1-oxopropyl)-1,5-dihexadecyl ester [succinic acid (SA)], to the liposome composition. The addition of a small amount of PEG to this liposome surface also appeared to modestly enhance the already high uptake of the liposomes in the marrow. Imaging was used to determine the most ideal concentration of PEG.
to have on the surface of these SA-liposomes for a maximized bone marrow uptake. On the basis of imaging studies, the uptake in the marrow was very rapid, with more than 60% of the infused dose taken up by the marrow at 6 h post intravenous administration. Although the precise mechanism by which these liposomes accumulate in marrow is unknown, electron microscopy studies as well as fluorescently labeled liposome confocal microscopy studies have demonstrated that the liposomes in the marrow are located in marrow macrophages. It is believed that the special surface modification of the liposomes results in specific targeting of the scavenger receptors on the surface of the bone marrow macrophages. The specific targeting of therapeutic agents to bone marrow using these SA-liposomes may be a promising approach for the delivery of drugs to the bone marrow.

**Intracavitary Liposomes**

Liposomes with high retention in body cavities and in the lymph nodes that drain from these cavities have been developed for drug delivery applications using imaging as a tool. These liposomes contain biotin on their surface, and when these biotin-coated liposomes are injected into a body cavity such as the peritoneum or the pleural space within 2 h of the injection of avidin into the same cavity, the liposomes will have high retention in this cavity. The mechanism by which this retention occurs is thought to be due to the aggregation of the liposomes by the high affinity that the multivalent avidin has for the biotin liposomes. Liposome imaging using the $^{99m}$Tc-HMPAO methodology has been used to monitor the retention in the cavity and determine the best timing for the injection of the avidin.

When the avidin/biotin–liposome methodology is used, most of the liposomes administered in the body cavity are retained in that cavity for a prolonged time, whereas the same biotin–liposome formulation without the avidin is rapidly cleared from the cavity over a 4–6 h period during which time the liposomes return to the blood from the lymphatics that drain the cavity. Figure 3 depicts a 3-D volume reconstruction SPECT image of a nude rat with an intraperitoneal ovarian cancer xenograft, acquired 4 h after intraperitoneal injection of $^{99m}$Tc-biotin–liposomes and 3.5 h after an intraperitoneal injection of avidin. The SPECT image is superimposed on a bone window CT image. As can be seen from the figure, much of the dose of $^{99m}$Tc-biotin–liposomes is trapped in the peritoneum. Without the avidin/biotin trapping system, nearly all liposomes would have been cleared from the peritoneum by 4 h post intraperitoneal administration.

This avidin/biotin–liposome approach has great potential for the intracavitary retention of therapeutic agents encapsulated in liposomes. One potential use for these liposomes is in the treatment of ovarian cancer in which the cancer cells are generally disseminated into the peritoneal cavity and lymph...
nodes that drain this cavity at the time of diagnosis. Another use could be in the treatment of lung cancer in which the cancer frequently drains into the mediastinal nodes. Imaging studies using the avidin/biotin–liposome procedure have shown that a large liposome dose can be targeted to the mediastinal nodes following administration of biotin liposomes along with avidin into the pleural cavity.

**Studies of Liposomes for Cancer Therapy**

Biodistribution studies in rats of the clinical formulation of Doxil labeled using the previously described 99mTc-BMEDA method have been performed by the authors.12 The ability to directly label commercially available liposome products may have important clinical uses.

Imaging has also been used to study the efficacy of specifically targeted liposomes. Several studies have used imaging to show the promise of modifying the surface of liposomes with molecules specifically targeted for the treatment of cancer.8,81 In these studies, liposomes were formed to contain both a polychelating amphiphilic polymer (PAP) and a specific targeting antibody on their surface. The specific polychelating polymer was composed of hydrophilic blocks carrying multiple side chains of the metal chelating agent, DTPA. For specific targeting, a monoclonal antibody against nucleosomes was also attached to these liposomes. Tumor-to-muscle ratio for the liposomes with the specific antinucleosome antibodies was 13.9 at 24 h versus lower ratios determined for the nonspecifically targeted liposomes that were either modified with nonspecific polyclonal antibodies (4.3) or liposomes with an unmodified surface (3.0) in rats that had Lewis lung carcinomas implanted on their thighs. This comparison with both types of control liposomes provides convincing evidence of the specificity of this antibody for targeting the tumor. This same antibody was also shown to appear to diffuse through the interstitial space of a tumor with a prolonged high retention. The degree of diffusion may depend on the characteristics of the particular liposome formulation injected. Liposome intratumoral diffusion should result in improved solid cancer therapy owing to a more homogeneous distribution throughout the tumor and a high intratumoral retention.

A significant advantage of liposomes for use in intratumoral injection is that they have the potential to move into the lymphatic vessels that drain from the solid tumors where they can deliver anticancer therapy to lymph nodes and other lymphatics that drain from the tumor. It is these lymph nodes that often contain tumor micrometastasis at the time the tumor is diagnosed.

**LIPOSOMES AS CARRIERS OF THERAPEUTIC RADIONUCLIDES FOR TUMOR THERAPY**

Liposomes are also promising carriers of therapeutic radionuclides for treatment of cancer. They can carry either therapeutic β-emitting or α-particle-emitting radionuclides for cancer therapy.86 Several therapeutic radionuclides have similar chemical features with diagnostic radionuclides and therefore can share the similar radiolabeling methods [for example, 111In and yttrium-90; 67Ga and holmium-166; 99mTc and 188Re, 186Re; radioactive halogens such as 123I, 124I, 131I and astatine-211, (211At)]. Table 3 contains the physical characteristics of β-emitting therapeutic radionuclides that have been used for labeling liposomes.

Table 4 contains a list of therapeutic radionuclides and methods that have been reported for labeling liposomes with therapeutic radionuclides. In certain cases, the methods currently used to label liposomes with diagnostic radionuclides could easily be adapted for the labeling of liposomes with therapeutic radionuclides. To date, this avenue of cancer therapy...
with liposomes is mostly theoretical, with few studies reported in the literature using liposomes as carriers of therapeutic radionuclides in cancer therapy.86,90–92

Previous theoretical dosimetry studies have addressed the potential use of radiotherapeutic liposomes for treatment of tumors via intravenous90,91,98 and intraperitoneal injection.99 There are some significant advantages of using the intratumoral delivery route for liposomes containing therapeutic radionuclides compared to intravenous injection, such as the much lower radiation doses delivered to liver, spleen, kidneys and other normal tissues, as well as the potential of simultaneous treatment of metastatic lymph nodes that drain from the region of the tumor.100 Another significant advantage of intratumorally administered radiotherapeutic liposomes is that perfect homogeneity of distribution within the solid tumor is not required, as the β-particle path length treats a field of tumor cells surrounding the location of the radiolabeled liposomes. This means that the therapeutic radionuclides carried with liposomes do not necessarily have to come directly into contact with every cancer cell. In addition, release of the radionuclide from the liposomes is not required for effective therapy. This field effect of β-particle emission is illustrated in Figure 4.

One possible use of radiotherapeutic liposomes is to treat residual tumor in the intraoperative situation. In many cases, the surgeon is unable to remove all of the cancer during surgery so that the margins of the resected tumor are positive. This generally means that there is cancer remaining at the operative site, which severely compromises patient survival. This positive margin can frequently be

<table>
<thead>
<tr>
<th>Radionuclide (abbreviation)</th>
<th>Half-lifea</th>
<th>Average Energy (MeV)a</th>
<th>Mean Range (mm)a</th>
<th>γ-ray Energies (Abundance) (keV)a</th>
<th>Production</th>
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<tbody>
<tr>
<td>Iodine-131 (131I)</td>
<td>8.0 days</td>
<td>0.182</td>
<td>0.91</td>
<td>284 (5.8) 364 (82) 637 (6.5)</td>
<td>Reactor</td>
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<tr>
<td>Lutetium-177 (177Lu)</td>
<td>6.7 days</td>
<td>0.133</td>
<td>0.67</td>
<td>113 (7)</td>
<td>Reactor</td>
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<tr>
<td>Rhenium-186 (186Re)</td>
<td>3.8 days</td>
<td>0.362</td>
<td>1.8</td>
<td>208 (11)</td>
<td>Reactor</td>
</tr>
<tr>
<td>Rhenium-188 (188Re)</td>
<td>16.9 h</td>
<td>0.764</td>
<td>3.5</td>
<td>155 (15)</td>
<td>Reactor and generator</td>
</tr>
<tr>
<td>Yttrium-90 (90Y)</td>
<td>2.7 days</td>
<td>0.935</td>
<td>3.9</td>
<td>None</td>
<td>Reactor and generator</td>
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aData derived from Ref. 87–89.

<table>
<thead>
<tr>
<th>Radionuclide (abbreviation)</th>
<th>Labeling Mode</th>
<th>Labeling Method</th>
<th>Cite</th>
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<tr>
<td>After-loading</td>
<td>Iodinated activated esters with arginine-containing liposomes</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Lutetium-177 (177Lu)</td>
<td>Bilayer intercalation</td>
<td>3-cholesteryl NTA hexyl ether</td>
<td>93</td>
</tr>
<tr>
<td>Rhenium-186 (186Re)</td>
<td>Encapsulation</td>
<td>Rephos chelator</td>
<td>94</td>
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<tr>
<td>After-loading</td>
<td>BMEDA with cysteine/GSH-liposomes</td>
<td>43</td>
<td></td>
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<tr>
<td>Rhenium-188 (188Re)</td>
<td>Encapsulation</td>
<td>Rephos chelator</td>
<td>94</td>
</tr>
<tr>
<td>After-loading</td>
<td>BMEDA with ammonium sulfate gradient</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Yttrium-90 (90Y)</td>
<td>Surface chelation</td>
<td>DTPA-phospholipid containing liposomes</td>
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</tr>
<tr>
<td>After-loading</td>
<td>BMEDA with ammonium sulfate gradient</td>
<td>95,96</td>
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**TABLE 3** | Physical Characteristics of Therapeutic Radionuclides Described for Radiolabeling Liposomes

**TABLE 4** | Methods of Labeling Liposomes with Therapeutic Radionuclides
determined during the operation. Radiotherapeutic liposomes that target residual tumor could be injected in the region of the positive tumor margin to sterilize the surgical margin of tumor cells. Because the liposomes will drain through the lymph nodes, they would also have the potential to treat micrometastasis in those nodes. These liposomes could contain a therapeutic radionuclide for radionuclide therapy, a chemotherapeutic drug, or a combination of both. Intraoperatively applied liposomes could, therefore, provide an additional tool for the surgeon, particularly when the margins of the tumor are positive.

LIPOSOMES LABELED WITH RHENIUM-186 AND RHENIUM-188

Our group has developed a novel method of labeling liposomes with the radionuclides of rhenium. This method uses BMEDA to post-load 99mTc, 188Re, or 186Re into liposomes.70 A second research group has also used this same BMEDA chemistry to label liposomes with 188Re.95,96

One of the advantages of rhenium therapeutic radionuclides compared with some other therapeutic radionuclides is that they emit a low ratio of photons in the ideal range of gamma camera imaging. For every 10 and 6.5β-emissions, both 186Re and 188Re radionuclides emit a γ-photon. This is an ideal ratio of β-to γ-emissions. The photon energy of both rhenium radionuclides is in the range of the photon energy of 99mTc (140 keV) so that the radiolabeled liposomes can be tracked continuously over time while performing therapy. This ability to image the distribution of 186Re-liposomes provides a significant advantage as compared to other commonly used therapeutic radionuclides such as yttrium-90 (90Y) and phosphorus-32 (32P), which are pure β-emitting radionuclides without γ-photons for imaging. A second significant advantage of 188Re and 186Re compared with many other therapeutic radionuclides such as 90Y and 32P is that they have almost no affinity for bone. Bone uptake occurs when the radioisotope eventually becomes separated from its chelator during metabolism of its carrier in the body. High bone uptake, which results in the suppression of hematopoietic cells in bone marrow, is generally the dose-limiting factor with current antibody-based radionuclide therapies. In contrast, rhenium radionuclides are mainly cleared through the liver and kidneys in a similar manner as 99mTc.

Previous theoretical dosimetry studies have addressed the potential use of radiotherapeutic liposomes for treatment of tumors via intravenous injection.101–103 In a recent experimental study with long-circulating pegylated liposomes radiolabeled with 188Re using the BMEDA method, 188Re liposomes had much higher uptake in the tumor as compared with 188Re-BMEDA alone. 188Re-liposomes were found to have a 7.1-fold higher tumor-to-muscle ratio as compared to intravenously administered unencapsulated 188Re-BMEDA in a C26 murine colon carcinoma solid tumor animal model.95

In addition to this intravenous investigation of 188Re-liposomes, our group has investigated the potential use of 186Re-liposomes for intratumoral therapy.70 High-resolution SPECT/CT images reveal the intratumoral distribution of therapeutic liposomes in Figure 5. The SPECT image was obtained from the
FIGURE 5 | High-resolution SPECT/CT images revealing intratumoral distribution of therapeutic liposomes.

137 keV \( \gamma \)-photons emitted by \( ^{186}\text{Re} \). The fusion of the SPECT image with the higher-resolution CT image provides improved anatomic detail of the intratumoral distribution of the \( ^{186}\text{Re} \)-liposomes. The ability to image the precise location of radionuclides during therapy can provide image-based feedback for further image-guided delivery of \( ^{186}\text{Re} \)-liposomes to ensure complete tumor coverage of therapeutic liposomes. There are some significant advantages of using the intratumoral delivery route for \( ^{186/188}\text{Re} \)-liposomes compared to the intravenous injection route, such as the much lower radiation dose delivered to liver, spleen, kidneys and other normal tissues, and the potential of simultaneous targeting of metastatic lymph nodes that drain from the region of the tumor as we just addressed.\(^\text{100}\)

Recent initial results with intratumorally administered \( ^{186}\text{Re} \)-liposomes by our group have demonstrated effective local control of 1.5-cm-diameter solid tumors in a rat head-and-neck xenograft tumor model.\(^\text{104}\)

CONCLUSION

The ability to track the uptake of liposomes in humans and research animals on a whole body basis is providing researchers with an excellent tool for developing targeted liposome-based drug delivery agents. The attachment of therapeutic radionuclides to liposomes also has great promise in cancer therapy. Many of the radiolabeling and noninvasive imaging technologies developed for nanosized liposomes will also be useful for the tracking of other nanoparticle systems under development as diagnostic and therapeutic agents.

REFERENCES


FURTHER READING


RELATED ONLINE ARTICLES

Liposome activated delivery.
Liposomes as pharmaceutical nanocarriers.
Liposome- and immunoliposome-based cancer therapeutics.
Advanced Review

Magnetic resonance susceptibility based perfusion imaging of tumors using iron oxide nanoparticles

Arvind P. Pathak1,2∗

Abundant preclinical and preliminary clinical data have convincingly supported antiangiogenic therapy as an effective strategy for the inhibition of tumor growth. This has led to an acute need for developing biological markers (biomarkers) of vascular remodeling that can be monitored in vivo, at repeated intervals in large numbers of patients with a variety of tumors in a noninvasive manner. Recently, magnetic resonance (MR) perfusion imaging with iron oxide nanoparticles has demonstrated the potential to be such a surrogate endpoint, that is, a biomarker intended to substitute for a clinical endpoint and predictive of clinical benefit. Consequently, both US Food and Drug Administration (FDA) and the National Cancer Institute (NCI) have major initiatives underway to improve the development of cancer therapies and the outcomes for cancer patients via biomarker development and evaluation. The biophysical principles, physiological relevance and range of imaging techniques underlying the success of susceptibility based contrast MR perfusion imaging with iron oxide nanoparticles as such a biomarker, are the subject of this review.

The dependence of tumor growth and metastasis on the vasculature has made angiogenesis a promising target for therapy.2-3 Strategies for inhibiting angiogenesis comprise a broad spectrum of antiangiogenic agents which include endothelial toxins directly targeted to endothelial antigens, growth factor antagonists that silence angiogenic growth factor signaling, protease inhibitors that inhibit proteases crucial to tumor invasion, and endogenous antiangiogenic compounds.4 As classified by Siemann et al., strategies for abolishing extant tumor vessels comprise an array of vascular disrupting agents that include tumor endothelium targeted antibodies conjugated to various moieties that induce thrombosis, and small molecule drugs that induce extensive vascular collapse by disrupting the cytoskeleton of proliferating endothelial cells.5 Several of these agents are currently in various phases of clinical trials.6 Recent results from the vanguard of antiangiogenic therapy such as the demonstration of the clinically significant normalization of tumor vessels in recurrent glioblastoma patients by daily administration of AZD2171, an oral tyrosine kinase inhibitor of vascular endothelial growth factor (VEGF) receptors,7 and the enhanced progression-free survival in chemotherapy-naïve metastatic or recurrent breast cancer patients treated with bevacizumab, a VEGF-specific antibody, in conjunction with chemotherapy compared to patients treated with standard chemotherapy alone,8 have heralded an urgent need for biomarkers to guide antiangiogenic monotherapy as well as combination therapy.9 These developments have collectively made it imperative to identify in vivo biomarkers of angiogenesis to: (1) direct the dosing and scheduling of antiangiogenic agents, (2) obtain early measurable signs of their therapeutic efficacy, (3) aid in the identification of the tumor vascular ‘normalization’ window, and (4) enable the testing of novel therapeutic regimens and agents. ‘Vascular normalization’ was described in...
1972 by Le Serve and Hellmann\textsuperscript{10} and more recently defined by Jain as the restoration of the blood vessel architecture (i.e., vessel density and caliber, fractal dimensions, pericyte coverage or basement membrane development) and consequently restoration of vascular function assessed in terms of a decrease in blood vessel permeability to macromolecules (and thus alleviated interstitial fluid pressure) and hypoxia, and improved blood perfusion,\textsuperscript{11} all of which resulted in enhanced drug delivery to the tumor and eventually improved patient outcome.\textsuperscript{11}

The plethora of available contrast mechanisms, in conjunction with its superior dynamic functional range, bestow on magnetic resonance imaging (MRI) the potential to be a noninvasive, \textit{in vivo} biomarker that can circumvent the drawbacks of traditional biomarkers of angiogenesis, which include tissue biopsies and microvessel density (MVD).\textsuperscript{12} Although widely used, both biopsies and MVD only provide static 'snap-shots' and do not lend themselves to dynamic \textit{in situ} assessments of the status of the tumor microvasculature, nor can they be used for noninvasive, \textit{in vivo} monitoring at repeated intervals in large cohorts of patients.\textsuperscript{13} Although there exist a wide array of MR contrast mechanisms, the exquisite sensitivity of susceptibility based MRI to the underlying vascular architecture gives it the potential to be a formidable tool in the noninvasive, \textit{in vivo} assessment of tumor angiogenesis.\textsuperscript{14} This review briefly highlights the basic concepts underlying the susceptibility based contrast mechanism, with a specific focus on perfusion MRI of cancer using iron oxide nanoparticles. It also calls attention to some of the novel techniques investigators have employed for assessing tumor angiogenesis, efficacy of antiangiogenic therapy and vascular normalization, and concludes with caveats to bear in mind when employing these techniques.

\section*{Susceptibility Based Magnetic Resonance Contrast}

A material’s tendency to interact with and distort an applied magnetic field is quantified in terms of its magnetic susceptibility. Specifically, the magnetization ($M$) induced in a sample is related to the applied field ($H$) by the susceptibility ($\chi$) of the material which is given by: $M = \chi H$. The majority of tissues relevant to human MRI are diamagnetic (i.e., $\chi < 0$), while paramagnetic and ferromagnetic contrast agents exhibit susceptibilities of $\chi > 0$ and $\chi \approx 1$, respectively.\textsuperscript{13} Often, distortions in the applied magnetic field such as those caused by surgical staples, biomedical implants and internal susceptibility differences at various tissue interfaces, lead to unwanted artifacts in MRI. Then again, susceptibility is also an intrinsic tissue property and regional variations in susceptibility can be exploited to extract important physiological information about healthy and pathological tissue by the judicious use of different MR pulse sequences and/or MR contrast agents.\textsuperscript{13}

Since Rosen et al.\textsuperscript{16,17} demonstrated in the early 1990s that a bolus of a high dose of gadolinium (Gd)-chelated contrast agent produces a transient decrease in signal intensity that can be converted into a concentration-time curve from which the relative cerebral blood volume (rCBV) can be computed using tracer kinetic principles,\textsuperscript{18} there has been a dramatic increase in the use and development of susceptibility contrast-based MRI. This has not only resulted in the development of elegant models for elucidating the biophysical phenomena involved,\textsuperscript{19–23} but has also resulted in the development of novel imaging strategies\textsuperscript{24} and contrast agents,\textsuperscript{25} including superparamagnetic iron oxide (SPIO) nanoparticles.\textsuperscript{26}

Specifically, when a paramagnetic contrast agent is restricted to the vascular compartment, each blood vessel perturbs the local magnetic field that water protons experience (Figure 1). As a consequence, each diffusing water proton experiences a slightly different magnetic field, and thus resonates at a different frequency. As protons diffuse through the microscopic field heterogeneities they lose phase coherence because of their random Brownian motion through these heterogeneous field distributions, eventually resulting in the attenuation of the MR signal\textsuperscript{20,21} (Figure 1). It should be pointed out that even without the diffusive movement of water, there exists a heterogeneity of resonant frequencies because of the presence of microscopic field inhomogeneities within an imaging voxel, which in turn affects the MR signal intensity (in gradient echo images) by causing intravoxel dephasing. When the passage of a (super)paramagnetic contrast agent bolus is tracked dynamically using either $T_2$- or $T_2^*$-weighted MR pulse sequences,\textsuperscript{16} it is known as dynamic susceptibility based contrast (DSC) MRI, which is discussed in an ensuing section.

The effect of magnetic field inhomogeneities on transverse relaxation can be characterized as:\textsuperscript{19,20}

$$\frac{1}{T_2} = \frac{1}{T_{2,0}^*} + \frac{1}{R_2}$$

(1)

The relaxation rate $1/T_2^*$ ($R_2^*$) is the rate at which the gradient-echo (GE) signal amplitude decays or the ‘effective’ $T_2$ and $1/T_2$ ($R_2$) is the rate at which the spin-echo (SE) amplitude decays or the
The vessel diameter), and the variation of the Larmor radius ($\gamma$) of the induced magnetic field inhomogeneity, the relaxation rate contribution attributable to magnetic susceptibility based contrast (DSC) magnetic resonance imaging (MRI). (c) Surface plot illustrating the three-dimensional aspects of mathematically simulated microscopic magnetic field gradients induced around a microvessel. The orientation of the applied field ($B_0$) and the axis along which the normalized field change ($\Delta B/B_0 \Delta \chi$) is plotted are shown in the inset. (Reprinted, with permission, from Ref.14. Copyright 2004 Elsevier).

