Nanobiosystems

Article *in* Wiley Interdisciplinary Reviews Nanomedicine and Nanobiotechnology · September 2009

DOI: 10.1002/wnan.49 · Source: PubMed

Advanced Review

Nanobiosystems

Mary-Margaret Seale-Goldsmith[1] and James F. Leary[2]*

'Nanobiosystems' is a relatively new term describing objects in the size range below 150 nm and having structures or functions that link to biological functions. Key features are that these nanosized objects typically self-assemble, are not capable of self-replication, and have functions that take advantage of its size. Nanobiosystems can be made entirely of biological or organic molecules that are organized into nanoparticles (e.g., liposomes, dendrimers) or be totally inorganic (with the exception of surface coatings used for biocompatibility) nanoparticles (e.g., gold, iron oxide, quantum dot nanocrystals). More complex nanobiosystems are inorganic/biologic hybrid composites that may include complex multilayered structures with targeting molecules (e.g., peptides, antibodies, aptamers), cell entry-promoting molecules (e.g., HIV-tat peptide sequence), drugs (small molecules), genes (therapeutic genes, reporter genes), and core nanomaterials (e.g., gold, quantum dot, iron oxide) that give the nanobiosystems sometimes unique detection capabilities by a variety of optical and non-optical modalities (fluorescence, surface plasmon resonance, magnetic resonance imaging). *WIREs Nanomed Nanobiotechnol* 2009 1 553–567

Nanobiosystems are non-existing in nature, but their properties have great potential for new advances in research and medicine. Their unique nanomaterial structures can have toxicities and *in vivo* distribution properties that are somewhat unpredictable. This will necessitate new research in the field of nanotoxicity, where size as well as composition can significantly alter the toxicity properties of these systems. Since these nanobiosystems are small enough to cross cell membranes, and even the blood–brain barrier, new methodologies need to be developed to safely contain them.

Nanobiosystems are devices designed and constructed to interact with biological systems at the nanolevel. Numerous biological and biomedical phenomena occur at the nanometer level, and the current research focus of many fields is nanotechnology. Nanobiosystems provide the ability to probe the sub-optical, molecular level and are becoming powerful tools to study biomolecular processes. Moreover, many uses and specific applications for nanobiosystems have been elucidated. For example, biosensors that can detect and capture molecules or pathogens beyond the limits of detection for current devices are being developed. Nanobiosystems also hold great promise for the field of nanomedicine, where nanostructures are designed to diagnose and provide therapy at the single-cell level.

Numerous examples of nanobiosystems and nanostructure components exist in the literature to date. The development of liposomes and dendrimeric polymers has greatly influenced the field of nanobiosystems and has provided materials and concepts for the development of nanobiosystems. Core nanoparticles have been a common platform for the nanobiosystem construction, notably in examples where the core nanoparticle displays properties that aid in detection or manipulation of the nanobiosystem. Advanced construction of nanomaterials has been a significant more recent research area, and scientists are constructing reproducible, highly controlled nanostructures with elaborate geometries and functions. Combining these advanced nanostructures and nanomaterial building blocks, construction and application of multifunctional nanobiosystems are being performed today. Multifunctional nanobiosystems will perform a variety of biological functions at the molecular level, and these systems will interact with biological systems through natural pathways to probe sub-optical phenomena and direct cellular processes.

*Correspondence to: jfleary@purdue.edu

[1]Weldon School of Biomedical Engineering, Purdue University, W. Lafayette, IN 47907, USA

[2]Department of Basic Medical Sciences, School of Veterinary Medicine and Weldon School of Biomedical Engineering, Bindley Bioscience Center and Birck Nanotechnology Center, Purdue University, W. Lafayette, IN 47907, USA

DOI: 10.1002/wnan.049

With the development of nanobiosystems, many challenges and questions have arisen regarding characterization and assessment of their function. For example, the detection and visualization of nanobiosystems remain a current challenge today. However, many tools are being utilized for the study of nanobiosystems. Nanobiosystems made of metallic nanomaterials can often be imaged by electron microscopy and light-scattering effects. Also, surface science techniques, such as X-ray photoelectron spectroscopy (XPS), have provided insightful data regarding the chemical composition of nanobiosystems. Nanobiosystem detection *in vitro* and *in vivo* has been greatly aided by the incorporation of fluorescent or radiolabels as well as sensitive imaging techniques, such as magnetic resonance imaging (MRI).