FIGURE 1 | Schematic illustrating the origins of susceptibility based Magnetic resonance (MR) contrast. (a) In the absence of any susceptibility difference between blood ($\chi_1$) and the surrounding tissue ($\chi_2$), no microscopic magnetic field gradient is set up and diffusing water protons experience the same local magnetic field. (b) When a susceptibility difference ($\Delta \chi$) arises between the intravascular space and the surrounding tissue, say as a result of the presence of superparamagnetic iron oxide (SPIO) contrast agent, a microscopic field gradient (~) is set up that perturbs the local magnetic field, and diffusing water protons experience different local magnetic fields, leading to loss of phase coherence, and MR signal attenuation that can be followed dynamically using either $T_2^*-$ or $T_1^*$-weighted MR pulse sequences. This constitutes the basis of dynamic susceptibility based contrast (DSC) magnetic resonance imaging (MRI). (g) Surface plot illustrating the three-dimensional aspects of mathematically simulated microscopic magnetic field gradients induced around a microvessel. The orientation of the applied field ($B_0$) and the axis along which the normalized field change ($\Delta B/B_0 \Delta \chi$) is plotted are shown in the inset. (Reprinted, with permission, from Ref.14. Copyright 2004 Elsevier).

‘true’ $T_2$. The relaxation rate, $1/T_2^*$ ($R_2^*$), is the relaxation rate contribution attributable to magnetic field inhomogeneities. In the presence of a magnetic field perturber, that is, contrast agent bearing tumor vessel, the relative $R_2$ and $R_2^*$ relaxation rates depend on the diffusion coefficient ($D$) of spins in the vicinity of the induced magnetic field inhomogeneity, the radius ($R$) of the magnetic field perturber (i.e., tumor vessel diameter), and the variation of the Larmor frequency at the surface of the perturber.19,20,22,27 The two physical characteristics $R$ and $D$ can be collapsed into the proton correlation time $\tau_D$:

$$\tau_D = \frac{R^2}{D}$$

(2)

The variation in Larmor frequency ($\delta \omega$), at the surface of the tumor vessel is given by:

$$\delta \omega = \gamma (\Delta \chi) B_0$$

(3)

where $\gamma$ is the proton gyromagnetic ratio (42.58 MHz/T), $\Delta \chi$ the susceptibility difference between the tumor vessel and its background tissue ($\sim$ 1–10 ppm for SPIO15,28), and $B_0$ the strength of the applied magnetic field. The relative magnitudes of $\tau_D$ and $\delta \omega$ determine the magnitude of the susceptibility induced relaxation effects or contrast, which can broadly be classified into three regimes.17,19,20,22,27 The first regime is one in which the rate of diffusion ($1/\tau_D$) is substantially greater than the frequency variation ($\delta \omega$), that is, $\tau_D \delta \omega \ll 1$. In this ‘fast exchange’ regime, the fast diffusion rate of the spins causes them to experience a similar range of field inhomogeneities within an echo time (TE), resulting in minimal loss of phase coherence and similar loss of phase coherence between gradient- and SE sequences (Figure 2(a)). This is also called the ‘motional averaged’ regime as the susceptibility induced local magnetic field gradients are averaged out.19–21 The second regime is one in which the rate of diffusion ($1/\tau_D$) is substantially smaller than the frequency variation ($\delta \omega$), that is, $\tau_D \delta \omega \gg 1$. In this ‘slow exchange’ regime, the phase that a proton accumulates as it passes a perturber is large, that is, the effect is the same as it would be for the case of static field inhomogeneities. Because of the absence of motion averaging, the GE relaxation rate tends to be greater than the SE relaxation rate (Figure 2(a)). There will be no signal attenuation on a $T_2^*$-weighted scan because the 180º pulse during the SE sequence refocuses static magnetic field inhomogeneities, while intravoxel dephasing still occurs in a GE sequence (because of the absence of a similar refocusing RF pulse). The third regime is one in which $\tau_D \delta \omega \sim 1$, that is, water diffusion is neither fast enough to be in the motionally narrowed regime, nor slow enough to be approximated as linear gradient characteristic of the slow regime, making descriptions of the susceptibility induced contrast more complex.
In this ‘intermediate exchange’ regime, SE relaxation is maximum and the GE relaxation is similar to what it would be in the slow exchange regime (Figure 2(a)). In this regime, analytic solutions to estimate signal loss in the presence of diffusion become invalid and numerical simulations are required.19–21,27

From this phenomenological description of susceptibility based contrast, it is apparent that SE and GE sequences have greatly differing sensitivities to the size and scale of the field inhomogeneities, resulting in a differential sensitivity to tumor vessel size. Monte Carlo simulations have demonstrated that (Figure 2(b)) the SE relaxation rate change ($\Delta R_2$) increases, reaches a maximum for capillary-sized vessels (~4–5 μm), and then decreases inversely with vessel radius.21 The GE relaxation rate change ($\Delta R_2^\ast$) increases and then plateaus to remain independent of vessel radius beyond capillary-sized vessels (Figure 2(b)). A consequence of this result is that the SE relaxation rate changes are maximally sensitive to the microvascular blood volume, while the GE changes are more sensitive to the total blood volume. This resulting contrast has been exploited in different ways by various investigators as will become apparent in the sections that follow.

**Iron Oxide Nanoparticles in Magnetic Resonance Imaging**

When the magnetic dipole moments of closely packed neighboring atoms interact and preferentially align parallel to each other, such materials demonstrate a magnetic ordering that results in regions or ‘domains’ that are always spontaneously magnetized.29 If one decreases the size of such multidomain ferro- or ferrimagnetic particles, eventually one ends up with a single magnetic domain particle that can exhibit unique magnetic properties known as superparamagnetism25,29 (Figure 3(a)). Superparamagnetic particles have very high magnetic susceptibilities and can be magnetized to saturation even in weak applied magnetic fields (Figure 3). However, when this external field is removed, the magnetic dipole moments of individual superparamagnetic particles become randomly oriented as a result of thermal motions and no net magnetization is retained by the sample, unlike in ferro- or ferrimagnetic materials which exhibit remnant magnetization14 (Figure 3(b–c)).

![Figure 2](image)

**FIGURE 2** | (a) Schematic illustrating the three different regimes of susceptibility induced relaxation effects and the differential sensitivity of gradient-echo (GE) ($\Delta R_2$) and spin-echo (SE) ($\Delta R_2^\ast$) relaxation rates to vessel caliber. This sensitivity to vessel size constitutes the basis for imaging macro- and microvascular blood volume as well as imaging vessel-size. (b) Size dependence of $\Delta R_2$ and $\Delta R_2^\ast$ for fractional volume = 2% and $\Delta \chi$ = $1 \times 10^{-7}$. $\Delta R_2$ peaks for microvessels, while $\Delta R_2^\ast > \Delta R_2$ for all radii and plateaus for macrovessels. (Reprinted, with permission, from Refs.14,21).
FIGURE 3 | Schematic illustrating: (a) random orientations of the magnetic domains in a superparamagnetic iron oxide (SPIO) particle in the absence of any applied magnetic field, and (b) application of an external magnetic field $B_0$ causing the magnetic domains of the SPIO particle to orient along $B_0$. (c) Superparamagnetic particles (S) (open arrows) have a very high magnetic susceptibility and can be magnetized (M) to saturation ($M_S$ = saturation magnetization) even in weak external magnetic fields (H). Unmagnetized ferromagnetic and ferrimagnetic materials (F) (solid arrows) also become magnetized along this curve. However, once magnetized, ferromagnetic and ferrimagnetic materials retain their magnetization ($M_R$ = remnant magnetization), even if the external field is reduced to zero. However, at ambient temperatures superparamagnetic materials do not retain their magnetization in the absence of an applied field. (Reprinted, with permission, from Ref. 25 Copyright 2001 Springer and Ref. 30 Copyright 1972 Addison-Wesley).

Josephson et al. also demonstrated that the iron in colloids made from para-, ferro- and SPIOs had vastly different effects on both $R_1$ and $R_2$ relaxivities. They observed that the larger the average iron oxide core size, the greater the $R_2/R_1$ ratio, and thus proposed a classification scheme for MR contrast agents on the basis of the relative magnitudes of $R_1$ and $R_2$, rather than the susceptibility of the agent. According to their classification, a type I agent would be one with $1 \leq R_2/R_1 \leq 2$, such as paramagnetic chelates; a type II agent would be one with $2 < R_2/R_1 \leq 25$, such as dispersed superparamagnetic oxides e.g., AMI-25; and a type III agent would be one with $R_2/R_1 > 25$, such as ferromagnetic iron oxides.

In most cases the susceptibility induced MR signal attenuation produced by SPIOs is exploited to obtain strong $T_2$- and $T_2^*$-weighted contrast. However, when particles of smaller size are employed in conjunction with $T_1$-weighted MR pulse sequences, strongly $T_1$-weighted images can be obtained. In addition to the abovementioned factors, image contrast in SPIO enhanced MRI is not only dependent on the biodistribution of the agent but can also be profoundly affected by the spatial distribution or clustering of these particles.

Several different MRI applications of SPIOs have been realized depending upon the size and composition (or coating) of the nanoparticles employed. These applications have been summarized in an excellent review by Corot et al. and include imaging macrophage uptake in a gamut of disease models ranging from stroke to infection, metastatic lymph node imaging, MR angiography, molecular imaging, and cellular label imaging. The ensuing sections will briefly describe the different techniques in which SPIO perfusion imaging has been employed in the study of cancer and the phenomenon of tumor angiogenesis.

THE SIGNIFICANCE OF ASSESSING PERFUSION IN CANCER

Over a century ago, Virchow recognized that the tumor stroma exhibited a distinct capillary network. Since then, studies of tumor vascular morphology have identified a variety of structural and functional differences between tumor and normal vasculature (a comprehensive review is given in Ref.35). Tumor-induced blood vessels are typically sinusoidal, fragile and highly permeable with discontinuous basement membranes. Other characteristics of the tumor vasculature include (1) spatial heterogeneity and disorganized branching hierarchies, (2) arterio-venous shunts, (3) acutely and transiently collapsing vessels, (4) poorly differentiated and leaky vessels lacking in smooth muscle cell lining, and (5) an inability to match the metabolic demand of rapidly proliferating cancer cells, resulting in areas of hypoxia and necrosis.

The structural anomalies of the tumor vasculature not only result in areas of hypoxic tissue within the tumor mass but also in altered hemodynamics, blood rheology, and blood flow with profound consequences on conventional treatment modalities, pharmacokinetics, radiosensitivity, proliferation rate, invasive and metastatic potential, and the metabolic microenvironment ($pO_2$, pH, energy...
status). This makes it imperative to have noninvasive, in vivo indices of tumor perfusion and of the remodeled tumor vascular geometry, to enable us to optimize tumor perfusion related parameters and facilitate successful chemo-, immuno-, radio-, thermo- or targeted-therapy.

In addition, as mentioned above the dependence of tumor growth and metastasis on the vasculature has made angiogenesis a promising target for therapy. Strategies for inhibiting angiogenesis or normalizing the extant vasculature are currently in various phases of clinical trials with recent results indicating an urgent need for biomarkers to guide such therapies. As some of the pre-clinical and clinical approaches described below will indicate, by providing an index of changes in both vascular structure (vessel size index, permeability and blood volume) and function (blood flow or perfusion), susceptibility contrast (SPIO) enhanced perfusion MRI demonstrates the potential to be such a biomarker.

### TYPES OF PERFUSION-RELATED MAGNETIC RESONANCE IMAGING

Perfusion MRI with SPIOs can be broadly classified into three categories: (1) dynamic susceptibility contrast MRI, (2) steady-state susceptibility contrast MRI, and (3) vessel-size imaging. A brief description of each of these applications follows.

#### Dynamic Susceptibility Contrast Magnetic Resonance Imaging

Several investigators have acquired rCBV maps from first-pass DSC studies, with good spatiotemporal resolution. With this technique, preliminary results indicate that MRI-derived rCBV may better differentiate histologic tumor types than conventional MRI and provide information to predict tumor grade. To quantitatively measure rCBV or cerebral blood flow (CBF), regional changes in signal intensity versus time need to be converted into concentration versus time curves. As mentioned above, both empirical data and modeling indicate that for a given TE, the $T_2^*$ rate change ($\Delta R_{2*} = 1/T_2^* - 1/T_{2\text{precontrast}}$) is proportional to the brain tissue concentration:

$$\Delta R_{2*} = \frac{1}{T_{2\text{postcontrast}}} - \frac{1}{T_{2\text{precontrast}}} = k[\text{conc.}]$$  \hspace{1cm} (4)

---

### TABLE 1 | Classification of Superparamagnetic Iron Oxide (SPIO) Agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>Particle Size</th>
<th>Class of Agent</th>
<th>Trade Name</th>
<th>Developer</th>
<th>FDA Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMI-227</td>
<td>300 nm</td>
<td>Oral SPIO</td>
<td>Lumirem (EU)</td>
<td>Guerbet S.A.</td>
<td>Approved</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gastromark (US)</td>
<td>Advanced Magnetics</td>
<td></td>
</tr>
<tr>
<td>OMP</td>
<td>3.5 µm</td>
<td>Oral SPIO</td>
<td>Abdoscan</td>
<td>Amersham Health (now GE Healthcare)</td>
<td>Approved</td>
</tr>
<tr>
<td>AMI-25</td>
<td>80–150 nm</td>
<td>SSPIO</td>
<td>Endorem (EU)</td>
<td>Guerbet</td>
<td>Approved</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Feridex IV (US)</td>
<td>Bayer Healthcare Pharmaceuticals</td>
<td></td>
</tr>
<tr>
<td>SHU-555A</td>
<td>62 nm</td>
<td>SSPIO</td>
<td>Resovist (EU)</td>
<td>Bayer Schering Pharma AG</td>
<td>Phase III complete</td>
</tr>
<tr>
<td>AMI-277</td>
<td>20–40 nm</td>
<td>USPIO</td>
<td>Sinerem (EU)</td>
<td>Guerbet S. A.</td>
<td>Discontinued</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Combidex (US)</td>
<td>AMAG Pharmaceuticals</td>
<td>Phase III</td>
</tr>
<tr>
<td>NC100150</td>
<td>20 nm</td>
<td>USPIO</td>
<td>Clariscan (US)</td>
<td>Amersham Health (now GE Healthcare)</td>
<td>Discontinued</td>
</tr>
<tr>
<td>–</td>
<td>2–5 nm</td>
<td>MION</td>
<td>–</td>
<td>–</td>
<td>Experimental</td>
</tr>
</tbody>
</table>

Adapted from Ref. 25 with permission.

### TABLE 2 | Pharmacokinetic Properties of Superparamagnetic Iron Oxide (SPIO) Agents

<table>
<thead>
<tr>
<th>Type of Agent</th>
<th>Surface Coatings</th>
<th>Half-life</th>
<th>Biodistribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral SPIO</td>
<td>Siloxane, polystyrene</td>
<td>Oral</td>
<td>Gut</td>
</tr>
<tr>
<td>SSPIO</td>
<td>Dextran, carbodextran</td>
<td>2–6 h</td>
<td>Liver and spleen</td>
</tr>
<tr>
<td>USPIO</td>
<td>Dextran, carbohydrate-polyethylene glycol</td>
<td>24–36 h</td>
<td>Lymph nodes</td>
</tr>
<tr>
<td>MION</td>
<td>Dextran</td>
<td>~ 4 h</td>
<td>Targeted imaging</td>
</tr>
</tbody>
</table>

Parameters for this table obtained from Refs 24, 25.
where $k$ is a tissue-specific, MR pulse sequence and field strength dependent, calibration factor.\textsuperscript{16,42} Assuming monoexponential signal decay, the signal intensity change following SPIO injection is

$$S(t) = S_0 e^{-TE(\Delta R_2^* t)}$$ (5)

yielding:

$$\frac{-1}{TE} \ln \left( \frac{S(t)}{S_0} \right) = kC(t)$$ (6)

where $S_0$ is the signal intensity before administration of the contrast agent, $S(t)$ the tissue signal with contrast, $TE$ the echo time, and $C(t)$ the concentration-time curve of the tissue. Without knowledge of the arterial input function (AIF) it is still possible to determine relative values of CBV and the mean transit time (MTT), which in turn yield relative values of the CBF.\textsuperscript{43} For example, one can determine rCBV by computing the area under the concentration-time curve, that is, $\Delta R_2^* t$ curve by directly integrating on a voxel-wise basis:

$$rCBV = \int_0^t \Delta R_2^* t \, dt = \frac{-1}{TE} \int_0^t \frac{S(t)}{S_0} \, dt$$ (7)

The relative MTT (rMTT) is then given as:

$$rMTT = \frac{\int_0^t t \Delta R_2^* t \, dt}{\int_0^t \Delta R_2^* t \, dt}$$ (8)

And the relative (rCBF) as:

$$rCBF = \frac{rCBV}{rMTT}$$ (9)

However to quantify the absolute blood volume and perfusion, one must know the concentration of the tracer in the arterial blood supply $C_a$. As outlined by Ostergaard,\textsuperscript{44} for an infinitely short bolus of contrast agent, the tissue concentration as a function of time may then be written as

$$C_i(t) = CBF \times C_a \times R(t)$$ (10)

where $R(t)$ is the tissue residue function that describes the fraction of tracer present in the voxel at time $t$ post injection. Thus, the residue function is a decreasing function of time, wherein $R(0) = 1$, and if the tracer does not bind to the vasculature, $R(\infty) = 0$. In Eq. 10, the product $CBF \times R(t)$ is known as the tissue impulse response function. However, since most injections are of finite duration, the observed concentration-time curve is the convolution of the ideal tissue-transit curve with the AIF $C_a(t)$.\textsuperscript{44}

$$C_i(t) = CBF \times C_a(t) \otimes R(t)$$ (11)

In order to extract the absolute CBF from Eq. 11, the tissue impulse response needs to be determined by deconvolution, essentially fitting $CBF \times R(t)$ from the experimental data. Typically, Eq. 11 is solved for $CBF \times R(t)$ using a transform approach or by a linear algebraic approach.\textsuperscript{44} Briefly, in the Fourier transform (FT) approach, the convolution theorem of the FT is utilized, that is, $F(f \otimes g) = F(f)F(g)$. Hence Eq. 11 can be solved as follows:

$$F[C_i(t)] = F[CBF \times R(t) \otimes C_a(t)]$$
$$= F[CBF \times R(t)] \times F[C_a(t)]$$
$$\Rightarrow F[C_i(t)] = F[CBF \times R(t)]$$
$$\Rightarrow F^{-1}\left\{ F[C_i(t)] \right\} = CBF \times R(t)$$ (12)

where $F$ and $F^{-1}$ denote the discrete and inverse discrete FTs, respectively. Since $R(0) = 1$, the CBF can be determined from the initial height of the tissue impulse response function, and the CBV from:

$$CBV = \int_0^t \frac{C_i(t)}{R(t)} \, dt$$ (13)

For an instantaneous bolus injection, the central volume principle states that $CBF = CBV/MTT$, where MTT is the mean transit time of contrast agent through the vascular network.\textsuperscript{18} A detailed exposition of the principles of cerebral perfusion imaging, the linear algebraic solution to Eq. 11 and technical issues associated with the solutions of Eq. 11, have been presented in an excellent review by Ostergaard.\textsuperscript{44} In order to reduce reperfusion effects on assessments of CBV and CBF, $C_i(t)$ and $C_i(t)$ are corrected for recirculation, which is usually accomplished by fitting them to a gamma-variate function with a recirculation cut-off.\textsuperscript{45} An obstacle to the application of the central volume principle for the calculation of blood flow is the direct measurement of the MTT. Weisskoff et al. have demonstrated that MTT is not the first moment of the concentration-time curve for MR of...
intravascular tracers, but does provide a useful relative measure of flow.46

There have been several DSC studies of tumor xenograft models using SPIOs that employ the principles described above. These include a study that developed the appropriate stereologic correlates for histologically validating MR-derived cerebral blood volume maps in a brain tumor model using MION.38 Another demonstrated the efficacy of DSC MRI in tracking the morphological and functional changes induced by the antiangiogenic agent SU11657 in a brain tumor model.47 As mentioned above, the SE relaxation rate changes are maximally sensitive to the microvascular blood volume, while the GE changes are more sensitive to the total blood volume. On the basis of this observation, SE sequences have been used in many tumor studies with the assumption that tumor angiogenesis is primarily characterized by an increase in the microvasculature.39 However, given the large (>20 µm) tortuous vessels usually found in tumors,38 whether SE or GE methods are most appropriate remains to be determined.

Steady-State Susceptibility Contrast Magnetic Resonance Imaging

As demonstrated in the section above, measurement of perfusion necessitates the use of a dynamic MRI pulse sequence that can sample the first-pass of the SPIO with the appropriate temporal resolution. However, more recently it has been demonstrated by several investigators that the rCBV can be mapped under ‘steady-state’ conditions, that is after the first-pass of the contrast agent and without prior knowledge of the AIF.42,48,49 This has only been made possible with the development of ‘blood pool’ contrast agents with very long intravascular half lives such as USPIOs and MION.26,50 Pre- and post-contrast T2* or T2-weighted images can be acquired at high-resolution with a high signal-to-noise ratio from which the steady-state \( \Delta R_2^* / \Delta R_2 \) or \( \Delta R_2^* \) can be computed reliably, because unlike DSC protocols the temporal resolution requirements for steady-state susceptibility MRI are less stringent, and USPIOs and MION have very strong susceptibility effects. When an intravascular contrast agent such as USPIO or MION achieves steady-state in the intravascular space after mixing, the rCBV can be calculated on a voxel-wise basis according to:

\[
\text{rCBV} \propto \frac{1}{\text{TE}} \ln \frac{S_{\text{precontrast}}}{S_{\text{postcontrast}}}
\]

where TE is the echo time, \( S_{\text{precontrast}} \) the GE signal before administering the SPIO contrast agent and \( S_{\text{postcontrast}} \) the GE signal after the contrast agent has achieved steady-state throughout the blood pool. A similar relationship can be used to calculate the rCBV from the SE signal. As before, the differential sensitivity of the gradient- and SE sequences can be exploited to give a ‘macrovascular’ rCBV and a ‘microvascular’ rCBV (i.e., weighted towards capillary-sized vessels), respectively. The steady-state approach has been elegantly validated using radiotracer measurements and intravital microscopy wherein it was demonstrated that there was a good correlation between techniques in terms of their ability to assess ‘angiogenic burden’

FIGURE 4 (a) Post-Gd MR image of a 9L gliosarcoma bearing rat brain illustrating the tumor ROI (yellow hatched ellipse). (b) Post-monocrystalline iron oxide nanoparticle (MION) ratio \( \Delta R_2^* / \Delta R_2 \) map of a 9L gliosarcoma bearing rat brain wherein one can clearly see the elevated ratio values in the angiogenic tumor rim (yellow hatched ellipse). (c) A histogram of the \( \Delta R_2^* / \Delta R_2 \) data showing that for the tumor ROI (over all slices), \( \Delta R_2^* / \Delta R_2 \) is shifted to the right i.e., larger caliber vessels with respect to the contralateral brain ROI (over all slices) \( \Delta R_2^* / \Delta R_2 \), a difference that is also apparent from (d) the histogram of the stereologically calculated vessel radii. (Reprinted, with permission, from Ref.62. Copyright 2001).
in a range of tumor models.\textsuperscript{52} This technique has been employed to characterize tumor angiogenesis in various experimental brain tumor models.\textsuperscript{28,42,43} In conjunction with blood oxygenation level dependent (BOLD) MRI, another susceptibility contrast based technique,\textsuperscript{53} it has been employed to simultaneously glean information about the tumor vascular architecture and its functional hemodynamic status.\textsuperscript{54} More recently the feasibility of using steady-state USPIO-enhanced MRI for early quantitative monitoring of antiangiogenic and vascular disrupting therapy was demonstrated in a murine fibrosarcoma model\textsuperscript{55} and a rat prolactinoma model,\textsuperscript{56} respectively. One study employed the USPIO NC100150 [Clariscan; Amer sham Health] to assess the effects of overexpressing dimethylarginine dimethylaminohydrolase (DDAH), which metabolizes the endogenous inhibitors of NO synthesis of asymmetric dimethylarginine and N-monomethyl-L-arginine, on tumor vascular morphogenesis in a glioma model \textit{in vivo}.\textsuperscript{57} Another study utilized MION to assess the antiangiogenic effects of VEGF receptor tyrosine kinase inhibitors in a drug-resistant colon carcinoma model.\textsuperscript{58} USPIO enhanced MRI can be especially useful in delineating the ‘co-opted’ (i.e., recruitment of preexisting vessels) vascular phenotype exhibited by low grade gliomas which does not enhance with conventional gadolinium based DCE MRI.\textsuperscript{59} Such measurements have also been used to assess tumor response to patupilone, a potent microtubule stabilizer and vascular disrupting agent\textsuperscript{60} and more recently to interrogate the effects of tumor-derived platelet-derived growth factor (PDGF) on tumor angiogenesis.\textsuperscript{61} Several of these steady-state applications of USPIOs in animal models of disease are well summarized in Ref. 51.

**Vessel-Size Magnetic Resonance Imaging**

As mentioned above, the compartmentalization of a superparamagnetic contrast agent within the vasculature induces magnetic field perturbations that extend far into the tissue (Figure 1), increasing its relaxation rates ($R_1$ and $R_2$). Assuming a monoexponential signal delay this enhancement in relaxation rates ($\Delta R_2$ and $\Delta R_1$) is given by Eq. 4. Also, as pointed out above no analytical solutions exist for the intermediate exchange regime, wherein one must resort to Monte Carlo-type numerical simulations for predicting the effect on $\Delta R_2$ and $\Delta R_1$. These results are shown in Figure 2(b), which shows that $\Delta R_2$ increases and peaks at a vessel radius of $\sim 4\text{–}5 \mu m$, while $\Delta R_1$ increases and then plateaus to remain independent of vessel size for vessel radii $\sim 5\text{–}6 \mu m$.\textsuperscript{21} Since Boxerman et al. had demonstrated computationally that both $\Delta R_2$ and $\Delta R_1$ exhibit an almost linear response with the fractional vascular volume and $\Delta R_2$, Dennie et al. proposed taking the ratio $\Delta R_1/\Delta R_2$ as a means of minimizing this dependence of the relaxation rates on these usually unknown quantities.\textsuperscript{49} For a given SPIO dose, Dennie et al. demonstrated that this ratio $\Delta R_1/\Delta R_2$ increased linearly with vessel size and could be employed as a metric of the average vessel size within a
voxel, and also demonstrated that ‘vessel-size’ maps obtained in a rat glioma model using an intravascular superparamagnetic iron-oxide nanoparticle (MION) contrast agent compared favorably to the predicted ratio using histologically determined vessel sizes. An example of such a ratio map is shown in Figure 4. On the basis of the GE signal predicted by the Yablonskiy and Haacke model in conjunction with the SE signal predicted by the model proposed by Kiselev and Posse, Tropres et al. formulated an alternative expression for the vessel size index (R):\[ R = 0.425 \left( \frac{D}{\gamma \Delta \chi B_0} \right)^{1/2} \left( \frac{\Delta R_2}{\Delta R_2} \right)^{3/2} \] where absolute measurement of R requires measurement of the diffusion coefficient (D) and of the increase in blood susceptibility (\( \Delta \chi \)) after SPIO administration. Finally, on the basis of the same susceptibility models, Jensen and Chandra formulated the relaxation rate shift index (Q) that is independent of the contrast agent dose (above a certain threshold dose) and given by:\[ Q \equiv \frac{\Delta R_2}{(\Delta R_2)^{2/3}} \]

Wu et al. demonstrated the feasibility of imaging the mouse brain microvasculature using this approach, and were able to show significant differences between various cortical areas in agreement with the histologically assessed MVD. While there have been several clinical studies with SPIOs for a variety of targets and pathologies, clinical susceptibility based perfusion studies of tumors with SPIOs remain limited. With several different SPIOs in various stages of clinical trials, it is a matter of time before we see widespread application of susceptibility contrast enhanced MRI biomarkers in the clinical setting for a wide array of diseases exhibiting anomalous vasculatures, including cancer.

CAVEATS AND ISSUES
Finally, all DSC measurements are made assuming that the calibration factor ‘k’ (Equation 4) is the same for different tissue types and independent of tissue pathology. However, a recent study has shown that k was the same for brain gray and white matter but not the same for normal brain and tumor tissue for the GE pulse sequence (Figure 5). This difference was attributable to the grossly different vascular morphologies of tumors because.

**FIGURE 6** (a) Post-Gd MR image of a 9L gliosarcoma bearing rat brain illustrating the contralateral (C) and tumor (T) ROIs employed for MR relative cerebral blood volume (rCBV) histogram analyses. (b) rCBV map computed from the first-pass of monocrystalline iron oxide nanoparticle (MION), overlaid on a high-resolution anatomical image: note elevated rCBV in the tumor, a finding consistent with a larger fractional blood volume (FV) relative to the contralateral brain. (c) Post-Gd MR image illustrating the contralateral (C) and tumor (T) grids that were sampled for stereological analyses. H&E stained tissue section of vessels perfused with Microfil (a silicone injection compound) at 20× magnification of (d) tumor tissue and (e) normal brain. (f) A histogram of the GE rCBV data showing that the tumor rCBV is shifted to the right with respect to the contralateral rCBV, a difference that is also apparent from (g) the histogram of the stereologically calculated FV. (Reprinted, with permission, from Ref. 35. Copyright 2000 Springer Verlag).
of tumor angiogenesis, compared to normal brain and/or possibly differing blood rheological factors such as hematocrit. Consequently, the sensitivity to blood volume differences between tumor and normal brain tissue may be underestimated when using GE susceptibility contrast agent methods. Additionally, as summarized by Kiselev, susceptibility effects can be considered to operate over various spatial scales to induce relaxation,70 and range from the spin-spin interactions between the water protons and the contrast agent ions, that is, the molecular or microscopic scale, to the scale at which protons are sensitive to the geometry of the susceptibility induced magnetic field gradients, which in turn are defined by the microvascular architecture and/or hematocrit of the vessels, that is, the mesoscopic scale, and finally the scale at which there exist inhomogeneities in the magnetic field due to imperfect shimming and so on, that is the macroscopic scale. This dependence of contrast agent relaxivity on the microvascular architecture is especially relevant to the context of tumor angiogenesis wherein the aberrant vessel morphology can drastically affect the observed image contrast in DSC protocols42 (Figure 6). This makes it imperative to develop simulation methodologies and models that take the ‘de facto’ microvascular architecture into account, such as those proposed in Refs 71,72. Finally, for the steady-state imaging approaches, care must be taken to ensure that contrast agent is not in its saturated regime or that the relationship between the concentration of the contrast agent and the relaxation rate is linear so that Eq. 4 is still valid. For example, empirical data49 have demonstrated that this linear relation holds true for low doses of MION (up to ~ 5 mg Fe/kg) but becomes sublinear for higher doses.28,51

CONCLUSION
As a result of its exquisite sensitivity to the underlying vasculature, in vivo susceptibility based contrast MRI is proving to be an important new tool for non-invasively elucidating the vascular remodeling that accompanies tumor angiogenesis, vascular normalization and antiangiogenic therapy. Perfusion imaging with SPIO nanoparticles has been an indispensable tool in the characterization of these phenomena. However, it should be borne in mind that the mechanisms underlying this contrast mechanism are complex and still being elucidated. A better understanding of the complex interplay between the microvascular architecture and biophysics of the susceptibility induced MR signal will result in the development of physiologically more relevant biomarkers.

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MR relaxation properties of superparamagnetic iron oxide particles.
Optical nanoparticle sensors for quantitative intracellular imaging

Yong-Eun Koo Lee* and Raoul Kopelman*

Real-time measurements of biological/chemical/physical processes, with no interferences, are an ultimate goal for in vivo intracellular studies. To construct intracellular biosensors that meet such a goal, nanoparticle (NP) platforms seem to be most promising, because of their small size and excellent engineerability. This review describes the development of NP-based optical sensors and their intracellular applications. The sensor designs are classified into two types, based on the sensor structures regarding analyte receptor and signal transducer. Type 1 sensors, with a single component for both receptor and transducer, work by mechanisms similar to those of ‘molecular probes’. Type 2 sensors, with a separate component for receptor and transducer, work by different mechanisms that require the presence of specific NPs. A synergistic increase in optical signal or selectivity has been reported for these second type of NP sensors. With ongoing rapid advances in nanotechnology and instrumentation, these NP systems will soon be capable of sensing at the single-molecule level, at the point of interest within the living cell, and capable of simultaneously detecting multiple analytes and physical parameters.

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Intracellular imaging of the biochemistry and biophysics of live cells has been of prime interest for decades. Numerous approaches have been proposed to achieve real-time, noninvasive analysis of chemical and physical properties at unperturbed cellular physiological status. Significant progress in the visualization of biological processes has been achieved by the dramatic advances in imaging processing technologies with high-performance computer systems as well as by the continuous development of new analyte-specific fluorescent molecular probes. These molecular probes, however, have several drawbacks that limit the indicator dyes available for reliable intracellular measurements. The indicator molecules have to be in a cell-permeable form, which often requires properly derivatized indicator molecules. The measurement is often skewed by intracellular sequestration to specific organelles inside the cell, or by nonspecific binding to proteins and other cell components. The cytotoxicity of the available dyes is sometimes a problem, as the mere presence of these dye molecules may chemically perturb the cell. Furthermore, the dye is usually not ‘ratiometric’, i.e., has only a single spectral peak, which then requires technologically more demanding techniques, such as picosecond lifetime resolution or phase-sensitive detection. We note that just loading into the cell a separate reference dye, for ratiometric measurements, is not a solution, because of the aforementioned sequestration and nonspecific binding.

In an attempt to solve these problems, while maintaining minimal physical interference, a new type of sensor has been developed, utilizing nanoparticles (NPs) as platforms for immobilizing the sensor chemistry. The NP sensor is physically noninvasive owing to its small size. An NP sensor of 20–600 nm in diameter takes up only 1 ppm to 1 ppb of a mammalian cell’s volume.¹ There are also standard methods for delivery of NPs into cells, such as via a gene gun, pico-injection, liposome incorporation, or endocytosis. This prevents unnecessary modification (derivatization) of the indicator dyes. The inert protective matrix of the NPs eliminates interferences such as protein binding and/or membrane/organelle sequestration.² The NP matrix also obviates the toxicity problem by protecting the cellular contents from the indicator dyes and vice versa. The cell
viability after NP sensor delivery is about 99%, relative to control cells, indicating negligible physical and chemical perturbation to the cell. Moreover, a ratiometric sensor can be easily constructed by co-loading of the indicator and reference components within the NP matrix. We note that the NP sensors can also be attached with specific molecular targeting moieties, enabling the measurements of analytes at specific cells or organelles of the cells.

The NP has a high surface-to-volume ratio that allows high accessibility of analytes to the indicator dyes/receptors as well as targeting factors towards specific cells or components of cells. Each NP can be loaded with a high amount of components (single or multiple) within the NP matrix as well as on the surface. High loaded amounts of dyes in close proximity to each other either within the restricted NP volume or on the NP surface allow multiple interactions with the sensing components, resulting in signal amplification. It is noteworthy that similar amplification effects have been reported for targeting efficiency by NPs with multiple targeting moieties on the surface.

Since the first NP sensor called PEBBLE (Photonic Explorer for Biomedical use with Biologically Localized Embedding) was reported by Kopelman and colleagues, a number of possibilities have been proposed for immobilizing the sensor chemistry within various kinds of NP matrixes, so as to construct NP sensors for specific intracellular applications. It is also noted that optical detection has remains the most widely used method for sensing and imaging of biological systems.

This review focuses on the uses of synthetic NPs of 1–1000 nm in diameter for the design of optical sensors and their applications to intracellular measurements. It covers only untethered, that is free, NP sensors which are suitable for in situ measurements in three dimensions; it does not cover mechanically fixed sensors like fiber-tip or film on glass slide, even when they utilize NPs.

**OPTICAL NP SENSOR DESIGN**

The basic structure of a sensor requires two components: an analyte recognizer that binds the target analyte, and a transducer that signals binding.

**Optical Transduction Modality**

Fluorescence is a highly sensitive, specific means for monitoring cell activity, and a number of fluorescent reporters can be analyzed simultaneously. Fluorescence has been and will be a major transduction modality but has limiting factors such as photobleaching and interference due to autofluorescence from cellular components.

Surface-enhanced Raman scattering (SERS) is a recently evolving optical modality for intracellular NP sensors and is complementary to fluorescence. It was found that the SERS effect is provided by a very small number of molecules located at special sites in the gap between two nearly touching gold nanocrystals. Because of the high specificity of a Raman spectrum, minute amounts of chemicals inside living cells might be identified by their unique fingerprint spectra. SERS requires the so-called ‘SERS-active substrates’ such as nanometer-sized silver or gold structures, which target molecules that get attached to them. Surface plasmon resonance (SPR) is another metal NP–based optical transducer that draws much interest in biological detection, including immunoassay. SERS and SPR are free from photobleaching and self-quenching of the marker molecule. However, their sensitivity/reproducibility still needs validation.

**NP Matrix**

NP matrices should exhibit excellent chemical stability and biocompatibility. A variety of NP matrices have been utilized for the design of optical nanosensors, as listed in Table 1.

Polymeric NPs of various matrices and sizes with surface-located reactive functional groups can be prepared by various synthetic methods. The sensor components can be loaded into the NP matrix by various methods, including encapsulation, covalent linkage, physical adsorption, etc. The matrix for polymeric NP-based sensors is selected by the accessibility of an analyte to a recognition element and the loading efficiency, within NP matrix, of indicator/receptor and signal transducer.

Liposomes or micelles present limited utility for biological sensing within the membrane-rich cellular environments, as they tend to mix with the native cell membranes, degrading the sensor structure. However, polymer-capped stabilized liposomes or micelles have been utilized for designing sensors for intracellular measurements.

Semiconductor quantum dots (QDs) are brighter and more stable against photobleaching than organic fluorophores, allowing real-time and continuous monitoring. A study shows that the fluorescence emission of QDs remains bright and stable inside cells for at least 14 days. The biosensing applications of QDs are usually based on fluorescence resonance energy transfer (FRET).
TABLE 1 | Matrices for Optical NP Sensors

<table>
<thead>
<tr>
<th>NP type</th>
<th>Optical transduction modality</th>
<th>Matrix</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymeric NP</td>
<td>Fluorescence</td>
<td>Poly(acrylamide)</td>
<td>3,8,14–20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(decylmethacrylate)</td>
<td>21–24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(ethylene glycol)</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(methacrylate)</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(n-butyloacylate)</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polystyrene</td>
<td>28–30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dendrimer</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Latex</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Organically modified silica</td>
<td>33,34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica</td>
<td>35–39</td>
</tr>
<tr>
<td>Polymerized liposome</td>
<td>Fluorescence</td>
<td>1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposome with polymethacrylate shell</td>
<td>40</td>
</tr>
<tr>
<td>Polymerized micelle</td>
<td>Fluorescence</td>
<td>Silane-capped (polymerized) mixed micelle</td>
<td>41</td>
</tr>
<tr>
<td>Quantum dot</td>
<td>Fluorescence</td>
<td>CdS</td>
<td>42,43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZnS-coated CdSe</td>
<td>44–46</td>
</tr>
<tr>
<td>Metal</td>
<td>SERS, fluorescence</td>
<td>Gold</td>
<td>47–49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SERS</td>
<td>50</td>
</tr>
<tr>
<td>Metal/polymer hybrid</td>
<td>SERS, SPR</td>
<td>Gold nanoshell over silica</td>
<td>51,52</td>
</tr>
</tbody>
</table>

NP, nanoparticles; SERS, surface-enhanced Raman scattering; SPR, surface plasmon resonance

In a metallic or metal-coated polymer NP, incident light can couple to the plasmon excitation of the metal. This leads to enhanced optical detection schemes utilizing SERS and plasmon resonance. These metal (gold, silver, or gold-coated silver) NPs have been utilized to detect a wide range of biological molecules through binding events involving interactions with surface-coated specific molecules that offer distinct SERS and SPR. Gold NPs have also been utilized to construct an optical biosensor for DNAs.

Sensor Classification

The NP optical sensors that have been developed so far for intracellular measurements can be classified into two types (see Figure 1): (1) Type 1 sensor where the incorporated single component, usually fluorescent molecular probe, serves as an analyte recognizer as well as an optical signal transducer; (2) Type 2 sensor where the analyte recognizer and optical transducer are distinct. Type 2 sensors enable a synergistic signal and selectivity enhancement as well as sensitivity control that cannot be achieved with free molecular probes.

In a Type 1 sensor, fluorescent or Raman-active dyes are either encapsulated in or covalently linked to polymeric or metallic NPs. Upon binding with the analyte, the spectral change (fluorescence quenching/enhancement, fluorescence lifetime change or fluorescence peak shift, SERS) of the indicator dyes occurs. The sensitivity and selectivity of the sensor mostly depend on the incorporated indicator dye but are also affected by the NP matrix.

In a Type 2 sensor, nonfluorescent selective analyte-recognition elements or receptors (enzymes, antibodies, ligands, or aptamers) are either encapsulated in or covalently linked to the polymeric or metallic NPs. Binding of a specific analyte to the receptors produces an effect on the optical reporters that consist of co-loaded fluorescent dyes or the NP themselves (as for QDs or metallic NPs).

Both Type 1 and 2 sensors have been developed to detect a variety of intracellular analytes, as exemplified in the following sections.

NP SENSORS FOR ION SENSING

Type 1 Sensors

Fluorescent Sensors

Type 1 fluorescent sensors, also called direct ion measurement PEBBLEs, have been developed for sensing H⁺, Ca²⁺, Mg²⁺, Zn²⁺, Cu¹⁺/²⁺, and...
Optical nanoparticle sensors for quantitative intracellular imaging

Fe$^{3+}$.$^{3,8,14–18,61,62}$ The design includes a fluorescent indicator and a reference dye entrapped in or covalently linked to an NP. The polyacrylamide NP has been used exclusively for this type of ion sensor because of its neutral and hydrophilic nature, which allows ions to readily permeate the NP matrix and interact with the indicator dye. The indicator dyes are mostly fluorescent molecular probes, but analyte-sensitive biological molecules, such as a red fluorescent protein, have also been used.$^{17}$ Type 1 ion PEBBLEs have been applied successfully for intracellular measurements of pH, Mg$^{2+}$, and Ca$^{2+}$. As an example, ratiometric calcium nano-PEBBLEs, containing the ‘Calcium Green- 1’ (‘Molecular Probes’) dye as sensing component and the sulforhodamine dye as reference, have been used to measure calcium release from mitochondria upon introduction of toxins.$^{63}$ Figure 2$^{64}$ shows a confocal microscope image of C6 glioma cells containing these PEBBLEs, after their selective delivery by liposomes (to the cytosol only). The sulforhodamine fluorescence is red (reference peak) in the image, while that of Calcium Green is yellow/green. The ratio of the Calcium Green/sulforhodamine intensity gives a good indication of cellular (cytosolar) calcium levels, regardless of dye or PEBBLE concentration, or fluctuations in light source intensity. The toxin, m-dinitrobenzene (DNB), was introduced on the left side of the sample (microscope slide) and allowed to diffuse to the right. The effect of DNB is a severe disruption of the mitochondrial function, followed by uncontrolled release of calcium (onset of a mitochondrial permeability transition). This caused calcium PEBBLEs inside the cytosol of different cells to ‘light up’ from left to right as a function of time. As a result, high resolution in both the spatial and temporal domains was obtained.

Another interesting intracellular application of Type 1 PEBBLE sensors was made with the Mg$^{2+}$ PEBBLEs, to study the chemical changes induced inside human macrophage cells by invading salmonella bacteria.$^{65}$ The Mg$^{2+}$ measurements by the PEBBLE sensors showed conclusively that Mg$^{2+}$ is not an important contributor in the control of pathogens by macrophages, in contradiction to previous reports.$^{66}$

A different kind of direct ion NP sensor was designed using additional layers of polyelectrolytes on the surface of NPs for immobilizing indicator dyes.$^{67}$ In this work, the potassium ion indicator, potassium-binding benzofuran isophthalate, was immobilized within poly(styrene sulfonate)/poly(allylamine hydrochloride) films assembled on the surface of fluorescent europium NPs. The fluorescence from the (commercial) core nanoparticle serves as reference for a ratiometric measurement. The indicator retains its sensitivity to potassium ions after immobilization within

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**Figure 1** | Schematic presentation of two kinds of nanoparticles (NP) sensors: (a) Type 1 where a single component serves as receptor and transducer; (b) Type 2 where receptor and transducer are separated but they communicate in order to produce optical signal change upon binding.