Another focus area in the study of nanobiosystems is the interaction with biological systems. At the cellular level, nanobiosystems and their materials may exhibit unique responses because of size, shape, and metabolism of materials. These phenomena can translate into perturbation of cellular function and undesirable effects. However, much research is being performed to understand what materials and doses are biocompatible and well-tolerated by cells. For *in vivo* applications, large-scale toxicity and biodistribution effects are major areas of development. Currently, researchers are focusing on the selection of non-immunogenic materials and understanding dimensions needed for nanostructures in order to guide nanobiosystems to the desired site in a complex *in vivo* environment.

Overall, nanobiosystems have great potential to impact numerous fields in science and medicine. Nanobiosystems are being developed for a variety of applications, and their successful application will likely exceed the efficiency and sensitivity of many current processes and techniques. Numerous examples of nanobiosystems and a general understanding of their use will be presented in this review of nanobiosystems research.

TYPES OF NANOBIOSYSTEMS

Building Blocks

Nanobiosystems today are the result of building blocks that have been developed in the field of nanotechnology and biomaterials. Although numerous examples exist, some of the most notable and extensively studied nanostructures are liposomes and dendrimeric polymers. While liposomes were discovered through the study of cell membrane phospholipids in the early 1960s,[1] dendrimeric polymers development became prominent in the 1990s.[2] Also, metallic nanoparticles have become well-known for their ease of synthesis and numerous advantageous properties at the nanolevel. These examples are among the first structures developed that crossed the threshold from micro- to nanostructures for unique biological applications.

Development of Nanostructures

Liposomes are vesicles comprised of amphiphilic molecules, such as phospholipids that form bilayers and enclose upon themselves to form sphere-shaped particles. Although there is wide-range of size distributions, liposomes typically have diameters nearly 100–200 nm. One of the key advantages in liposome technology is that liposomes self-assemble, meaning that under controlled conditions and with the necessary materials, these vesicles assemble and encapsulate molecules for cellular delivery. Much of the liposome research has focused on development of liposomes with synthetic polyethylene glycol (PEG) phospholipids for the encapsulation of hydrophobic drugs. Some studies have investigated the effects of tumor targeting and gene delivery with antibody- or peptide-conjugated liposomes.[3] Currently, doxil® is an Food and Drug Administration(FDA)-approved liposomal form of the anti-cancer drug doxorubicin where doxorubicin is encapsulated within PEG-based liposomes.[4] While liposomes may be an ideal platform for hydrophobic drug delivery, some of the major drawbacks to liposomes as a platform for nanobiosystems are their short half-life in blood and difficulty monitoring *in vivo*. Further opportunities for liposomal nanobiosystems are likely to arise in the future as a result of the numerous hydrophobic pharmaceutical agents used clinically; however, the large size of liposomes and shorter circulation times *in vivo* are being challenged by smaller, more biologically stable nanoparticle devices.

Another important building block in the development of nanobiosystems is dendrimeric polymers. Highly branched polymers, such as polyamidoamine (PAMAM), have been studied extensively for their biocompatible and non-immunogenic properties. Specifically, these polymers have been investigated for their ability to transfect cells with genetic material because of their ability to cross cell membranes with minimal perturbation.[5,6] More recently, investigators have focused on the nanomaterial properties of dendrimeric polymers for radiolabeled tumor detection and antibody-mediated tumor targeting.[7,8] One of the key strengths with dendrimeric polymers is the very large surface area and variety of biomolecules that can be attached to the dendrimer. In addition, dendrimer

materials have been well-characterized and appear to be generally biocompatible and non-immunogenic. For example, PAMAM dendrimers were conjugated to an fluorescein isothiocyanate(FITC) fluorescent tag and a prostate-specific monoclonal antibody for cellular targeting *in vitro*.[8] Similar to liposomes, polymeric dendrimers will face similar challenges *in vivo* because of break down of the polymers by blood enzymes. On the other hand, it is highly likely that dendrimeric polymers will be a common theme in the development of nanobiosystems because of their multifaceted ability to incorporate drugs, targeting ligands, genetic materials, and labeling compounds all within one structure.[9,10] Other opportunities for dendrimeric polymers exist as coating materials for metallic core nanoparticles, especially as a means to enhance water solubility and increase surface area for functionalization. However, their use in other nanobiosystems will depend on the size constraints of the nanostructure because dendrimeric polymers are likely to increase the size of a nanostructure by approximately 10–100 nm in diameter, depending on the number of dendrimeric layers used.