**Figure 2** | Confocal microscope image (time snapshot) of three human C6 glioma cells that contain Calcium Green/sulforhodamine Photonic Explorer for Biomedical use with Biologically Localized Embeddings (PEBBLEs) (with m–dinitrobenzene (DNB) toxin diffusing from left to right). (Reprinted, with permission, from Ref. 64. Copyright 2003 Taylor & Francis Group).
the films and exhibits sensitivity toward increases in potassium concentration over a broad range.

**SERS Sensors**

An SERS pH sensor was developed with silver NPs (50–80 nm in diameter) functionalized with para-mercaptobenzoic acid (4-MBA).50 The SERS spectrum from the functionalized silver NPs shows a characteristic response to the pH 6–8 of the surrounding solution. There was a large variability in the measured pH, as the SERS spectrum was observed only from aggregated particle clusters. These sensors were delivered into living Chinese Hamster Ovary (CHO) cells by passive uptake. The NP sensors retained their robust signal and sensitivity to pH within a cell. The spectrum indicates that the pH surrounding the NP is below 6, which is consistent with the particles being located inside a lysosome (pH 5).

A similar SERS pH sensor was designed on the basis of a gold nanoshell/silica core NP coated with a layer of para-MBA.52 The nanosensor was capable of measuring pH in its local vicinity continuously over the range of 5.80–7.60 pH units.

**Type 2 Sensors**

Type 2 NP sensors for cobalt, copper, hydrogen, nickel, potassium, silver, sodium, zinc, and chloride ions have been developed.

NP sensors called ion-correlation PEBBLEs have been developed for Na\(^{+}\), K\(^{+}\), and Cl\(^{-}\) ions.21–23 The sensor is made of poly(decylmethacrylate) (PDMA) NPs embedded with three components: a nonfluorescent ionophore that binds selectively to the ion of interest, a fluorescent hydrogen ion-selective dye that plays the role of a reporter, and a lipophilic additive that maintains ionic strength. The operation of the entire system is based on having a thermodynamic equilibrium that controls ion exchange (for sensing cations) or ion co-extraction (for sensing anions), i.e. an equilibrium-based correlation between different ion species. The degree of protonation measured from the fluorescence change of the hydrogen ion-selective dye is related to the concentration of the analyte ion by the theory developed for optical absorption–based ion-correlation sensors.68,69 The hydrophobic PDMA matrix is selected to ensure a local chemical equilibrium among embedded components within NPs in the aqueous phase. The composition of the matrix, i.e. the cross-linker-to-monomer ratio, was found to affect the dynamic response range. Intracellular measurements of K\(^{+}\) and Cl\(^{-}\) ions were made by this type of PEBBLE sensors. The PDMA K\(^{+}\) PEBBLE sensors, as an example, were introduced into rat C6 glioma cells using a BioRad (Hercules, CA) Biologic PDS-1000/He gene gun system.21 The confocal images confirmed that the sensors were localized in the cytoplasm of the cells. The response of the PEBBLE sensors inside the cells to the addition of kainic acid, a K\(^{+}\)-channel-opening agonist, indicated an increase in K\(^{+}\) concentration, the expected trend. Another K\(^{+}\) NP sensor based on the same mechanism was designed with a different matrix, i.e. a poly(n-butyl acrylate) (PnBA) nano sphere of less than 200 nm in diameter.27 This study shows that the composition of the three sensor components (ionophore, hydrogen-sensitive dye, and lipophilic additive) affects the characteristics of the sensors, such as its dynamic range, selectivity, and response time. It should be noted that the selectivity of these two Type 2 K\(^{+}\) NP sensors23,27 is higher than that of Type 1 K\(^{+}\) NP sensor67 by a factor of 1000–10,000.

Several FRET-based Type 2 ion NP sensors have been designed.

A Type 2 pH nanosensor was developed by coating a pH-insensitive fluorescent polystyrene bead (200 nm in diameter) with a layer of polyaniline (PANI) of only a few nanometers thick.28 Plain PANI films display no fluorescence in the visible and near-IR range, but they do display characteristic pH-dependent absorption spectra that are due to protonation and deprotonation, respectively, of the emeraldine form of the PANI. Because of the fluorescence spectra of the beads being overlapped with the absorption spectra of PANI, the fluorescence intensity changes in accordance with the changes in pH.

Silica NPs have been utilized for two different designs of Type 2 fluorescence sensors for copper ions. In one design, the surface of silica NPs was covalently linked with a picolinamide subunit (selective Cu\(^{2+}\) ligand) and fluorescent dansylamide.36–38 The grafting of the ligand and the dye subunits to the NP’s surface not only ensures the intercomponent communication in the sensor but also induces cooperative processes in the binding of the substrate. The sensitivity of the sensors was tuned by changing the ligand-to-dye ratio. In another silica-based design, silica NPs were prepared from a monomer containing chemosensor-like unit (similar to a molecular probe) made by coupling polyamine chains (receptor) and dansyl units (fluorophore).5 These sensors may be classified as Type 1.5 sensors. The sensors were selective for copper, cobalt, and nickel ions and showed a greatly improved sensitivity from the occurrence of multicomponent cooperative photophysical processes.

Another Type 2 copper ion sensor was developed utilizing latex NPs. The hydrophobic fluorophore (BODIPY) is entrapped within the particle core, and the copper-chelating receptor (cyclam) is covalently
linked to the polymer backbone. The fluorescence of the dye is quenched upon binding Cu²⁺ to cyclam because of FRET between the dye and copper cyclam complexes. The response of the sensors is fast, with 90% quenching within 1 s.⁴²,⁷⁰

QDs have also been used for designing ion nanosensors. The ligands coated on the surface of QDs were found to have a profound effect on the luminescence response of QDs to physiologically important metal cations. l-Cysteine- and thioglycerol-capped CdS QDs were used to detect zinc and copper ions in physiological buffer samples, respectively. The detection limits were 0.8 µM for zinc (II) and 0.1 µM for copper (II) ions.⁴² Pentapeptide Gly-His-Leu-Leu-Cys-coated CdS QDs (2.4 ± 1.5 nm by transmission electron microscopy (TEM)) were designed to detect Cu²⁺ and Ag⁺ selectively, with high sensitivity, below 0.5 µM.⁴³

It is noted that the copper ion nanosensor has been the most studied among Type 2 NP sensors. Table 2 compares Type 1 and 2 NP sensors for copper ion that have been developed so far. It should be noted that, none of them has been applied for intracellular studies because the dynamic ranges of the developed sensors are above the normal unbound copper ion level, which is only femtomolar.⁷¹,⁷² These sensors may be applied for cells under stressed conditions that could increase the free copper ion concentration to micromolar levels.⁷³ In order to study the copper ion homeostasis under normal conditions, a sensor with higher sensitivity needs to be developed.

**NP SENSORS FOR SMALL MOLECULES**

**Dissolved Oxygen Sensor**

All the NP sensors that have been developed for detecting dissolved oxygen belong to Type 1. The first NP sensor for dissolved oxygen was developed using hydrophilic silica NPs paired with ruthenium indicator dyes, and reference dyes. The sensor was used successfully for the reliable oxygen imaging done inside live cells. NP sensors with enhanced sensitivity and dynamic range were developed using the more sensitive platinum-based oxygen-sensitive dyes and reference dyes, embedded in a hydrophobic matrix, organically modified silica (ormosil), or PDMA.⁴⁴ The hydrophobic matrix is usually better suited for oxygen sensing than the hydrophilic one because of its higher oxygen solubility. The embedded platinum (II) octaethylporphine ketone, an oxygen-sensitive dye, has infrared (IR) fluorescence and makes the sensors work in human plasma samples, unaffected by light scattering and autofluorescence. These PEBBLE nanosensors exhibit a perfectly linear Stern–Volmer calibration curve over the entire range of dissolved oxygen concentration, an ideal but previously unachieved goal for any fluorescent oxygen sensors. The sensitivity was very high with Q.DO of 97–97.5% is the quenching response to dissolved oxygen, defined by

$$Q_{DO} = \frac{(I_{N2} - I_{O2})}{I_{N2}} \times 100$$

where $I_{N2}$ is the fluorescence intensity of the indicator dye or the indicator/reference intensity ratio in fully deoxygenated water, and $I_{O2}$ is that in fully oxygenated water.

These oxygen nanosensors were also successfully applied for real-time imaging of oxygen inside live cells, monitoring metabolic changes inside live C6 Glioma cells.³³

Oxygen sensors with additional layers of polyelectrolytes on the NP surface have been developed. The polyelectrolyte layers are used either to control the dye loading or to systematically assemble

<table>
<thead>
<tr>
<th>Sensor type</th>
<th>Matrix</th>
<th>NP size (nm)</th>
<th>Recognition component</th>
<th>Signal producer</th>
<th>Optical signal</th>
<th>Detectable range</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Poly (acrylamide)</td>
<td>85</td>
<td>DsRed</td>
<td>DsRed</td>
<td>Fluorescence</td>
<td>200–5000 nM</td>
<td>17</td>
</tr>
<tr>
<td>Type 2</td>
<td>Silica</td>
<td>18–75</td>
<td>Picolinamide</td>
<td>Dansylamide</td>
<td>Fluorescence</td>
<td>4.7–200 µM</td>
<td>36–38</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>30</td>
<td>Polyamine</td>
<td>Dansyl unit</td>
<td>Fluorescence</td>
<td>50–1000 µM</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Latex</td>
<td>16</td>
<td>Cyclam</td>
<td>BODIPY derivative</td>
<td>Fluorescence</td>
<td>1 nM–5 µM</td>
<td>32,70</td>
</tr>
<tr>
<td></td>
<td>CdS QD</td>
<td>3.5</td>
<td>Thioglycerol</td>
<td>QD</td>
<td>Fluorescence</td>
<td>0.1–1600 µM</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>CdS QD</td>
<td>2.4</td>
<td>Peptide (Gly-His-Leu-Leu-Cys)</td>
<td>QD</td>
<td>Fluorescence</td>
<td>100 nM–2 µM</td>
<td>43</td>
</tr>
</tbody>
</table>

*Type 1 NP sensor based on DsRed is designed to sense both Cu²⁺ and Cu³⁺, while all Type 2 NP sensors are made for Cu²⁺ only. NP, nanoparticles; QD, quantum dots*
the sensors on the cell membranes. In one realization, commercial fluorospheres (100 nm) are coated with a multilayer of polyelectrolytes via layer-by-layer self-assembly, and then a ruthenium-based oxygen-sensitive fluorophore, (tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II), is post-loaded within the deposited polyelectrolyte multilayers. The fluorescent NPs act as physical scaffolds and provide a reference peak for a ratiometric measurement. The sensitivity was medium level, with QDO sensitivity was medium level, with QDO of 60%.

The sensors were successfully delivered to the interior of human dermal fibroblasts via endocytosis with no apparent loss in cell viability. In another design, the same ruthenium-based dye is entrapped in commercial polystyrene beads of 100 nm in diameter, and poly(ethyleneimine) (PEI) is covalently linked to the NP surface via glutaraldehyde chemistry. These nanosensors were assembled on individual Saccharomyces cerevisiae cells via electrostatic interactions between the positively charged PEI and negatively charged cell surfaces. This work demonstrates a proof of concept for self-assembly of nanosensors onto individual cell surfaces in a controlled manner for noninvasive examination of the oxygen concentration in the proximity of individual yeast cells.

Oxygen nanosensors were also developed on the basis of a nanometer-sized, polymerized phospholipid vesicle (liposome). The liposomes of 150 nm diameter were prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or DOPC doped with small (<1%) mole percentages of 1,2-dioleoyl-sn-glycero-3-phosphoethanol amine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-PE). These vesicles were then stabilized via a cross-linking polymerization of hydrophobic methacrylate monomers, partitioned into the hydrophobic interior of the DOPC bilayer. For oxygen detection, a ruthenium-based dye was encapsulated into the aqueous interior of the polymerized liposome. NBD-PE was used as a reference dye for ratiometric measurements. The Stern–Vomer plot provides a straight line over the entire dissolved oxygen range and the QDO is 76%.

The oxygen NP sensors described above have all utilized fluorescence intensity for measurements. Lifetime measurement based oxygen sensors were also constructed by encapsulating Pt(II)-tetrapentafluorophenylporphyrin (PtPFP) in polystyrene beads of 0.3–1µm in diameter. The sensors were injected into plant cells using glass microcapillaries, and an optical multifrequency phase-modulation technique was used to discriminate the sensor signal from the strong autofluorescence of the plant tissue. The same sensors were injected into the salivary glands of the blowfly to quantify the changes in oxygen content within individual gland tubules during hormone-induced secretory activity.

The NP sensors for dissolved oxygen are summarized in Table 3.

### NP Sensors for Reactive Oxygen Species

NP sensors have been developed for two molecular reactive oxygen species (ROS) (singlet oxygen and hydrogen peroxide) and one radical ROS (hydroxyl radical). These sensors were designed to show irreversible responses towards ROS, due to high reactivities and short lifetimes of the ROS.

#### Singlet Oxygen Sensor

Ratiometric NP sensors for singlet oxygen have been developed using ormosil NPs. These sensors incorporate the singlet oxygen-sensitive 9,10-dimethyl anthracene as an indicator dye and a singlet oxygen-insensitive dye, octaethylporphine, as a reference dye for ratiometric fluorescence-based analysis. The encapsulation of these dyes into the hydrophobic ormosil matrix results in a higher specificity toward singlet oxygen, as the matrix blocks the entry of short-lived polar ROS, such as OH and superoxide radicals. These nanoprobes have been used to monitor the singlet oxygen produced by ‘dynamic nanoplatforms’ that were developed for photodynamic therapy.

#### OH Radical Sensors

The hydroxyl radical is the most reactive ROS, presenting two problems for the construction of sensors: (1) inability to penetrate significantly into any matrix without being destroyed; (2) ability to oxidize (and photobleach) most potential reference dyes. A sensor was designed to get around these problems by attaching the hydroxyl indicator dye coumarin-3-carboxylic acid (CCA) onto the NP surface, while encapsulating the reference dye deep inside it. The detection of this probe was based on the irreversible hydroxylation of a nonfluorescent form of CCA, resulting in a fluorescent product (7-hydroxycoumarin-3-carboxylic acid). This nanoprobe demonstrates a proof of principle of a ratiometric hydroxyl radical probe, with good sensitivity and reversibility.

#### Hydrogen Peroxide Sensor

A poly(ethylene glycol) (PEG) hydrogel nanosphere (250–350 nm) with the encapsulated enzyme horseradish peroxide (HRP) was prepared and utilized as a sensor for hydrogen peroxide, based on the Amplex Red assay. In the presence of HRP, Amplex Red (10-acetyl-3,7-dihydroxyphenoxazin)
reacts with $\text{H}_2\text{O}_2$, in a 1:1 stoichiometry, to produce the red fluorescent oxidation product, resorufin. The response of the HRP-loaded PEG NPs changed as a function of $\text{H}_2\text{O}_2$ concentration in the presence of externally introduced Amplex Red, indicating that the enzyme activity of HRP was still maintained within the NPs. The HRP-loaded NPs were introduced via phagocytosis inside macrophages and were found to respond to exogenous $\text{H}_2\text{O}_2$ ($100 \mu\text{m}$) as well as endogenous peroxide induced by lipopolysaccharide (1 $\mu\text{g/mL}$).

**Glucose Sensor**

A poly(acrylamide) NP-based fluorescent glucose sensor was developed by incorporating glucose oxidase (GOx), an oxygen-sensitive ruthenium-based dye, and a reference dye.\(^{19}\) This is a Type 2 sensor in which the enzymatic oxidation of glucose to gluconic acid results in the local depletion of oxygen, which is measured by the oxygen-sensitive dye. It should be noted that the traditional ‘naked’ molecular probes cannot be used to achieve this kind of synergistic task. The dynamic range was found to be $\sim 0.3$–$8 \text{mM}$, with a linear range between 0.3 and 5 $\text{mM}$.

**Maltose Sensor**

Three different designs of QD-based maltose sensors have been reported with maltose-binding proteins (MBPs) as maltose receptors. Two of them utilize the $\beta$-cycloextrin-acceptor dye conjugates that are capable of binding within the saccharide-binding pocket of MBP and thus compete effectively with maltose, the MBP’s preferred substrate.\(^{44}\) In one configuration, a $\beta$-cycloextrin–QSY9 conjugate is bound to an MBP located on the QD surface, resulting in FRET quenching of the QD photoluminescence. Added maltose displaces the $\beta$-cycloextrin–QSY9, and the QD photoluminescence increases in a systematic manner. In another configuration, QDs were coupled with Cy3-labeled MBPs bound to $\beta$-cycloextrin-Cy3.5. In this case, the QD donor drives the sensor function through a two-step FRET mechanism that overcomes inherent QD donor–acceptor distance limitations. A ratiometric measurement was made on the basis of the emission peaks of Cy3 and Cy 3.5. In these two designs, the loss of displaceable quenchers may cause errors. A QD–MBP-based maltose sensor was developed without quencher molecules.\(^{45,46}\) In this design,
The cells were first incubated with either 0
positive) and UMSCC-38 cell (folate receptor negative).

nanosensors were applied for apoptosis measurements
in the range from 2
response to the analyte in the range from 2
the probe showed good selectivity and had a linear
sensors have higher photostability and lower toxicity
in comparison with free AEC. The results revealed that
sensors can measure the intracellular activities or analytes in the specific location selected by the
targeting moieties linked to the NP surface.

Metronidazole Sensor
A Type 1 nanosensor for detecting metronidazole, a drug for the treatment of anaerobic protozoan
and bacterium infections, was developed by covalent immobilization of indicator dye, 3-amino-9-
ethylcarbazole (AEC), in poly(methacrylate) NP of the size less than 100 nm in diameter.26 The obtained
sensors have higher photostability and lower toxicity in comparison with free AEC. The results revealed that
the probe showed good selectivity and had a linear response to the analyte in the range from 2.0 × 10⁻³
to 1.0 × 10⁻³ mol L⁻¹ with a detection limit of
9.0 × 10⁻⁵ mol L⁻¹

NP SENSORS FOR LARGE BIOLOGICAL MOLECULES
NP sensors have been used for detecting large biological molecules such as DNAs and proteins. The basic
design is composed of NPs functionalized with receptors (antibodies,52 DNAs,48 or aptamers77) for target-
specific detection. The analysis was done with various approaches including optical methods such as fluores-
cence, SERS, and SPR. These sensors have been developed for diagnostic assay, i.e. laboratory measure-
ments of the analytes in biological samples like blood, which is not really relevant for this review article focused on direct intracellular measurements. Readers interested in these sensors are referred to the literature
for reviews on NP-based diagnostic assay.49,78–80

NP SENSORS FOR CELLULAR ACTIVITY
Apoptosis Sensor
A nanosensor for detecting apoptosis of cells was developed by conjugating a caspase-specific FRET-
based apoptosis reagent (PhiPhiLux G1D2) to the G5 poly(amidoamine) (PAMAM) dendrimer for apopto-
sis detection and folic acid for specific targeting.33 The nanosensors were applied for apoptosis measurements in two different cell lines: KB cell (folate receptor positive) and UMSCC-38 cell (folate receptor negative). The cells were first incubated with either 0.45µM NP
sensors or phosphate-buffered saline (PBS) (untreated cells) for 30 min, added with either the apoptosis-
inducing agent staurosporine at a concentration of 0.5µM or PBS (control), and incubated again for an
additional 3 h. The apoptosis was observed on the basis of the fluorescence of the detached cells using a
flow cytometer. The cell death by apoptosis was not monitored. The apoptotic KB cells increased the fluo-
rescence intensity to a much greater degree, while the apoptotic UMSCC-38 cells did not show any increase in fluorescence intensity over the background fluorescence of stained control cells. These results suggest that the sensor can measure the intracellular activities or analytes in the specific location selected by the targeting moieties linked to the NP surface.

NP Sensor for Lipid Peroxidation
A nanosensor for detecting lipid peroxidation by chemiluminescence was designed by conjugating
Coumarin C343 (C₁₆H₁₅NO₄) to silica NPs (15 nm) and then entrapping these dye-linked silica in a sol-gel silica NP (~ 100 nm).39 Coumarine C343 is known to enhance the weak chemiluminescence associated with lipid peroxidation. The produced nanosensor enhanced low-level chemiluminescence by approximately 100%.

NP SENSORS FOR INTRACELLULAR PHYSICAL PROPERTIES
NP Sensors for Electric Field
Intracellular electric fields have been measured by voltage dyes or patch/voltage clamps. These tech-
niques frequently require lengthy calibration steps for each cell or cell type measured, and the measurements
are confined to cellular membranes. A nanodevice to determine electric field inside any live cell or cellular
compartment, called E-PEBBLE, was developed using polymerized micelles.41 The E-PEBBLE is prepared by encasing the fast-response, voltage-sensitive dye di-4-ANEPPS inside the hydrophobic core of a silane-capped (polymerized) mixed micelle, which provides a uniform environment for the molecules and therefore allows for universal calibration.

The E-PEBBLEs are calibrated externally and applied for in vitro E-field determinations, with no further calibration steps. The PEBBLEs were introduced into immortalized rat astrocytes, DITNC cells, by endocytosis and enabled, for the first time, complete three-dimensional electric field profiling throughout the entire volume of living cells (not just inside membranes). This new ability is expected to greatly enhance the understanding of the role
of cellular E-fields in influencing and/or regulating biological processes, with wider implications for cellular biology, biophysics, and biochemistry.

**NP Sensor for Local Viscosity Measurements**

A new type of sensors for local viscosity measurements has been developed with so called ‘MOONs’ (MOdulated Optical Nanoprobes). The MOONs are half-metal-capped fluorescent NPs whose fluorescence signals can be modulated according to their orientations, as the metal-coated side reflects the excitation light. The Brownian rotation or the rotational behaviors of the MOONs under an external magnetic field have been utilized to measure the local viscosity, which affects the rotation rate of the MOONs.81,82 We note that the same rotational behavior of MOONs also allows the sensor’s signal-to-noise (background) ratios (SNR) to be enhanced by up to 4000 times.83 So far, MOON-based sensors have been developed using a micron size particle owing to the size-related difficulties for efficient magnetization or high fluorescent intensity of individual sensor particle. With recent progress on nanotechnology and coating technology, such as molecular beam epitaxy, nanometer-sized MOONs are being developed. This sensor design is quite attractive, as adding a metal coating on one hemisphere of any NP sensor containing a fluorescent indicator allows the simultaneous measurement of the local viscosity as well as the concentration of a chemical analyte. It also increases tremendously the SNR of the chemical sensing part.

**CONCLUSION**

A variety of NP-based optical sensors have been developed in concurrence with advances in nanomaterials. These sensors provide minimal physical as well as chemical interferences owing to the combination of their small size and their protective NP matrix, or surface coatings. Some, but not all of these NP sensors have been successfully utilized for real-time measurements of important intracellular analytes. It has been reported that single-cell analysis has the potential for diagnosing diseases at an early stage, at which changes on a tissue level are not yet evident but chemical changes within cells are observable.84 Getting chemical or physical information from a single cell or a specific location within a single cell would be one of the important future applications of NP sensors. The following issues must be considered in order to improve the performance of the NP sensors for wider and more effective future intracellular applications:

**Sensitivity and Signal-to-Noise**

The goal will eventually be to enable single analyte molecule (ion) detection, in a single cell, in vitro or in vivo, despite the large background. Owing to the limited numbers of analyte molecules (ions) within a small volume single cell, instrumentation and sensing technology must meet stringent detection limits. One of the promising future sensor designs for enhanced sensitivity may be based on ‘MOONs’ that provide a background-free detection. This technique can be useful for samples with highly scattering and/or fluorescent backgrounds, or for experiments with several fluorescent probes.

**Selectivity**

The selectivity of the sensors toward the analytes of interest is mainly determined by that of the molecular probes or receptors. A higher level of selectivity can be obtained by locating the NP sensors at a specific location in a live cell, either through molecular targeting groups conjugated to the NP surface, or through remote steering means such as magnetic or laser tweezers. A recent study demonstrates the potential use of magnetic tweezers for remote control of the orientation and position of the NP sensors.85

**Multiplexing Capability**

Detection of multiple analytes can be made possible by a properly designed single NP sensor. An NP sensor containing multiple molecular probes or receptors that are specific to different analytes could be, for example, one in which the various optical signals are well resolved. The MOON-based fluorescent NP sensors provide another example of multitasking sensors that can measure the chemical property simultaneously with a physical property, such as local temperature or viscosity.73,74,81,82 A third example could be given in which confocal microscopy resolves the individual signals from a number of cell-embedded nanosensors.

**NOTES**

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Synthesis of poly(alkyl cyanoacrylate)-based colloidal nanomedicines

Julien Nicolas* and Patrick Couvreur

Nanoparticles developed from poly(alkyl cyanoacrylate) (PACA) biodegradable polymers have opened new and exciting perspectives in the field of drug delivery due to their nearly ideal characteristics as drug carriers in connection with biomedical applications. Thanks to the direct implication of organic chemistry, polymer science and physicochemistry, multiple PACA nanoparticles with different features can be obtained: nanospheres and nanocapsules (either oil- or water-containing) as well as long-circulating and ligand-decorated nanoparticles. This review aims at emphasizing the synthetic standpoint of all these nanoparticles by describing the important aspects of alkyl cyanoacrylate chemistry as well as the experimental procedures and the different techniques involved for the preparation of the corresponding colloidal devices.


Nanotechnology has emerged as a promising area of research in which scientists from both academia and industry put a lot of effort, hoping for the best with regard to life in future. It is a highly multidisciplinary field which consists of engineering functional systems at the molecular scale and covers applied physics, materials science, interface and colloid science, supramolecular chemistry as well as chemical, mechanical, and electrical engineering. One of the direct applications of nanotechnology is devoted to the medical and pharmacology areas, also called nanomedicine, the most famous example being nanoparticle drug delivery.

Indeed, a crucial impulse was given to nanomedicine with the development of various types of drug-carrier nanodevices, made possible by means of multidisciplinary approaches—organic and polymer chemistry, physicochemistry, pharmacology, etc. Among suitable nanodevices for drug delivery, nanoparticles on the basis of biodegradable poly(alkyl cyanoacrylate) (PACA) polymers have appeared as an established technology for colloidal nanomedicine.

Introduced more than 25 years ago in the field of pharmacology, PACA drug carriers have demonstrated significant results in numerous pathologies such as cancer, severe infections (viral, bacteriologic, parasite) as well as several metabolic and autoimmune diseases, well-reviewed in the recent literature.1–6 As a complementary work, the objective of the present review is to emphasize the synthetic aspect of these colloidal carriers by describing, as precisely as possible, the chemistry of the cyanoacrylate monomers, their polymerization as well as the different structures and morphologies of the corresponding nanoparticles. In particular, description of this PACA-based nanotechnology will start from the simplest nanocarriers to more sophisticated and ‘smart’ drug delivery devices. The reader who would like a more exhaustive point of view about the biologic and pharmaceutical aspects of PACA nanoparticles as well as the drugs successfully incorporated in such colloidal devices is referred to the above-mentioned references.

ALKYL CYANOACRYLATE MONOMERS AND RELATED POLYMERS

General Features of Alkyl Cyanoacrylate Monomers

Alkyl cyanoacrylates are widely known monomers, extremely appreciated for their very high reactivity.
and the excellent adhesive properties of the resulting polymers. On one hand, the famous Superglue (manufactured by Henkel), which contains short alkyl chain cyanoacrylates, is commonly employed by the general public for repairing and do-it-yourself activities, whereas longer alkyl chain cyanoacrylates have been developed for biomedical purposes such as surgical glue for the closure of skin wounds\textsuperscript{7–13} and embolitic material for endovascular surgery.\textsuperscript{10,11,14}

Indeed, several commercial products have emerged from the use of cyanoacrylates in the biomedical area, mainly devoted to tissue adhesion. For instance, methyl cyanoacrylate (MCA, Figure 1) is the main component of the Biobond tissue adhesive and longer alkyl ester chain cyanoacrylates, such as \textit{n}-butyl cyanoacrylate (\textit{n}-BCA, Figure 1) or octyl cyanoacrylate (OCA, Figure 1), were commercialized under the trademarks of Indermil, Liquidband, and Dermabond, respectively.

The synthesis of alkyl cyanoacrylate monomers has been described in the patent literature since 1949.\textsuperscript{15–18} Basically, the main strategy to achieve \(\alpha\)-cyanoacrylates comprises two steps. First, the corresponding alkyl cyanoacetate is reacted with formaldehyde in the presence of a basic catalyst, to form PACA oligomers (by the so-called Knoevenagel condensation reaction). The catalyst is a base, either inorganic (e.g., sodium or potassium hydroxide, ammonia) or organic (e.g., quilonine, piperidine, dialkyl amines). Then, pure alkyl cyanoacrylate monomer is recovered by a thermal depolymerization reaction of the previously obtained oligomers, using suitable stabilizers such as protonic or Lewis acids with small amounts of a free-radical inhibitors to prevent repolymerization (Figure 2).

From that moment on, the synthetic protocol remained almost unchanged. It was only slightly modified and improved essentially by playing with the nature of the solvent mixture,\textsuperscript{19,20} by applying a transesterification approach for making cyanoacrylates bearing longer alkyl ester chains,\textsuperscript{21} or by using a more efficient catalyst (namely pyrrolidine) for the condensation step.\textsuperscript{22}

**Polymerization of Alkyl Cyanoacrylates in Homogeneous Media**

On the fringe of typical vinyl monomers [styrenics, (meth)acrylates, etc.] is the alkyl cyanoacrylate family, which seems to be an exotic class of polymerizable compounds. Indeed, due to the presence of two powerful electro-withdrawing groups in the \(\alpha\)-carbon of the double bond, namely ester (COOR) and cyano (CN), alkyl cyanoacrylate monomers exhibit a remarkable reactivity toward nucleophiles such as anions (hydroxide, iodide, alcoholate, etc.) or weak bases (alcohol, amine, etc.), resulting in a very high polymerization rate. Even traces of one of the above-mentioned compounds in the reaction medium are sufficient to initiate such a fast polymerization. This explains why alkyl cyanoacrylates are extremely difficult to handle under their pure form and that batches of these monomers are usually maintain stable with a small amount of acidic stabilizers (e.g., SO\textsubscript{2}, sulfonic acid, etc.).

PACA can be synthesized according to three distinct types of polymerization: (1) anionic, (2) zwitterionic, and (3) radical (Figure 3). In practice, because of the exceptional reactivity of alkyl cyanoacrylate derivatives, anionic and zwitterionic polymerization mechanisms are by far predominant under conventional experimental conditions with
FIGURE 3 | Initiation and propagation steps involved during anionic (a), zwitterionic (b), and radical (c) polymerizations of alkyl cyanoacrylate monomer initiated by a base (B\(^-\)), a nucleophile (Nu), and a radical (P\(^\bullet\)), respectively.

respect to a pure radical process. This explains why studies on alkyl cyanoacrylates polymerization in both homogeneous (i.e., bulk or solution) and heterogeneous (i.e., emulsion, microemulsion) media were mainly devoted to anionic and zwitterionic processes.

**Synthesis of Homopolymers**

In this field, an extensive work has been accomplished by Pepper and coworkers to get a better understanding of the involved polymerization mechanisms depending on the experimental conditions.\(^{23-26}\) Indeed, the homopolymerization in solution of ethyl cyanoacrylate (ECA, Figure 1) and nBCA were initiated either by simple anions (CH\(_3\)COO\(^-\), CN\(^-\), I\(^-\), etc.) or by covalent organic bases (Et\(_3\)N, pyridine, etc.), leading to anionic or zwitterionic polymerizations, respectively.\(^{23}\) For zwitterionic polymerization of nBCA, the influence of the nature of the initiator as well as other experimental conditions (inhibiting species, presence of water, etc.) on both the main characteristics of the obtained polymer (number-average molecular weight, molecular weight distribution) and polymerization kinetics (monomer conversion, polymerization rate, etc.) were investigated through a small library of covalent organic bases such as phosphine,\(^{26-29}\) pyridine,\(^{24,27}\) and amine\(^{5,27,30}\) derivatives. Considering anionic polymerization, the same research group used tetrabutyl ammonium salts (hydroxide, bromide, acetate, and substituted acetates) as the initiating species for the polymerization of nBCA at 20–40 °C in tetrahydrofuran (THF) and reported a nearly ideal living polymerization in the case of the hydroxide-based initiator.\(^{31-33}\)

Even though anionic and zwitterionic mechanisms are more likely to occur for the polymerization of alkyl cyanoacrylates, free-radical polymerization was believed to be the main chain-extension process during homopolymerization\(^ {24-27}\) and copolymerization\(^ {27,38}\) carried out in bulk when a suitable inhibitor is introduced in the reaction medium. However, even under these specific inhibition conditions, anionic polymerization is not totally suppressed but is made negligible regarding the timescale of the polymerization reaction. In particular, Canale et al.\(^ {34}\) used in 1960, boron trifluoride–acetic acid complex while conducting free-radical bulk polymerization of MCA at 60 °C initiated by azobisisobutyronitrile (AIBN), whereas Bevington et al.\(^ {36}\) used propane-1,3-sultone as an efficient inhibitor against anionic polymerization for the free-radical polymerization of MCA in bulk or in 1,4-dioxane at 60 °C, initiated by AIBN or benzoylperoxide (BPO). In 1983, Yamada et al.\(^ {37}\) polymerized ECA in bulk at 30 °C with a small amount of acetic acid or propane-1,3-sultone and from their results, they extracted very high propagation rate constants: \(k_p = 1622 \text{ l mol}^{-1} \text{ s}^{-1}\) in the presence of acetic acid and \(k_p = 1610 \text{ l mol}^{-1} \text{ s}^{-1}\) in the presence of propane-1,3-sultone. As a
comparison, methyl methacrylate (MMA) which is considered as a highly reactive monomer gave $k_p = 450 \text{ mol}^{-1} \text{ s}^{-1}$ at 30°C.\textsuperscript{39}

**Synthesis of Copolymers**
Alkyl cyanoacrylates were also copolymerized with more ‘common’ vinyl monomers through a free-radical process (using trifluoride–acetic acid complex as an efficient inhibitor against anionic polymerization) to give different kinds of copolymers, depending on the nature of the comonomer.\textsuperscript{38} Random copolymers with MMA were achieved in bulk, whereas alternating copolymers with styrene were reported in benzene solution at 60°C under AIBN initiation. Considering bulk properties, random copolymers with 10% MMA exhibit physical properties similar to those of the PMCA homopolymer, whereas alternating copolymers with styrene had an enhanced thermal stability compared with random copolymers. Hall et al., who previously investigated the reactions of electron-rich olefins with electron-poor olefins,\textsuperscript{40–42} confirmed the previously investigated the reactions of electron-rich olefins with electron-poor olefins,40–42 confirmed the alternating copolymer starting from a 1:1 styrene : MMA mixture, either initiated by AIBN under UV light at 40°C in benzene solution or produced spontaneously at room temperature.\textsuperscript{43} However, when using other comonomers such as isobutyl vinyl ether, $p$-methoxystyrene or $\beta$-bromostyrene, copolymerizations with MCA led to mixtures of (co)polymers and/or small adducts.\textsuperscript{43}

In 1978, a comprehensive synthetic approach of bis(alkyl cyanoacrylate) was proposed by Buck starting from anthracene adducts.\textsuperscript{44} These difunctional alkyl monomers derived from cyanoacrylates were copolymerized with monofunctional alkyl cyanoacrylates such as MCA and isobutyl cyanoacrylate (IBCA, Figure 1), resulting in crosslinked macroscopic compositions exhibiting superior mechanical properties under both dry and wet environments than the noncrosslinked counterparts, which could be advantageously employed as pit and fissure sealant in dentistry.

More sophisticated macromolecular architectures as diblock and triblock copolymers comprising poly(ethylene glycol) (PEG) and PACA blocks were also synthesized in homogeneous media via zwitterionic polymerization.\textsuperscript{45} The synthesis involved the preparation of triphenylphosphine end-capped mono-hydroxyl and dihydroxyl PEGs, giving the corresponding monofunctional and difunctional macrozwitterionic initiator. The polymerization of IBCA was then initiated with each one of the macroinitiators in THF at ambient temperature to afford PIBCA-$b$-PEG diblock and PIBCA-$b$-PEG-$b$-PIBCA triblock copolymers with tuneable compositions in good match with the initial stoichiometry.

Synthesis of poly[[hexadecyl cyanoacrylate]-co-methoxypoly(ethylene glycol) cyanoacrylate] [P(HDCA-co-MePEGCA)] comb-like copolymers exhibiting amphiphilic properties was reported by Peracchia et al.\textsuperscript{46} This original approach derived from Knoevenagel condensation reaction where corresponding cyanoacetates, namely hexadecyl cyanoacetate and PEG monomethyl ether cyanoacetate, were reacted with formaldehyde in the presence of dimethylamine as the catalyst (Figure 4). Thanks to the slow, in-situ formation of the cyanoacrylate monomers, it allowed the polymerization process to be better controlled compared with a direct anionic polymerization. Besides, the composition of the copolymer (and thus its hydrophilicity/hydrophobicity) can be adjusted simply by varying the initial cyanoacetates feed ratio.

**POLY(ALKYL CYANOACRYLATE)-BASED NANOPARTICLES**

**General Consideration on the Synthesis of Poly(alkyl cyanoacrylate) Nanoparticles**
Nanoparticle is a collective name for two different types of colloidal objects, namely nanospheres (NS) and nanocapsules (NC), which can be separately obtained depending on the preparation process. Basically, nanospheres are matrix systems constituted by the polymer in which the drug is physically and uniformly dispersed, whereas nanocapsules are vesicular systems in which the drug is solubilized in a liquid core, either water (w-NC) or oil (o-NC), surrounded by a thin polymer layer (Figure 5).

During the last 25 years, an important breakthrough in this field has been witnessed with the development of PACA nanoparticles as colloidal drug carriers. Polymerizations in heterogeneous media (i.e., emulsion, dispersion, miniemulsion,
Synthesis of Nanospheres

In 1979, Couvreur et al. first developed a simple process to directly generate stable MCA or ECA nanospheres, consisting of a dropwise addition of the monomer into a vortexed HCl solution (2 < pH < 3) containing a nonionic or a macromolecular surfactant. Since then, numerous studies aiming at establishing relevant parameters governing the polymerization kinetics as well as the characteristics of the macromolecules and the nanospheres have been reported. It has been shown that the nature and the concentration of the surfactant played a significant role on the particle size, whereas the type of both the monomer and the surfactant strongly influenced the molar mass of the obtained polymer. Besides the monomer concentration, the pH of the reaction medium and the concentration of sulfur dioxide (acting as a polymerization inhibitor) were also crucial parameters which strongly affected the macromolecular and/or colloidal properties of the nanospheres. The size of the colloidal objects which can be obtained usually ranged from 50 to 300 nm, which is a well-adapted window for colloidal drug delivery devices, especially by intravenous administration.

For a more fundamental standpoint, several tentative mechanisms have been postulated. It has been reported that the emulsion/dispersions polymerization in acidic medium is not that trivial and proceeds via a stepwise, anionic mechanism comprising reversible propagation and reversible termination steps (Figure 6). Basically, PACA oligomers are formed in the monomer droplets and are reversibly terminated by the acid-inhibiting agents present in the monomer. This step is followed by a re-initiation reaction of terminated species by still living chains, leading to further polymerization until a molecular weight balancing is reached, similar to depolymerization/repolymerization events. One should be aware that in all these mechanisms, the polymerization is postulated to be initiated by the hydroxyl ions from the aqueous phase independently of other reactants existing in the polymerization medium.

On the basis on an interfacial polymerization mechanism, Limouzin et al. polymerized nBCA in emulsion and miniemulsion in the presence of dodecylbenzenesulfonic acid (DBSA) acting as both surfactant and terminating agent (also termed tersurf). By releasing protons at the water/oil interface, DBSA allowed the interfacial, anionic polymerization to be drastically slowed down through a (reversible) termination reaction and to proceed under a fairly controlled fashion leading to stable high solids content (20%) PnBCA nanospheres. The miniemulsion technique was also used by Weiss et al. for the preparation of PnBCA nanospheres. By varying the concentration of the surfactant (SDS), and by adding sodium hydroxide as the initiating species, high solids content dispersions up to 10% with average diameters ranging from 110 to 360 nm were obtained.

Synthesis of Nanocapsules

Nanocapsules are reservoir-type nanoparticles in which drugs can be encapsulated according to their
The organic phase, and the inhibition of the (micro)emulsion, using polysorbate, sorbit monoleate counterparts. They are usually prepared by water-soluble polymers that are not chemically reactive. The nature of the dispersed phase involved in a heterogeneous polymerization process, usually emulsion or microemulsion. Basically, the macromolecular shell is formed by the spontaneous anionic polymerization of alkyl cyanoacrylate occurring at the interface between the dispersed and the continuous phase. Historically, oil-containing nanocapsules were first developed by Gallardo et al. through a simple protocol: a solution of monomer and oil in a water-miscible solvent (usually ethanol) is poured into an aqueous solution of surfactant (usually Poloxamer 188) under vigorous stirring, leading to small oil/monomer droplets at the interface of which the polymerization is initiated by hydroxide ions present in water. Gallardo et al. reported that the crucial parameters for achieving nanocapsules lies: (1) in the diffusion behavior of the organic solvent (acting as a monomer support) within the aqueous phase, which ultimately governs the reservoir nature of the nanoparticles, and (2) in the simultaneous precipitation of the polymer at the water/oil interface (i.e., the polymer should be insoluble in both the aqueous and the organic phase). Usually, nanocapsules exhibit average diameter ranging from 200 to 350 nm, the latter being governed by several physicochemical parameters such as the nature and the concentration of the monomer and encapsulated drug, the amount of surfactant and oil as well as the speed of diffusion of the organic phase within the aqueous phase. However, Altinbas et al. have demonstrated that when a miniemulsion is applied instead of an emulsion, nanocapsules of an average diameter below 100 nm can be obtained.

The main drawback often encountered in this approach is the contamination of the nanocapsule population by a substantial amount of nanospheres, resulting from a partial polymerization in the organic phase. However, it has been shown that an optimized ethanol/oil ratio, the acidification of the organic phase, and the inhibition of the polymerization in the organic phase by aprotic solvents (acetonitrile, acetone) each avoided the formation of matrix-type nanoparticles. Water-containing nanocapsules have been developed more recently than were the oil-containing counterparts. They are usually prepared by water in oil (w/o) (micro)emulsion, also called an inverse (micro)emulsion, using polysorbate, sorbit monoleate or poly(ethylene oxide) lauryl ester (Brij 35) as surfactants. Basically, the alkyl cyanoacrylate monomer is added to the preformed (micro)emulsion and, in a similar way to that of oil-containing nanocapsules, spontaneous anionic polymerization occurred at the water/oil interface to form a thin PACA layer surrounding an aqueous core. Depending on the nature of the surfactant and the starting system (emulsion or microemulsion), which are parameters governing the surface properties of these colloidal objects, this process led to 50–350 nm diameter, stable nanocapsules.

However, because the inverse (micro)emulsion processes conduct to water-containing nanocapsules dispersed in oil (which are suitable for oral route administration), intravenous injection cannot be directly performed with a nonaqueous dispersing medium. To circumvent this limitation, a recent method aiming at transferring the nanocapsules from an oil-dispersing medium to a water-dispersing medium was recently suggested by Couvreur and coworkers and consisted in a centrifugation step of the nanocapsules onto an aqueous layer.

To synthesize nanocapsules with preformed polymers, homopolymer of alkyl cyanoacrylate are required and synthesized separately, for instance by dripping the monomer in pure water, the polymer being subsequently recovered by lyophilization. The nanocapsules preparation method, also called interfacial deposition, consists of the addition of a solution of the homopolymer and a small amount of oil, for instance Miglyol (which will constitute the oily core of the nanocapsules), into an aqueous phase. The oil-containing nanocapsules form instantaneously by precipitation of the homopolymer at the oil/water interface, which precipitate as a macromolecular shell. In general, a surfactant is added in the aqueous phase to ensure colloidal stability of these nanocapsules.