Solid Core Nanoparticles

Another approach common among nanobiosystem development is construction around a core nanoparticle, where the core material displays unique properties for stability and/or detection of the system. Core nanoparticles typically range in diameter from several nanometers up to 100 nm, and nanoparticles of many different shapes and composite formulations have been developed. In the design of the nanobiosystem, biomolecules are incorporated either within the core nanoparticle or onto the nanoparticle via surface coatings.

One of the most extensively studied core nanoparticle material is metals. Gold, silver, iron, cobalt, nickel, platinum, and various metal composite nanoparticles have been developed and studied extensively in the development of nanobiosystems. Some of the most notable advancements have been made with gold, iron oxide, and composite metal nanoparticles. Gold nanoparticles have been utilized extensively because of their ease of detection by electron microscopy, plasmon resonance properties, and photothermal effects. In addition, conjugation of biomolecules to gold surfaces is readily performed with thiol-containing molecules in aqueous buffers.[11] For example, Thaxton et al.[12] reported a DNA barcode detection method that relies on DNA probes attached to gold nanoparticles. Target DNA is detected by the absorbance shift of the nanoparticles upon binding target DNA or the magnetic separation by hybridizing the bound target DNA with magnetic microparticles. In comparison, iron oxide nanoparticles provide similar density properties for detection by electron microscopy, but their magnetic properties for magnetic resonance contrast and cell separation are unique as compared to other nanoparticle materials. Researchers to date have focused on magnetic nanoparticles as a platform for drug/gene delivery,[13,14] rare cell detection and manipulation,[15] and MRI enhancement *in vivo*.[16,17] Metallic nanoparticles provide stability and enhanced detection capabilities of the nanobiosystem; however, some metals are toxic in elemental form. Nanotoxicology is an embryonic field and the dynamics and toxicity of these nanomaterials *in vivo* are not well-understood at this time. Toxicity of nanomaterials may well vary with nanoparticle size, and toxicity is difficult to evaluate with the masking presence of hydrophilic biocoatings used to coat these generally hydrophobic nanostructures. Toxicity may increase if these biocoatings are removed *in vivo* or inside cells. Metallic nanoparticle toxicity remains a largely unresolved issue in the field of nanoparticle research, and other biodegradable nanobiosystems threaten the development of metallic nanoparticles as nanobiosystems, especially *in vivo*. Opportunities exist for FDA-approved formulations of metallic nanoparticles, such as dextran-iron oxide nanoparticles, to be developed as a platform for nanobiosystems.

Another commonly studied core nanoparticle is the 'quantum dot', named for its unique optical properties and high quantum yield. Quantum dots are typically comprised of semiconductor materials, including cadmium–selenium, cadmium–tellurium, as well as indium–phosphorous.[18] The exceptionally bright and photostable properties, as well as their different emission spectra for quantum dots of the same nanomaterials based on their size, of quantum dots are the reasons for their use as fluorescent nanobiosystems. Quantum dot research has revolved around sensitive optical molecular imaging and monitoring, and quantum dot technology has great potential to improve the sensitivity of diagnostics and molecular detection assays.[19] The main drawback to quantum dot based nanobiosystems is their toxic elemental materials, namely cadmium. Quantum dots have the greatest potential for *in vitro* and small animal nanobiosystem development because of their fluorescent properties. But the ability to detect these fluorescent nanoparticles is limited to typically a few millimeters of depth through skin and tissue making their use limited to near the surface of tissue or if detected by endoscopic analysis. Overall, core nanoparticles are an attractive material for the construction of nanobiosystems because nanoparticle

materials provide enhanced sensitivity for detection and interaction with the biological environment by means of electron density, magnetic properties, light scattering, or fluorescence.