Synthesis of Poly(alkyl cyanoacrylate) Nanoparticles with Controlled Surface Properties

In this topic, the major breakthrough is undoubtedly the grafting of PEG, a nonionic, flexible, and hydrophilic polymer, onto nanoparticles (which also applies for other colloidal drug carriers such as liposomes). This approach, termed ‘PEGylation’, represented a milestone in the drug delivery area. Indeed, non-PEGylated nanoparticles are quickly eliminated from the bloodstream because of the adsorption of blood proteins (opsonins) onto their surface, which triggers the recognition of the mononuclear phagocyte system (MPS) by the macrophages.
As a consequence, these nanoparticles are ineluctably accumulated in MPS organs such as the liver and the spleen, restricting the therapeutic activity of the entrapped compounds to liver diseases (i.e., hepatic primary hepatocarcinoma or metastasis as well as liver intracellulare infections). In contrast, when covered by PEG chains, the obtained nanoparticles are able to efficiently escape this recognition system, resulting in long-circulating, colloidal devices, also called ‘stealth’ nanoparticles.82,83

After it has been demonstrated that PACA nanoparticles can be seen as very promising biodegradable drug carriers (the BioAlliance Pharma spin-off company is now producing doxorubicin-loaded PACA nanoparticles for clinical use in phase II/III trials with resistant liver hepatocarcinoma as main indication), their complexity was further increased by performing appropriate tuning of their surface properties in order to control their in vivo fate.

### Surface Modification of Nanospheres

First attempts concerning surface modification of PACA nanospheres logically concerned the ‘PEGylation’ concept, either via a simple adsorption of PEG chains onto the nanoparticles or by a covalent linkage of PEG chains with PACA polymers. However, the adsorption approach does not fit the covalent linkage criteria and is not really suitable as long as it has been demonstrated that these kinds of assemblies (PACA nanoparticles on which poloxamer 388 or poloxamine 908 was adsorbed) are not stable during in vivo administration, resulting in a loss of coating and no significant influence on the biodistribution pattern.84 Thus the covalent bond of the PEG chains at the surface of the nanoparticles is a prerequisite for this kind of application.

Basically, different types of hydrophilic molecules have been anchored, on purpose, to the surface of PACA nanoparticles (Figure 7). Efficient surface modification of nanospheres can be achieved either in situ during the polymerization in aqueous dispersed media or from preformed amphiphilic copolymers during emulsification processes.

Concerning previous studies about anionic/zwit terionic emulsion polymerization of alkyl cyanoacrylate, the hydrophilic molecules introduced in the recipes (SDS, dextran, poloxamer, Tweens, cyclodextrins, etc.) were solely used as stabilizing agents for investigating their effect on the stability, the average diameter, and the particle size distribution. However, it was not fully understood at this time that some of them, especially those containing nucleophilic functional groups, might take part in the initiation of the polymerization, leading to a partial formation of surface-active macromolecules. This point is of great importance since nanoparticles with covalently anchored stabilizing moieties at their surface would behave differently in a biologic medium than those with adsorbed surfactants. As a consequence, this is only later on that researches have been strictly devoted to surface engineering of PACA nanoparticles in order to investigate any subtle change of the surface properties of the nanoparticles on their in vivo fate.

However, almost unmarked, early works by Douglas et al. postulated that dextran or β-cyclodextrin may also initiate the polymerization of butyl cyanoacrylate (BCA) resulting in the formation of amphiphilic copolymers, helping to stabilize the nascent nanospheres.54 This approach was revisited by Peracchia et al. using different linear PEGs acting as stabilizers and initiators for the emulsion polymerization of IBCA85,86 (Figure 7(a) and (b)). It was demonstrated that PEG chains exhibited different conformations at the surface of the nanospheres: (1) hairy nanospheres with PEG monomethyl ether due to a single initiation site (Figure 7(a)) or (2) long loops using PEG due to the divergent chain growth (two initiating sites) during the polymerization of IBCA87 (Figure 7(b)). In the same spirit, the use of polysaccharides, such as dextran, dextran sulfate, chitosan, and thiolated chitosan, as stabilizing/initiating agents under similar experimental conditions also led to stable nanospheres in the 100–500 nm range, exhibiting different surface properties; for instance, positively charged with chitosan61,88,89 and from rather neutral to negatively charged with dextran derivatives.88,90,91

So far, anionic (mini)emulsion polymerization was the most widespread and straightforward technique to synthesize PACA nanospheres. Even though, in that case, the mechanism is on the basis of anionic propagating species,63,64 Chauvierre et al. recently adapted Couvreur’s original protocol to a free-radical emulsion polymerization process, thanks to the polysaccharide/cerium IV (Ce4+) ions redox couple as the initiator62 (Figure 8). Because of the fast radical initiation rate, anionic polymerization is negligible regarding the timescale of the experiment which makes way for a free-radical chain growth process. This technique was also employed for the emulsion polymerization of alkyl cyanoacrylate using different kinds of polysaccharides,89,90,91,94 allowing a direct comparison with nanospheres obtained from anionic emulsion polymerization. The first difference is the conformation of polysaccharide chains at the surface of the nanospheres in direct relation with the structure of the copolymer. Indeed, anionic emulsion polymerization led to grafted copolymers, whereas linear block
copolymers were achieved under redox radical initiation (Figure 8), leading respectively to compact loops (Figure 7(d)) and hairy polysaccharide chains (Figure 7(e)) at the surface of the nanospheres.88,93

The size of the polysaccharide-decorated nanospheres was in the 80–800 nm range and depended on: (1) the molecular weight of the polysaccharide, where a minimum value of about 6000 g mol\(^{-1}\) was required for ensuring an efficient colloidal stability88,89 and (2) on the nature of the polysaccharide: dextran-decorated nanospheres exhibited an average diameter below 300 nm, dextran sulfate and chitosan led to a larger average diameter of about 350–600 nm,88 whereas the use of heparin conducted to 90-nm nanospheres.95,96

Another crucial difference resulting from the surface conformation of the hydrophilic chains, for either PEG derivatives or polysaccharides, concerns the measure of the complement activation,87,88 which is known to play a significant role in the non-specific recognition events of the immune system. Indeed, according to Peracchia et al., nanospheres bearing big loops because of \(\alpha,\omega\)-dihydroxyl PEG (Figure 7(b)) were shown to better prevent complement consumption than do the hairy nanoparticles obtained from PEG monomethyl ether87 (Figure 7(a)). Besides, Bertholon et al. demonstrated that, for both dextran and chitosan, an increase of the length of the compact loops (Figure 7(d)) resulted in an increase of complement activation, whereas the opposite effect was obtained by increasing the length of the hairy polysaccharide chains88 (Figure 7(e)), which clearly demonstrated that complement activation is highly sensitive to any change of the surface chain conformation. In a recent work, it was also suggested that the conformation of the coating material also affects the cytotoxicity profile of PACA nanoparticles.97
Recently, an interesting synthetic pathway to functionalize PACA nanospheres using amino acids was proposed by Weiss et al. The authors used a miniemulsion process to prepare a stable pH 1 dispersion of nBCA nanoparticles stabilized by SDS as the surfactant. Polymerization was then triggered by the addition of nucleophilic compounds such as amino acids (for instance, glycine), leading to functionalized, stable nanospheres (as already discussed earlier, the similar miniemulsion process has been applied to nonfunctionalized nanospheres when sodium hydroxide was added as the initiator). This method allowed: (1) the solids content to be increased up to 10 wt% with average diameter ranging from 80 to 350 nm, depending on the amount of surfactant as well as the nature of the amino acid and (2) a convenient surface functionalization by amino acid moieties (Figure 7(f)).

The preparation of ‘PEGylated’ nanoparticles from preformed polymers is a well-established technique which first requires the synthesis of amphiphilic copolymers with PEG segments. PIBCA-b-PEG diblock and PIBCA-b-PEG-b-PIBCA triblock copolymers were synthesized from phosphine end-capped PEG macroinitiators. With diblock copolymers, unimodal size distribution and stable nanoparticles in the range of 100–700 nm were obtained by nanoprecipitation or emulsification/solvent evaporation, the average diameter being controlled mainly by the amount of organic solvent and by the composition of the polymers. However, the presence of phosphine groups within the synthesized polymers may be a toxicological issue.

The amphiphilic, biodegradable copolymers comprising poly(hexadecyl cyanoacrylate) hydrophobic units and methoxypoly(ethylene glycol) cyano acrylate hydrophilic units (Figure 4) were used to prepare the corresponding P(HDCA-co-MePEGCA) nanospheres exhibiting a biodegradable PACA core and a shell of excretable PEG chains.
FIGURE 9 | Concentration of radioactivity in right hemisphere (a), left hemisphere (b), and cerebellum (c), after intravenous administration of 60 mg kg$^{-1}$ of $^{[14]}$C]-P(HDCA-co-MePEGCA) nanoparticles, poloxamine 908-coated $^{[14]}$C]-PHDCA nanoparticles, polysorbate 80-coated $^{[14]}$C]-PHDCA nanoparticles, and uncoated $^{[14]}$C]-PHDCA nanoparticles (mice at 1 h postinjection).

(N Figure 7(c)). Nanoprecipitation or emulsification/solvent evaporation techniques employing P(HDCA-co-MePEGCA) polymers led to very stable ‘PEGylated’ nanospheres with average diameters in the 100–200 nm range and monomodal size distributions.98 These materials showed a reduced cytotoxicity toward mouse peritoneal macrophages, and the presence of the PEG segments was found to increase the degradability of the polymer in the presence of calf serum.98 Besides, as a result of the PEG coating, an extended circulation time in the bloodstream was demonstrated.100

The impressive result deriving from the use of these stealth nanoparticles is their ability to significantly cross the blood–brain barrier (BBB) compared with non-PEGylated counterparts and those with preadsorbed surfactants such as polysorbate 80 or poloxamine 9082,101–104 (Figure 9).

This unique feature suggested that P(HDCA-co-MePEGCA) nanospheres exhibited appropriate properties for entering the central nervous system (CNS) via the BBB. Even though a passive diffusion because of an increased permeability of the BBB (when locally disrupted at the tumor site) may not be ruled out, the mechanism by which those nanoparticles preferentially crossed the healthy BBB was assigned to a specific adsorption of apolipoprotein E and B-100 (Apo E and B-100) onto P(HDCA-co-MePEGCA) nanospheres leading to their translocation mediated by low-density lipoprotein receptors (LDLR).105–107

The involvement of Apo E on the translocation through the BBB of polysorbate 80-covered PACA nanoparticles was also reported by Kreuter’s group who hypothesized the formation of lipoprotein particle mimics recognized by the LDLR gene family in the brain endothelial cells of the BBB.108

Synthesis of ‘PEGylated’ Nanocapsules

To the best of our knowledge, the only examples of ‘PEGylated’ PACA nanocapsules were reported by Brigger et al.81 and Li et al.,109,110 both using P(HDCA-co-MePEGCA) copolymers.46 Although Brigger et al.81 prepared the corresponding stealth, oil-containing nanocapsules by the interfacial deposition technique, Li et al. used a water-in-oil-in-water (w/o/w) double emulsion process to achieve ‘PEGylated’, water-containing nanocapsules as tumor necrosis factor-α carriers.109,110 This two-step emulsification protocol started by the emulsification of the aqueous phase containing the drug into the organic phase in which the P(HDCA-co-MePEGCA) copolymer was dissolved (w/o), followed by its addition into an aqueous PVA solution (w/o/w). Stable nanocapsules of about 140–150 nm in diameter were then collected by centrifugation.

Addressed Poly(alkyl cyanoacrylate) Biodegradable Nanoparticles

For the forthcoming years, the most exciting challenge in drug delivery, irrespective of the nature of the drug carriers (i.e., liposome, nanoparticles), will be undoubtedly the synthesis of efficient ligands-decorated colloidal devices for achieving specific cells targeting, on the basis of molecular recognition processes. Indeed, the main drawback of previous generation of drug carriers is their non-specific drug release behavior. Nanoparticles are indeed unable to be efficiently addressed to the desired cells and the therapeutic activity of the encapsulated drug may be partly hampered. Even for the remarkable case of brain-targeted P(HDCA-co-MePEGCA) nanospheres,2,101–104 the linkage of a judicious ligand at their surface would certainly result in a strongly higher extravasation yield across the BBB.

Thus, if a great deal of effort has been already devoted to this area, a lot of works remain due to be done. The only example of the so-called third-generation PACA nanoparticles involves folate-decorated P(HDCA-co-MePEGCA) nanospheres to target the folate receptor, which is overexpressed at the surface of many tumor cells. For this purpose, the synthetic route for P(HDCA-co-MePEGCA) copolymers46 was adapted to the synthesis of a poly[hexadecyl cyanoacrylate]-co-aminopoly(ethylene glycol) cyanoacrylate]...
[P(HDCA-co-H\textsubscript{2}NPEGCA)] copolymer, starting from a protected aminopoly(ethylene glycol) cyanoacetate\textsuperscript{111}.

Then, the corresponding nanospheres were obtained by nanoprecipitation showing a narrow size distribution for an average diameter of 80 nm. The conjugation with N-hydroxysuccinimide–folate (NHS–folate) occurred via an amidation pathway directly at the surface of the nanospheres bearing available amino groups (Figure 10). The specific interaction occurring between the folate-conjugated nanospheres and the folate-binding protein was demonstrated by surface plasmon resonance. The apparent affinity of the folate bound to the nanospheres appeared 10-fold higher than the free folate in solution, because of the multivalency of the folate-decorated nanoparticles.

Biocompatibility and Biodegradation of Poly(alkyl cyanoacrylate) Polymers

The degradation and toxicity of PACA nanoparticles are a crucial point, especially for biomedical applications. Indeed, a drug carrier device is suitable for \textit{in vivo} applications only if it is made of biocompatible, possibly biodegradable, or at least excretable (e.g., by the kidneys) materials. In fact, PACAs are bioerodible polymers for which different degradation pathways have been reported so far (Figure 11).

The predominant mechanism occurs via the hydrolysis of their side chain ester functions\textsuperscript{55,112,113} producing the corresponding alkyl alcohol and poly(cyanoacrylic acid) as the degradation products, the latter being fully water-soluble and readily eliminated by kidney filtration (Figure 11(a)). This hydrolysis, which is believed to be the main degradation mechanism \textit{in vivo}, proceeds typically in a couple of hours for PACA nanoparticles and is strongly affected by: (1) the length of the alkyl side chains; the longer the alkyl side chains, the lower the toxicity but the slower the hydrolysis\textsuperscript{55,114,115} and (2) the surrounding environment as it can be strongly catalyzed by esterases from serum, lysosomes, and pancreatic juice.\textsuperscript{116,117} However, a complete excretion of these
materials would occur only for low-molecular-weight PACA polymers, typically below 10,000 g mol$^{-1}$.

It has been postulated that the ‘unzipping’ depolymerization reaction, initiated by a base, could also take part in the biodegradation pathway of PACA, especially in biologic media where it can be theoretically induced by amino acids of proteins (Figure 11(b)). Following the depolymerization of parent polymers, instant repolymerization to form lower-molecular-weight polymers would occur, even if no clear description of this mechanism has been shown yet, possibly because of its too fast occurrence to be unambiguously observed.

Finally, another suggested mechanism for the degradation of PACA polymers is on the basis of the well-known inverse Knoevenagel condensation reaction, which produces the corresponding alkyl cyanoacetate and formaldehyde (Figure 11(c)), even though the release of formaldehyde might also result from hydrolysis of the α-hydroxyl functions of the polymer chains, provided the hydroxyl ions have been initially used as an initiator (Figure 11(d)). However, the inverse Knoevenagel condensation reaction has been reported to a lesser extent in aqueous solution at physiological pH and too slow to compete with the above-mentioned enzyme-catalyzed hydrolysis mechanism.

**CONCLUSION**

Even though the chemistry of alkyl cyanoacrylates is not as straightforward as for other ‘common’ vinyl monomers, the convergent involvements of organic chemistry, polymer science, and physicochemistry made possible the development of more and more sophisticated, biodegradable PACA-based nanoparticles, successfully used as drug delivery devices. Indeed, from the pharmacology standpoint, PACA nanoparticles fulfill important requirements of ideal drug delivery systems: ease and reproducibility of preparation, ease of storage and administration in a sterile form, satisfying drug-loading capacity, low toxicity, excellent biodegradability, and feasibility for scale-up production. By playing with experimental conditions (nature and amount of reactants, process of preparation, etc.), various types of PACA nanoparticles can be obtained, each of them exhibiting specific features regarding the nature of the drug and/or the way the drug is encapsulated: nanospheres (matrix-type nanoparticles; oil-soluble drug) or nanocapsules (reservoir-type nanoparticles; either oil-soluble or water-soluble drug), nonsurface-modified (mainly devoted to MPS organs) or ‘PEGylated’ nanoparticles (long-circulating drug carriers) as well as ligand-decorated nanospheres (addressed drug delivery devices). As a result of constant efforts in this field, PACA nanotechnologies have thus opened exciting perspectives for the discovery of novel and more efficient nanomedicines.

**NOTES**

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Synthesis of PACA-based colloidal nanomedicines


**RELATED ONLINE ARTICLES**

Polyalkylcyanoacrylate nanoparticles for delivery of drugs across the blood–brain barrier.
Hydrogel mediated delivery of trophic factors for neural repair

Joshua S. Katz¹ and Jason A. Burdick∗

Neurotrophins have been implicated in a variety of diseases and their delivery to sites of disease and injury has therapeutic potential in applications including spinal cord injury, Alzheimer’s disease, and Parkinson’s disease. Biodegradable polymers, and specifically, biodegradable water-swollen hydrogels, may be advantageous as delivery vehicles for neurotrophins because of tissue-like properties, tailorsability with respect to degradation and release behavior, and a history of biocompatibility. These materials may be designed to degrade via hydrolytic or enzymatic mechanisms and can be used for the sustained delivery of trophic factors in vivo. Hydrogels investigated to date include purely synthetic to purely natural, depending on the application and intended release profiles. Also, flexibility in material processing has allowed for the investigation of injectable materials, the development of scaffolding and porous conduits, and the use of composites for tailored molecule delivery profiles. It is the objective of this review to describe what has been accomplished in this area thus far and to remark on potential future directions in this field. Ultimately, the goal is to engineer optimal biomaterials to deliver molecules in a controlled and dictated manner that can promote regeneration and healing for numerous neural applications.


Disruption of central nervous system (CNS) or peripheral nervous system (PNS) tissues such as the spinal cord, optic nerve, and motor neurons can severely affect a patient’s motor, sensory, and autonomic functions, and depending on the severity of the injury, the patient’s quality of life can decline dramatically.¹–³ Unfortunately, current clinical treatment options are severely limited for many of these injuries and diseases and are unable to restore complete function to these patients. For instance, in the spinal cord, one significant barrier to regeneration is the extremely complex cascade of events (e.g., inflammation, glial scarring, release of inhibitory molecules) that occurs after injury that must be addressed to restore functional recovery to the patient.⁴,⁵ However, one promising therapy is the delivery of neurotrophins that can influence the local function of cells within and surrounding the injury site.

Neurotrophins have been widely investigated for their influence on cell mortality, differentiation, and function in both the CNS and the PNS.⁶ These neurotrophins include factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and glial derived neurotrophic factor (GDNF). Neurotrophins bind to tropomyosin-related kinase (Trk) receptors (TrkA for NGF, TrkB for BDNF and NT-4/5, and glial derived neurotrophic factor (GDNF). Neurotrophins bind to tropomyosin-related kinase (Trk) receptors (TrkA for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3) and the pan-neurotrophin receptor p75.⁷ The functions of neurotrophins in vivo are many and include controlling neural cell growth and survival, influencing glial development, and functions in non-neural tissues such as in the cardiovascular and immune systems.⁸–¹² Additionally, neurotrophins can mediate axon signals or act on myelinating glia to influence the remyelination of axons.¹³,¹⁴ For example, neurotrophins can promote axonal growth, neuronal survival, and plasticity after injury to the spinal cord.¹⁵ Lu and coworkers¹⁶ recently illustrated the ability of NT-3 in combination with cyclic adenosine monophosphate to induce regeneration of sensory axons past a spinal cord lesion. Additionally, the overexpression of neurotrophins after injury induced sprouting of corticospinal tract...
axons past the injury site. Techniques such as gene therapy, delivery via stem cells, and polymeric delivery vehicles are being investigated for the supplementation of neurotrophins to injured neural tissues.

There are several methods which have been explored for the delivery of neurotrophins and drugs to the nervous system, including mini-pumps, genetically modified cells, and polymer formulations. Hydrogels are water-swollen insoluble polymer networks that have a wide range of chemical compositions and properties. Hydrogels can be formed through a variety of mechanisms, including both physical (e.g., ionic or hydrogen bonding) and chemical gelation (e.g., covalent bonding). Through alterations in the chemical structure, important properties such as swelling, degradation (e.g., hydrolytic or enzymatic), and mechanics can be controlled. The delivery of large molecules, such as neurotrophins, is typically accomplished by encapsulating the molecules during gelation, which are subsequently released via diffusion and degradation mechanisms. This process is relatively complex and dynamic as the hydrogel mesh size changes as the material degrades and swells. With recent advances in polymer synthesis and our understanding of biological polymers, our ability to control hydrogels, and consequently, molecule delivery is constantly improving.

There are numerous factors that make hydrogels ideal delivery vehicles for neurotrophic factors and repair of neural tissue. First, this approach does not introduce either live tissue (e.g., grafts) or viral vectors, eliminating potential issues with graft rejection and adverse responses. Next, hydrogel delivery eliminates the need for devices like pumps and catheters that can malfunction. Finally, hydrogels can provide constant and tailorable delivery of either one or numerous molecules to a desired in vivo location. Because of the short in vivo half life of neurotrophins, sustained delivery to the injury site results in significantly better recovery compared to a single injection. Because of the complexity of injuries, the appropriate delivery profile depends on the injured tissue and timing of therapies. Several hydrogels have been investigated for the controlled delivery of neurotrophins and it is the objective of this review to outline past work in this area and look forward to future directions. The hydrogels investigated have ranged in composition from purely synthetic (e.g., poly[ethylene glycol] (PEG)) to purely natural (e.g., collagen), and in physical structure from uniform gels to porous scaffolds and composite materials.

INJECTABLE HYDROGELS

Hydrogels are made injectable through numerous means including free-radical polymerizations (i.e., thermal, photo, or redox initiation), self-assembly of materials, or ionic crosslinking. One of the biggest advantages to using injectable materials for these applications is the non-invasiveness of hydrogel delivery, which can limit further tissue damage. For instance, disruption of the dura cover to many tissues (including the brain and spinal cord) results in the loss of many potentially stimulatory molecules, which could be avoided with injection through the dura. Additionally, many imaging techniques could be used in combination with the injection procedures to potentially deliver these hydrogels in a closed surgery. Several clinically used biomaterials are already injected in vivo, such as poly(methyl methacrylate) bone cements and photocurable resins for filling dental caries, and neural applications could benefit from similar procedures. This section focuses on the various injectable hydrogels that have been explored for delivery of growth factors for neurological applications.

Agarose is a polysaccharide derived from seaweed and comprised of repeating galactopyranose units. It has been used for a variety of biomedical applications and can be thermally induced to form a hydrogel through intermolecular hydrogen bonding interactions. At elevated temperatures when hydrogen bonds cannot form, agarose solutions do not gel. However, as the solution is cooled, hydrogen bonds begin to form, leading to gelation. Jain and coworkers used cooled nitrogen gas to gel solutions of agarose in situ. Following injury to the spinal cord, a solution of agarose containing BDNF-loaded microtubules was pipetted into the injury site. The solution was then cooled by nitrogen gas which was passed over a bath of dry ice to produce a gel. A schematic of this cooling system is shown in Figure 1(a). The presence of BDNF greatly enhanced the regeneration of axons and their ability to penetrate into and through the scaffold.

Another natural polymer, collagen, crosslinks at physiological conditions through ionic interactions with salts present in solution. Hamann and coworkers injected aqueous solutions of collagen containing growth factors (epidermal growth factor (EGF) and/or FGF-1) into the intrathecal space surrounding the spinal cord as a drug delivery system. Rather than acting as a mechanical support for axonal regeneration as seen in many other systems, this system chemically supports the regenerative response to spinal cord injury (SCI) by the delivery of therapeutic agents directly and locally to the site of injury.
Examples of techniques for in situ gelation of hydrogel solutions. (a) For agarose, nitrogen is passed from the tank through a bath of dry ice and acetone (box) through an aluminum rod surrounded by dry ice to cool the solution and induce gelation. (b) Shear forces, induced through syringe injection, allow methylcellulose and acetate-modified hyaluronan (HAMC) gels to flow and then re-gel at physiological temperatures in vivo, potentially in the intrathecal space.

Significantly higher levels of cavitation were observed in animals that did not receive the growth factors at the site of injury. However, in a functional recovery test with this specific delivery system, there was no significant difference between the treated animals and the controls.

To address some of the issues associated with the injectable collagen system, such as slow gelation and cell infiltration within the dura, Gupta and coworkers created mechanically-reversible gels consisting of a blend of methylcellulose and acetate-modified hyaluronan (HAMC). Methylcellulose (MC) is a modified natural material that gels with increasing temperature. As hydrogen bonds break, hydrophobic interactions force the MC to gel. Hyaluronan (HA) is a naturally occurring polysaccharide that may spontaneously gel in water though hydrophilic associations with the water and has been explored widely for biological applications. However, when sheared, the gel breaks as the molecules align with the direction of the shear. One limitation of using unmodified HA alone is that it quickly disperses when injected in vivo because of its high water solubility. HAMC blends gel at room and physiologic temperatures, but flow when subjected to shear stresses (Figure 1(b)). A volume of 10 µL of HAMC was injected into the intrathecal space following a clip compression injury to rats. While functional recovery of the animals was better with the injection of the HAMC than with an injection of artificial cerebrospinal fluid (aCSF) into the intrathecal space, the difference was not significant after 1 week. However, no growth factors were injected with the HAMC in this study, and the presence of such factors could potentially cause significant improvement in the animal’s recovery.

PEG has been widely investigated as a biomaterial for many years, primarily because it is a relatively inert material. As protein adsorption to PEG hydrogels is minimal, non-specific protein binding and cellular interactions can be avoided. Hubbell and coworkers modified PEG hydrogels with both degradable units and then reactive groups to form biodegradable hydrogels based on PEG. The chemical structure of this material is shown in Figure 2(a). These synthetic macromers form a hydrogel via a radical polymerization, which typically is initiated using a photoinitiation process. For the encapsulation and release of a growth factor (e.g., neurotrophin), the PEG macromer is dissolved in a buffer solution containing the photoinitiator and the growth factor. This solution is exposed to light to form a hydrogel and the growth factor is released through a combination of diffusion and degradation. This hydrogel has been explored extensively for both tissue engineering and drug delivery applications, primarily because of the control that is afforded over the temporal material properties. For instance, degradation can be altered through parameters such as the molecular weight of the PEG, the type (e.g., lactic vs caproic acid) of degradable groups, the number of degradable groups, and the concentration of the macromer in solution.

For the delivery of neurotrophins, Burdick and coworkers monitored the release kinetics of several factors from PEG hydrogels. Example release profiles are shown in Figure 2(b) for a range of neurotrophins. They found that the release kinetics of these factors were controlled by changes in the network crosslinking density, which influences neurotrophin diffusion and subsequent release from the gels, with total release times ranging from weeks to several months. The release and activity of one neurotrophic factor, ciliary-neurotrophic factor (CNTF), was assessed with a cell based proliferation assay and an assay for neurite outgrowth from retinal explants. CNTF released from a degradable hydrogel above an explanted retina was able to stimulate outgrowth of a significantly higher number of...
neurites than controls without CNTF. Finally, unique microsphere/hydrogel composites were developed to simultaneously deliver multiple neurotrophins with individual release rates.48

This system was also exploited for application to the injured spinal cord.49 The failure of injured axons to regenerate in the mature CNS can have significant implications in spinal cord injury. However, the delivery of neurotrophins can promote axon growth in injured CNS and thus, the delivery of NT-3 was used in an attempt to alter anatomical and behavioral outcomes. NT-3 was delivered to a dorsal hemisection lesion in adult rats using PEG hydrogels by injecting the macromer and initiator solution directly on the lesion and exposing to visible light (using a dental curing lamp) for 60 s for gelation. The treated animals showed improved recovery in both an open-field BBB test and in a horizontal ladder walk test compared to untreated rats. Also, the treated animals showed much greater axon growth in both the corticospinal and raphespinal tracts over control animals. Thus, this provides great scope to the use of injectable hydrogels for trophic factor delivery to influence outcomes in injured patients.

POROUS SCAFFOLDS AND GUIDANCE CHANNELS

While the primary goal of injectable hydrogels is to chemically support axonal regeneration through neurotrophin delivery locally at the site of injury, it may be advantageous to also physically support cellular function and growing axons. To date, the majority of this support has come through the development of nerve guidance channels, which aim to template regenerating axons through the injury site.50 While originally designed from non-degradable, non-hydrogel materials, more recent scaffolds have begun to incorporate many features common among tissue engineering scaffolds, such as porosity and swelling.

Poly(hydroxyethyl methacrylate) (poly(HEMA)) hydrogels have been widely explored as a biocompatible, non-degradable material for drug delivery and tissue engineering applications.51,52 Gels form through the free-radical polymerization of the methacrylate units in the presence of a crosslinker (i.e., dimethacrylate). These materials have been used recently for the delivery of neurotrophins to cells for axonal regeneration. Piotrowicz and Shoichet53 synthesized nerve guidance channels from copolymers of HEMA and methyl methacrylate (MMA). They incorporated NGF into the channels either by adding poly (lactic-co-glycolic acid) (PLGA) microspheres to the formulation or by adding a second layer of HEMA containing NGF to the interior wall of the channel. Sustained release of NGF was observed for both systems over a 30-day period, but the release was much higher for the HEMA/NGF coated nerve guidance channels. Shoichet and coworkers54,55 also induced concentration gradients of NGF and NT-3 into macroporous HEMA nerve guidance channels using a gradient mixer. Neurite outgrowth within the channels was observed to follow the gradient of NGF. Additionally, they found that NGF and NT-3 work synergistically, with less NGF required to successfully guide neurite growth when NT-3 was present.

Belkas and coworkers56 filled HEMA-co-MMA nerve tubes with collagen and implanted them into rats that had 10 mm sections of the sciatic nerve removed. A bimodal response was observed in which approximately 60% of the rats improved in comparison to rats which received autografts in place of the nerve tube, while approximately 40% saw no significant healing, potentially as a result of channel collapse. In an expansion of this work, Tsai and coworkers57 filled HEMA-co-MMA hydrogel guidance channels with Matrigel™, MC, fibrin, or type I collagen. Some channels also contained either FGF-1 or NT-3. Channels were implanted into rats
that had undergone a complete spinal cord transection (Figure 3). The presence of the growth factors altered the density and improved the orientation of the growing axons. Two channel formulations (fibrin and multiple channels within a larger channel) led to consistent improvement in recovery as measured by BBB score.

To better mimic the mechanical properties of the spinal cord, Bakshi and coworkers\textsuperscript{58} designed HEMA microporous gels containing 85\% water. These gels had compressive moduli of 3–4 kPa, similar to that of the spinal cord. The flexibility of the gels allows them to easily fit into a defect caused by spinal cord injury, and the presence of the micropores allows for cells to migrate and grow through the gels. Gels were implanted into injured rats and after 1, 2, and 4 weeks, the rats were sacrificed and examined for response to the gels. All gels prompted a moderate inflammatory response, though there was little scarring. In gels soaked in BDNF prior to implantation, axon regeneration was observed, though only transiently (2 weeks). In order to make the nerve conduits bioactive, Yu and Shoichet\textsuperscript{59} copolymerized HEMA with 2-aminoethyl methacrylate, which can be easily modified with peptides. In this work, two peptides derived from laminin (i.e., YIGSR and IKVAV) were used. Peptide-modified conduits templated on polycaprolactone (PCL) fibers (which were subsequently removed, yielding hollow channels) were much more conducive to cell growth and neurite extension compared to unmodified channels.

In a recent study, Bryant and coworkers\textsuperscript{60} used a photomask and ultraviolet light to selectively gel certain regions of a precursor solution, creating channels within poly(HEMA) hydrogels. The gels were created by pouring a HEMA solution containing a biodegradable crosslinker and photoinitiator over templated poly(methyl methacrylate) (PMMA) spheres, which is a technique to create highly-ordered porous hydrogels.\textsuperscript{61} Selective blocking of the UV light and exploitation of reaction behavior was used to create open channels of several hundred microns in diameter, while the rest of the gel contained open, highly interconnected pores of approximately fifty microns in diameter. While the authors did not address the potential application of this system to neural regeneration, it is easy to postulate that within the system, the large pores could facilitate axonal growth and guidance, while the smaller surrounding pores enabled the rapid transport of nutrients to and from the growing cells.

Stokols and Tuszynski\textsuperscript{62,63} created agarose scaffolds with linearly-oriented channels by growing ice crystals along a temperature gradient through a solution of agarose followed by freeze drying. A representative image of the scaffolds is shown in Figure 4, showing longitudinal pores with a honeycomb structure cross-section. BDNF was incorporated into the gels by swelling the freeze-dried gels in the presence of the protein. Prior to implantation in rats, the channels were also filled with collagen. The presence of a growth factor allowed for 2–3 times as many axons to penetrate through the channels compared to negative controls (no BDNF), and the immune response was minimal. However, the authors do not report on the functional recovery of the animals. Stokols, Tuszynski, and coworkers\textsuperscript{64} also templated agarose channels on polystyrene fibers, yielding a scaffold with uniform channels following...
polystyrene dissolution. Prior to implantation, the authors filled the channels with bone marrow stromal cells (MSCs) or MSCs engineered to produce BDNF. BDNF production by the MSCs in vivo greatly enhanced the ability of regenerating axons to penetrate into the scaffold and span the length of the scaffold. However, the functional recovery of the animals was not reported.

PATTERNED AND COMPOSITE HYDROGELS

To provide spatial chemical cues and to enhance material properties, numerous techniques such as patterning and the use of composite materials have been investigated. As demonstrated by Nomura and coworkers, both the mechanical and chemical properties of implants are important for the development of a successful implant. Loading one material within another material allows for further tuning of both types of properties in tandem with each other.

The 2-nitrobenzyl moiety is well-known as a photo-protecting group, and recently has found a niche in the development of functional biomaterials which can be patterned in multiple dimensions. In a pioneering work, Luo and Shoichet conjugated agarose polymers with 2-nitrobenzyl cysteine. Following gelation, selected portions of the gels were exposed to ultraviolet light, liberating the thiol sidechain of the blocked cysteine. Further conjugation of the liberated cysteine residues allowed for biofunctionalization of the gels with small peptides or proteins. Rat dorsal root ganglia were then seeded on the gels and seen to grow through the columns of gel containing the peptide, as patterned via the light activation. This work reports the spatial control of only two dimensions or at best a gradient in the z direction. However, it is easy to see how such a system could easily be expanded to three dimensional patterning using advanced microscopy and laser techniques and could incorporate the delivery of neurotrophic factors.

To study how cells move along a molecular gradient, Dodla and Bellamkonda immobilized laminin-1 (LN-1) in an agarose gel. Gradients of different degrees were created by diffusion of LN-1 through the gel followed by photoimmobilization. Interestingly, the lower concentration gradients of LN-1 did a better job of promoting directionality in neurite extension and growth. These results are potentially useful for the development of regenerative materials that could be modified with gradients of neurotrophins and used in vivo for axonal growth and guidance.

Fibrin is another natural polymer that has been widely used in the biomaterials field. Sakiyama-Elbert and coworkers have shown progress in the controlled release of neurotrophins from fibrin gels through interactions with heparin or peptides. Heparin interacts non-covalently with various neurotrophins, such as NGF, BDNF and NT-3. Heparin was attached to fibrin gels that contained immobilized heparin binding peptides within the matrix to influence the release of neurotrophins from the fibrin gels. A schematic of this process is illustrated in Figure 5. Decreased release rates from the gels were observed and reported for both NGF and NT-3. and were observed to significantly improve neurite extension in vitro. In in vivo studies, regeneration of...
axons was significantly improved by the inclusion of heparin into the fibrin matrices to control the release of NGF or NT-3. As another method to slow the release of NGF from fibrin matrices, phage display was used to positively select an NGF binding peptide, which was then immobilized in the fibrin matrix. An improved response in neurite extension was observed relative to free NGF in a fibrin matrix and the response was both dose and pH dependent.

Towards another composite structure, Nomura and coworkers carried out a study similar to that carried out by Belkas and coworkers (discussed above) with HEMA or HEMA-co-MMA channels loaded with fibrin, acidic fibroblast growth factor (aFGF), and heparin and reinforced with a PCL coil. They employed the coil to alleviate the problems of tube collapse that was observed in the previous study. The coil was successful in preventing collapse of the nerve tubes; however, no axonal regeneration was observed as a result of the development of syringomyelia (a cyst which causes nerve degeneration) and migration of the rostral stump. This work emphasizes the importance of mechanical properties of the hydrogel towards the successful regeneration of the tissues.

Combining synthetic polymers as drug releasing devices within a natural polymer matrix, Goraltchouk and coworkers incorporated PLGA 50/50 microspheres containing bovine serum albumin (BSA) or epidermal growth factor (EGF) within chitosan/chitin nerve guidance channels. PLGA is an example of a poly(α-hydroxy ester) that has been widely explored as a degradable material for tissue engineering and drug delivery. They observed controlled release of protein from the microspheres into solution over an 84-day period compared to only 70 days for the free microspheres. The released EGF from the nerve guidance channels was able to successfully promote the formation of neurospheres in culture for the first 14 days of release. However, by day 21, the released EGF was no longer biologically active. The lack of availability of bioactive EGF is a very serious potential limitation that must be addressed for the development of sustained release implants. Others have entrapped numerous neurotrophins in microspheres for delivery in vivo. Yu and Bellamkonda loaded poly(sulfone) guidance channels with LN-1-modified agarose and lipid microtubules loaded with NGF for slow release. The channels were implanted in a 10-mm defect in the sciatic nerve of rats. Recovery and regeneration were statistically the same as autografts, though neither group returned to ‘normal’ function. As an additional example, Yang and coworkers formed porous nerve conduits of PLGA 75/25 containing NGF (illustrated in Figure 6). Successful release of NGF was observed and release could be sustained up to a 40-day period by changes in porosity, mechanism of NGF incorporation, and polymer molecular weight. Others have used non-porous scaffolding for neural regeneration applications but this is beyond the scope of this review.

**FUTURE DIRECTIONS**

As reviewed above, a substantial amount of work has been performed in the area of hydrogel delivery of trophic factors. This work has led to significant advances in the development of potential therapeutics for individuals with damage to their neural system. However, recent advances in the engineering of materials, polymer synthesis, and neural biology open up further avenues for research in this area. Several important and developing technologies are outlined below.

Stimuli responsive hydrogels are being developed for numerous applications, where some external stimuli (pH, temperature, light) are used to alter hydrogel properties, and consequently, molecule release. For instance, poly(N-isopropylacrylamide) hydrogels respond and deswell with increased temperatures, which can lead to the release of growth factors. Das and coworkers recently incorporated light-sensitive gold nanoshells into these hydrogels, where the gold nanoshells absorb light at a predefined wavelength, heat up, and initiate the hydrogel thermal response. In this system, light can be transmitted transdermally to trigger release, leading to a well defined release profile. Stayton and coworkers have developed several novel copolymer systems that are responsive...
to pH. These polymers are used as gene delivery vehicles which swell at low pH and facilitate release from the endosome, allowing more specific, local release of the gene. Such a polymer could be potentially useful for intracellular delivery or delivery around the injury where the pH has been shown to be slightly lower than surrounding tissues.\textsuperscript{89} Thus, advances in polymer synthesis and processing are leading to novel approaches for growth factor, and potentially, neurotrophin delivery.

In a different area, Mi and coworkers identified pleiotrophin (PTN, also known as heparin binding neurotrophic factor) as a neurotrophic factor that aids in the recovery of damaged motor neurons in the spinal cord.\textsuperscript{90} Following nerve injury, a significant upregulation of PTN was observed in recovering nerves near the site of injury for up to 1 month. \textit{In vitro} studies showed that PTN could induce axonal growth in a culture of spinal cord explants in the direction of the PTN source. Following a sciatic nerve transection, HEK–293\textsuperscript{PTN} cells were delivered in a silicone tube to the site of injury. After 8 weeks, there was a ten-fold increase in axon density compared to control, and some functional recovery was observed. Additionally, when HEK–293\textsuperscript{PTN} cells were transplanted in gelfoams to the site of facial nerve injury in mouse pups, 63\% of the facial motor neurons recovered compared to 12\% of the controls. The emergence of PTN as another neurotrophic factor that plays a role in the recovery of injured neurons should further aid in the search for the ideal delivery system/delivery agent(s) combination.

Finally, recent trends are towards combinatorial approaches for the delivery of multiple stimulatory factors that together can have an additive effect on regeneration. One example by Lu and coworkers\textsuperscript{16} illustrated that the delivery of both cAMP and NT-3 provided elevated recovery in animals with spinal cord injury over the delivery of only one of the molecules. Similar results were also seen in the work done by Moore and coworkers with NT-3 and NGF.\textsuperscript{55} Biomaterials, and specifically, hydrogels, are ideal candidates for the delivery of multiple factors, each with individual release profiles, as a result of our excellent control over polymer behavior. Additionally, various stem cells and novel scaffolding may be combined in combination with neurotrophin delivery to accelerate healing and recovery.

**CONCLUSION**

The application of synthetic and natural materials for neurotrophin delivery has only recently been used and is finding widespread success. While no single system explored has yet to provide what many would consider to be complete recovery, the results from many of these studies are promising and for many of these applications, incremental improvements correlate to significant enhancement in quality of life. Continued research in this field will shed new light on the role each neurotrophin plays in the healing of neural tissues, as well as on the role that the physical material
can play in the delivery of the neurotrophin and support of growing axons at the injury site. Recent trends and expected directions in this field promise even more advanced systems of responsive materials, novel trophic factors, and combinatorial approaches that will allow for promising treatment methods for patients with debilitating neurological conditions.

REFERENCES


RELATED ONLINE ARTICLES

Neuroregenerative scaffolds for CNS repair.
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Mesoporous silica-based nanomaterials for drug delivery: evaluation of structural properties associated with release rate

Maria Strømme,* Ulrika Brohede, Rambabu Atluri1 and Alfonso E. Garcia-Bennett1

We present here a study of the controlled release of amino acid-derived amphiphilic molecules from the internal pore structure of mesoporous nanoparticle drug delivery systems with different structural properties, namely cubic and hexagonal structures of various degrees of complexity. The internal pore surface of the nanomaterials presented has been functionalised with amine moieties through a one-pot method. Release profiles obtained by conductivity measurements are interpreted in terms of specific structural and textural parameters of the porous nanoparticles, such as pore geometry and connectivity. Results indicate that diffusion coefficients are lower by as much 4 orders of magnitude in two-dimensional structures in comparison to three-dimensional mesoporous solids. A fast release in turn is observed from mesocaged materials AMS-9 and AMS-8, where the presence of structural defects is thought to lead to a slightly lower diffusion coefficient in the latter. We conclude that the use of single or mixed phases of these porous systems can be utilized to provide sustained release over long time periods and expect their use in a variety of formulations.

INTRODUCTION

Drug delivery systems (DDS) are used to facilitate the delivery of pharmaceuticals or other therapeutics (below called drugs). They are devices created by combining knowledge of formulation technology and material science with knowledge of chemical interactions and biological processes. Traditional DDS usually administer drugs with an initial burst of drug release. As the amount of drug falls below the minimum effective serum concentration, the therapeutic effect is lost. In order to keep the concentration within the therapeutic range, several administrations are usually required. Therefore, to avoid frequent administration, controlled DDS are needed. Such systems can either speed up or slow down the in vivo drug uptake. Systems providing sustained, extended or immediate release, as well as depot delivery, fast dissolving and chewable tablets are all variations of rate-controlling technologies that might be used to help deliver drugs. There is no ‘one-type-fits-all’ drug delivery technology that fulfils every need or desire to deliver a drug in a particular way.

In the sustained-release category drug delivery vehicles based on polymer and polysaccharide matrixes, coated reservoirs and other traditional dissolution or diffusion controlled systems1 are totally dominating the market. However, as stated by one of the editors of Modern Drug Discovery, ‘Innovative pharmaceutical treatments require innovative methods of administration’,2 and therefore innovative DDS.

In general, the smaller the size of the drug-carrying vehicle, the better is the absorption in the body. With new therapeutic agents such as proteins,
nucleic acids and poorly soluble drugs, new nanotype carriers may offer not-yet-realized possibilities to drug administration.\(^3\)

One of the most promising categories of nanoparticles and microparticles for which an internal ordered pore structure may be tailored to host differently sized drug molecules as well as to create predetermined release profiles are the mesoporous, amorphous silica–based materials. Such materials have been shown to be able to incorporate high dosages of drugs in the internal pore system\(^8\) owing to their large internal surface areas and pore volumes as well as their tuneable pore sizes. It has already been shown that a sustained drug release over several days can be obtained using mesoporous particles.\(^3\) In that seminal study, a mesoporous material, MCM-41, with cylindrical pore geometry was used to host and release ibuprofen, with the drug release over 3 days. These materials offer further interesting strategies for more precise control of the chemical surface properties determining both the drug release and transport properties of the carrier. These may include surface functionalisation and geometrical design of the pores for specific drug candidates.

Recently, mesoporous silicas with controlled nanoparticle size have been prepared using anionic amino acid–derived amphiphiles and alkoxysilane co-structure-directing agents (CSDAs), denoted AMS-\(n\) mesoporous silicas.\(^6\)–\(^8\) Briefly, the synthesis mechanism of AMS-\(n\) mesoporous materials is thought to be governed by the interaction of the CSDA with the self-assembling (micellar) amphiphiles. Using well-established sol–gel techniques, a silicate wall is then condensed surrounding the micellar–CSDA complex, and a mesoporous solid is eventually achieved through the preferential calcination (or solvent extraction) of the organic amphiphile from the condensed silica particle. The micellar ‘liquid crystal’ phase of the anionic amphiphile is hence reproduced or templated into silica form. The use of CSDAs has the double advantage, as it not only acts as a structural directing agent but, if the amphiphile is removed through solvent extraction, it also leads to a homogeneous coating of organic functional groups on the internal surface of the pores. A schematic diagram of the synthesis mechanism and the final material composition is shown in Figure 1.

In our investigations of the silicate particles as DDS, we have utilised the incorporated amphiphilic molecules—which are anchored via electrostatic interactions to the internal surface of the mesopores—as the model drugs. Considering the synthesis mechanism of AMS-\(n\) materials, we can obtain a very well defined and consistent (reproducible) interaction between model drug and particle surface for all the structures in this report, as well as certainty of the position of the amphiphilic molecule within the porous matrix. Furthermore, the loading amount of the molecule is predetermined from the synthesis mixture and can be confirmed through conventional thermogravimetric analysis. It has already been shown that the incorporation of model drugs molecules can be achieved by direct synthesis through three distinct routes: (1) by utilizing the hydrophobic core of the amphiphile micelle in order to solubilise non-polar molecules;\(^9\) (2) through electrostatic interaction with the hydrophilic headgroup; and (3) by a simple addition to the synthesis gel leading to inhomogeneous incorporation and loss of porous structure.\(^10\) Post-synthetic methods in which functionalisation of internal pore surfaces has been utilized are also a useful and facile alternative for drug loading. In such a case, the ideal confirmation is an homogeneous functionalisation of the entire internal pore surface together with a stoichiometric concentration of drug molecules over such a surface.\(^3\) The use of amino acid–derived amphiphilic molecules in this study allows us to probe the release from such ideal confirmations, enabling us to for the first time study release from a variety of periodic porous structures.

In this study mesoporous nanoparticles and microparticles forming mesocaged cubic AMS-8 (\(Fd\overline{3}m\)), tetragonal AMS-9 (\(P4_1/mmm\)), cylindrical, bicontinuous cubic AMS-6 (\(Ia\overline{3}d\)) and hexagonal AMS-3 (\(p6mm\)) structures have been prepared and characterised extensively using N-lauroyl glutamic acid (N-Lauroyl glutamic acid) as a model drug. For comparison purpose, the use of the amphiphile as a model drug ensures complete drug loading and complete electrostatic interaction of the model drug with the internal porous surface.

\[\text{FIGURE 1} \quad \text{Schematic representation of the self-assembly mechanism of AMS-}\(n\) mesoporous materials and the use of the amino acid–derived amphiphile, N-lauroyl-glutamic acid, as a model drug. For comparison purpose, the use of the amphiphile as a model drug ensures complete drug loading and complete electrostatic interaction of the model drug with the internal porous surface.}\]
acid (C\textsubscript{12}-Glut) and N-lauroyl alanine (C\textsubscript{12}-Ala) anionic amphiphiles in combination with amnon-
propyl triethoxysilane (APES) as CSDA as previously described in the literature.\textsuperscript{11} The mesoporous
structures AMS-6 and AMS-3 are analogous to the well-known MCM-48 and MCM-41 materials. A recent study of the immunological response of AMS-6 and AMS-8 particles showed low tox-
icity profiles on monocyte-derived dendritic cells, and the particles provided a promising approach worth further development into drug/vaccine delivery systems.\textsuperscript{12}

The aim of the present work is to evaluate how the control over structural properties such as pore geometry, connectivity (two- or three-dimensional) and pore wall chemistry, within the context of mesoporous nanoparticles and microparticles, can be used to achieve well-defined, controlled release over extended time periods. An extended version of the present work will be published elsewhere.\textsuperscript{13}

In molecular terms, the release process may be considered as a random walk of molecules inside the pore system of the carrier. The first 60% of release curves has been shown to be adequately described by a semi-empirical power-law expression: \textsuperscript{14–18}

\begin{equation}
Q = a + b \cdot t^c
\end{equation}

for various carrier symmetries such as planar, cylindrical and spherical. Here \( Q \) is the amount of molecules released per unit exposed area of the carrier, \( t \) denotes time and \( a, b, \) and \( c \) are constants.

The power-law function, Eq. (1), is related to the Weibull function that has been suggested as a universal tool for describing release from both Euclidian and fractal systems, and may be considered as a short-
time approximation of the latter.\textsuperscript{19} The constant \( a \) takes initial delay and burst effects into account, and \( b \) is a kinetic constant.\textsuperscript{20} The power-law exponent, \( c \), also called the transport coefficient, characterises the diffusion process, and equals 0.5 for ordinary Case I–or carrier controlled–diffusion in systems for which no swelling of the carrier material occurs.\textsuperscript{21}

Diffusion-controlled release from a planar system, in which the carrier structure is inert, may be described by the Higuchi square-root-of-time law: \textsuperscript{14}

\begin{equation}
Q = \sqrt{D_{\text{eff}} C_m (2C_m - \varepsilon C_m)} \cdot t
\end{equation}

Here \( D_{\text{eff}} \) is the effective diffusion coefficient of the drug inside the carrier, and \( C_m \) is the total amount of drug present in the carrier per unit volume, \( \varepsilon \) is the total porosity of the carrier, defined as the volume fraction of pores when the drug material has been removed (here referred to as the porosity of the calcined samples), and \( C_s \) is solubility of the drug in the release medium. For model drugs that are amphiphiles, the critical micelle concentration (CMC) can be treated as the solubility limit for the molecularly dispersed species, especially when the aggregation number of the micelles is high\textsuperscript{19} as is the case for the amphiphiles used as model drugs in the present work.\textsuperscript{22}

For detailed expressions for \( D_{\text{eff}}, \varepsilon, C_m, \) and the total outer particle area \( A_{\text{tot}} \) exposed to the liquid in the release measurements see Supplementary Information 1 (SI1).

EXPERIMENTAL

Synthesis of mesoporous nanoparticles

The general synthesis of AMS-\( n \) mesoporous materials has been reported previously.\textsuperscript{7} For details on the synthesis conditions used in the present work, see Supplementary Information 2 (SI2).

Structural, textual and compositional characterisation

\( \text{N}_2 \) adsorption–desorption isotherms were measured at \(-196 \)°C on an ASAP2020 Micromeritics Instrument. The calcined mesoporous materials were out-
gassed for a period of 6 h at 200 °C and 0.3 kPa pressure. The BET specific surface area\textsuperscript{23} was evaluated from the adsorption data in the relative pressure range from 0.05 to 0.3. The total volume of intra-
particle pores \( V_{\text{tot}} \) was extracted from the adsorption data as the amount adsorbed at a relative \( \text{N}_2 \) gas pressure of 0.9. This upper pressure limit was chosen in order to exclude the inter-particle porosity contribu-
tion to the pore volume. Pore size distribution curves were derived from desorption curves using the non-
local density functional theory (NLDT) method\textsuperscript{24} assuming a spherical model of cages.

Scanning electron microscopy (SEM) images were recorded using a LEO 1550 SEM, equipped with a Schottky field-emission gun. The SEM was operated at 3 kV and at magnifications of between 20,000 and 50,000×.

Thermogravimetric (TG) analyses were performed on a Mettler TGA instrument by heating the samples from 25 to 900°C at a heating rate of 10°C/min on an alumina holder under the flow of air at 20 mL/min. From these data, the particle composi-
tion as well as the amount of amphiphilic molecules released could be assessed.
Release studied by conductivity measurements

Release measurements were performed on three to five samples each from the four types of as-synthesised (un-calcined) mesoporous particle types under study in 20 mL of distilled water as dissolution medium by using ac conductivity measurements as implemented by the alternating ionic current (AIC) technique. The water was kept at a temperature of 65 ± 3 °C during the entire release measurement by keeping the measuring cell in an incubator (incucell IC 55, BMT a.s., Brno, Czechia). This elevated temperature was used because of the rather poor solubility of alanine at room temperature. The experimental set-up used was identical to that described elsewhere, except that the cubic measuring cell was somewhat larger (4 × 4 × 4 cm³) and equipped with stainless steel electrodes covering two opposite surfaces of the cell. The cell was covered by a lid to prevent water evaporation during the entire measurement. A function generator (HP 3325A) applied an alternating voltage (1 VAMS, 10 kHz) to the electrodes of the cell, and the conductance of the cell was calculated from measurements of the voltage across the cell and the current that passed through it using two digital multimeters (Agilent 34401A). The number of molecules released was thus assessed from the conductance measured across the dissolution medium. This experimental set-up minimises delays caused by transport times to the measurement site, and therefore enhances the temporal resolution of the measurement. In order to assess the CMC of the two types of amphiphilic molecules used (C12Glut and C12Ala), conductivity versus concentration curves were recorded at the same conditions as for the release experiments. The CMC value was then extracted in the usual manner: as the concentration for which a discontinuity in the slope of the curve appeared.

RESULTS AND DISCUSSION

Analysis of the synthesised material

The N2 adsorption–desorption isotherms are displayed in Figure S3.1 of Supplementary Information 3 (SI3), together with insets showing the pore size distribution as obtained using the NLDFT approach assuming cylindrical and slit-type pore geometries (for cage structures). The textural properties of the samples are shown in Table 1. As evident from the adsorption isotherm curves, the relative pressure of 0.9 used for the total mesopore volume calculation is located well below the region where capillary condensation in inter-particle voids occurs for all particle types under study. The total pore volume for sample AMS-9 is considerably smaller than that reported previously in the literature and possesses a smaller pore size. This may be due to pore-blocking of cavities within the tetragonal structure. This effect has been observed previously and may be due to prolonged hydrothermal treatment. Smaller cage/cavity windows may influence the release rate considerably, providing a geometrical inhibition for the release of the molecules under study, and hence this sample was deemed adequate within the scope of this project.

Analysis of SEM images show that all synthesized particles are approximately spherical and fairly mono-dispersed (Figure 2). The AMS-3 sample prepared in this project, however, contains some rod-shaped particles of approximately the same diameter (but with a somewhat larger length) as the spherical ones (Figure 2(a)). The radii used for the assessment of the drug-releasing particle area, Aint, needed to obtain Q, were obtained by extracting the particle sizes from more than 10 SEM images of each sample. The radii are presented in Table 1, and, as can be seen, AMS-8 and AMS-9 samples consist of microparticles, while for AMS-3 and AMS-6 the particle radii are ~200 and 160 nm, respectively.

Analysis of the release process

The amphiphile CMC values (C in Eqs (2) and (S1.1)) obtained from conductivity measurements at 65 °C are shown in Table S1.1 in Supplementary Information 1 (SI1). As expected, the measured CMC values 65 °C are somewhat higher than corresponding values previously obtained at 40 °C.26,27 TG data normalized at 100 °C for an AMS-8 sample before and after a release experiment are displayed in Figure S3.2 in Supplementary Information 3 (SI3). All samples show TG curves characterised by an initial weight loss below ~100 °C owing to the loss of adsorbed moisture and solvent. Three further distinctive weight losses are seen at around 200 (step I), 400 (step II) and 600 °C (step III). The latter step has been previously associated with dehydration of framework silanol groups and is quite prominent in all materials synthesised in this study, with an average loss of 10 wt%. The loss in step II takes place over a broad temperature range (250–450 °C) and can be associated with the decomposition of both the anionic amphiphile and amino-functional groups bound to the pore wall. The decomposition of the surface-bound functional groups at higher temperatures than the ‘free’ functional group (measured at 200 °C for APES) is consistent
<table>
<thead>
<tr>
<th>AMS-( n )</th>
<th>Pore Diameter(^a) (DFT, Å)</th>
<th>Surface Area ( \varepsilon ) (BET, m(^2)/g)</th>
<th>Total Pore Volume ( C_m ) ((\text{cm}^3))</th>
<th>Average Particle Radius ( D_{\text{eff}} ) (SEM, nm)</th>
<th>Unreacted</th>
<th>Reacted</th>
<th>Amphiphile(^b) (TG, %)</th>
<th>Outer Particle Surface Area ( A_{\text{br}} ) (m(^2)/g)</th>
<th>Porosity ( \varepsilon )</th>
<th>Volume ( V_{\text{tot}} ) ( (\text{amphiphiles}/\text{cm}^2) )</th>
<th>( D_{\text{eff}} ) (cm(^2)/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cylindrical hexagonal AMS-3</td>
<td>41.0</td>
<td>559.3</td>
<td>0.561</td>
<td>200 ± 15</td>
<td>3.66</td>
<td>5.73</td>
<td>26.15</td>
<td>10.67 ± 1.5</td>
<td>0.602</td>
<td>8.45 × 10(^{26})</td>
<td>1.37 × 10(^{-17}) ±0.09 × 10(^{-17})</td>
</tr>
<tr>
<td>Cylindrical cubic AMS-6</td>
<td>52.0</td>
<td>520.0</td>
<td>0.569</td>
<td>160 ± 13</td>
<td>2.85</td>
<td>6.55</td>
<td>19.27</td>
<td>12.78 ± 2.7</td>
<td>0.582</td>
<td>6.459 × 10(^{26})</td>
<td>2.22 × 10(^{-14}) ±0.12 × 10(^{-14})</td>
</tr>
<tr>
<td>Caged cubic AMS-8</td>
<td>33.1</td>
<td>547.6</td>
<td>0.321</td>
<td>1135 ± 110</td>
<td>3.60</td>
<td>8.98</td>
<td>13.76</td>
<td>1.73 ± 0.32</td>
<td>0.481</td>
<td>3.985 × 10(^{26})</td>
<td>4.05 × 10(^{-12}) ±0.32 × 10(^{-12})</td>
</tr>
<tr>
<td>Caged tetragonal AMS-9</td>
<td>16.6</td>
<td>419.0</td>
<td>0.196</td>
<td>3045 ± 175</td>
<td>5.47</td>
<td>7.1</td>
<td>25.07</td>
<td>0.68 ± 0.07</td>
<td>0.349</td>
<td>7.024 × 10(^{26})</td>
<td>7.71 × 10(^{-11}) ±0.51 × 10(^{-11})</td>
</tr>
</tbody>
</table>

\(^{a}\)Based on a cylindrical pore geometry for AMS-3 and AMS-6 and a spherical pore geometry for AMS-8 and AMS-9.

\(^{b}\)Obtained from TG measurements of as-synthesised particles.

\(^{c}\)Obtained from TG and SEM data using Eq. (S1.2)
with other published works on amino and other functional groups. The weight loss in step I is therefore considered here to be due to the decomposition of the aminopropyl groups not taking part in the co-assembly process with the amphiphilic molecule. A plot of absolute values of the first derivative of the weight loss with respect to temperature is also included in Figure S3.2 of Supplementary Information 3. The amounts of APES and amphiphile present in the as-synthesised and the released particles have been estimated by subtracting the weight loss in step II from the total weight of APES added to the synthesis gel. In Table 1, the amount of APES and amphiphiles present in the as-synthesised samples as well as the weight loss estimated from step I is summarized, together with the $A_{tot}$ and $C_m$ values calculated from Eq. (S1.2) and (S1.4), respectively (Supplementary Information 1).

Figure 3 shows the initial part of the typical release curves for the different mesoporous structures under study. For clarity, log-lin plots of the full release curves have been included in the figure inset. The $Q$ values on the vertical axis have been obtained from TG analyses of each released sample and by using the $A_{tot}$ values (divided by 10 since 100 mg, not 1 g, was used in the release experiments) in Table 1. From release curves similar to those in Figure 3, an effective diffusion coefficient for the release process was extracted according to Eq. (S1.1). The results are summarized in Table 1.

None of the mesoporous particles released the total amount of amphiphilic molecules hosted in the structure during the time span of a release experiment. The cage-type AMS-8 and AMS-9 released 53 ± 5% and 51 ± 2%, respectively, of the total amphiphile content during the first 8 h of the release experiment. It should be noted that the significantly different values of $Q$ reached at the end of the release experiment for the AMS-8 and AMS-9 samples shown in Figure 3 do not imply that very different amounts of amphiphiles have been released from these structures; $Q$ is the released amount of molecules normalized by $A_{tot}$, and this area differs by a factor of ~2.5 between the structures (Table 1). There appears to be very little steric constraint imposed on the diffusion of molecules by the presence of a cage connecting window for both of these structures. Although there are considerable differences in the textural properties, these two structures are very similar crystallographically; both are assumed to form from cubic and tetragonal close packing of spheres with intricate 3D connectivities. The AMS-8 structure is known to be composed of two networks of cages of different sizes, while the AMS-9 structure is thought to be composed of three networks of cages of different sizes and has lower symmetry than AMS-8. Adsorption isotherm data measured for AMS-9 shows low textural properties, which might have resulted from a prolonged hydrothermal treatment. Furthermore, most samples of AMS-8 contain a certain amount of structural defects, the sample prepared here being no exception as determined by TEM. All these factors appear to have little effect on the amount released after 8 h; however, the diffusion coefficient for AMS-9 is approximately one order of magnitude higher than that of AMS-8, which could be attributed to presence of stacking fault defects in AMS-8 and the resulting more intricate system of cages (higher tortuosity). Unexpectedly, AMS-9 shows the fastest diffusion coefficient of all mesoporous structures studied here.

For the cylindrical type AMS-3 and AMS-6 structures, the corresponding amount released was 63 ± 1.5% and 39 ± 2% after about 57 and 18 h, respectively. When comparing the diffusion coefficients for C$_{12}$-Ala in the AMS-3 and AMS-6 structures, we find that $D_{eff}$ in AMS-3 is extremely low and more than 3 orders of magnitude smaller than in the AMS-6 structure. This significant difference between AMS-3 and AMS-6 can be explained in terms of the connectivity of the two cylindrical pore structures, since textural properties, morphology and amount of functionalisation are all in the same range (Table 1). The 2D hexagonal structure (AMS-3$^{13}$) shows the slowest diffusion coefficient of all AMS-$n$ materials presented here, while all 3D cubic structures (AMS-6 and AMS-8$^{13}$) show larger
diffusion coefficients and larger amounts released per unit time.

Clearly, the diffusion coefficients of C\textsubscript{12}-Glu in the AMS-8 and AMS-9 structures are several orders of magnitude larger than those for C\textsubscript{12}-Ala in the AMS-3 and AMS-6 structures. For comparison, the pore wall surface structures of mesoporous materials AMS-3, AMS-6 and AMS-8 are shown in Figure 4. The intertwined bicontinuous surface of AMS-6 and the 3D connectivity in the structure of AMS-8 are worth noting. From these models, it is possible to appreciate how a larger amount may be release from cage structures. There are numerous connections between cages and more connections to the external surface, which, although increase the tortuosity of the cage structure, may contribute largely to the higher diffusion coefficients calculated for these structures. In the case of the cylindrical pore systems, there is no connection between cylindrical channels, and diffusion is limited by the number of openings to the external surface. The nature of the interaction between two molecules C\textsubscript{12}-Glu and C\textsubscript{12}-Ala and the respective silica surface is clearly different, since C\textsubscript{12}-Glu contains two acid moieties in the head group, while C\textsubscript{12}-Ala contains only one. Functional groups on the surface of the mesopores can be considered as anchors for the drug molecules in a potential drug delivery system. TG analysis suggests that the molar ratio of surfactant to APES in the final material is close to 1:0.3, 1:0.4, 1:1 and 1:0.4 for the AMS-3, AMS-6, AMS-8 and AMS-9 structures, respectively. These values appear to support our findings for the cage containing structures, inasmuch as a higher concentration of surface functional groups would result in a slower release rate or \( D\text{eff} \), in particular if the model drug molecule contains two anchoring sites. However, this argument does not explain the apparent differences in \( D\text{eff} \) between the cage and cylindrical structures or, indeed, between the 2D and 3D cylindrical systems studied here.

**CONCLUSION**

In conclusion, a detailed study of the controlled release achieved from mesoporous materials containing cylindrical and cage pore systems of different textural and morphological properties has been conducted. Amino acid–derived amphiphilic molecules, used here as model drug molecules to probe the release, were
loaded directly into the mesoporous structures via the co-assembly of micelles with amino silanes and a silica source, ensuring that for all structural systems the amphiphile is electrostatically bound to the internal surface of the pores. This provides an ideal modelling tool for the investigations of the structure–function relation with respect to the potential use of mesoporous particles as drug carriers and in DDS. It is clear from this work that the connectivity (2D or 3D) of the pores is the key to controlling the release rate with an increase of several orders of magnitude in diffusion coefficient from 3D porous networks with similar textural properties. From this study, it can also be concluded that pore geometry can play an important role in controlling release rates, and we have shown release curves from cage-type ordered mesoporous systems determining that the degree of structural defects may contribute towards a slower diffusion coefficient, and that the presence of cage connecting windows may play an unexpected part in speeding up the release of molecules from the internal cavities as exemplified by the higher diffusion coefficients in the cage structures AMS-8 and AMS-9. Our results do not indicate any clear correlation between electrostatic binding and release rate and suggest that the control of structural properties such as connectivity or pore geometry may be a much more efficient strategy in order to control the release rate. The 2D hexagonal structure of AMS-3 showed the slowest diffusion coefficient of all mesoporous materials studied here as well as the smallest amount of amine functionalisation.

Likewise, samples with particle sizes below 500 nm show a lower release rate than those with larger particle size, although it is not possible to draw significant conclusions at this stage with respect to the effect of particle size on release rates from ordered mesoporous materials. This will form part of our future work.

Overall, we have shown the large, potential use of mesoporous materials with cylindrical and cage-type structures as controlled drug release systems using amino acid–derived amphiphilic molecules as model drugs. It is clear that the use of single phase or mixed phases of these porous systems can be utilized to provide sustained release over long time periods and we expect their use in a variety of formulations.

NOTES

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REFERENCES


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