Advanced Construction

In the past decade, many researchers have focused on synthesizing reproducible nanostructures with controllable sizes and geometries. A unique concept in the development of these nanobiosystems is that size and geometry of the nanostructure may dictate very critical biological and material effects. Therefore, many research groups have investigated nanostructures with alternative geometric features.

Nanostructure Geometry

One of the most studied structures with a unique geometric configuration is carbon nanotubes. Carbon nanotubes are lattices of carbon atoms that wrap into a tube with dimensions of 10–100 nm in diameter and up to several hundred microns in length,[20] and these lattices are typically single- or double-walled nanotubes. Although carbon nanotube research has been predominantly in the field of materials science and chemistry, recent advances for biological applications of carbon nanotubes have been made.[21,22] For example, Shim et al.[23] reported a coating method to immobilize functional PEG molecules and streptavidin onto the carbon nanotube surfaces for future applications of attaching biomimetic peptides and antibodies to these functional PEGs. Nanowires have a similar structure to nanotubes with diameters less than 100 nm and lengths up to several microns. Nanowires have been constructed for various applications, such as electrical biosensors for molecular detection. For example, Hahm et al.[24] developed a peptide nucleic acid coated Si nanowire for detection of DNA mutations as a model for detection of cystic fibrosis. Despite the meticulous construction of nanowires and nanotubes, these nanostructures have as yet few specialized applications as nanobiosystems and are not as versatile as other nanobiosystems.

Gold nanoshells and gold nanorods are nanostructures where geometric modifications have been studied extensively because of the substantial effect these modifications have on nanostructure properties. Gold nanoshells synthesized on silica core nanoparticles display high optical scattering and tunable absorption of near infrared (NIR) light.[25] These properties have been used for optical detection of tumor targeting and photothermal ablation of cancer cells.[26,27] In another variation of gold nanostructure geometry, gold nanorods demonstrate detectable single-particle plasmon wavelength shifts that have been utilized to measure target binding to antibody-conjugated nanorods.[28] In addition, nanorods hold great potential for nanobiosystem development because some research suggests that particles with higher aspect ratios will promote reduced phagocytic uptake[29] and longer circulation times *in vivo*.[30] Nanorods and nanoshells, in particular those made with gold, are highly advantageous for the construction of nanobiosystems because of their biocompatibility and optical detection properties.

Biologic Nanostructures

In addition to varying size and shape, some researchers have developed advanced nanostructures with biological materials. Biomolecules, such as peptides, RNA, and DNA, are generally biocompatible and readily integrated with other molecules for drug or gene delivery. In one approach, Falciani and colleagues synthesized branched peptides on a PEG backbone for tumor targeting to increase circulation time.[31] Other groups have designed DNA nanomotors for powering nanodevices,[32] while RNA hairpin probes have demonstrated mRNA detection in live cells.[33]

Nanomotors and nanotubes constructed of nucleic acid molecules are a predominant area of biologic nanostructure research. phi29 packaging RNA has been used as a nanomotor tool for targeted delivery of siRNA to cancer cells by Guo and colleagues.[34] This RNA nanomotor engineered from bacteriophage entry mechanisms is a versatile tool for delivery of environmentally sensitive genetic material. DNA has also been used for the construction of biological nanostructures, such as nanotubes.[35] O'Neill et al.[36]reported synthesis of a more thermally stable DNA nanotube because of T4 ligation. These recent advancements in the field of biologic nanostructures suggest that this biomolecular engineering will promote the use of DNA, RNA, and peptides for advanced applications in nanobiosystems. Despite the unique applications for biologic nanostructures, their complex functions may limit their application as nanobiosystems. For example, protection of nucleic acids is required for *in vivo* applications in order to evade enzymatic degradation. In addition, fluorescent or other tags must be incorporated with the biologic molecule to provide means for detection. On the other hand, biologic nanostructures may present new opportunities if combined with other platforms for nanobiosystems, such as more stable gold nanoparticles or quantum dots.

Nanobiosystems: Current Focus and Future Outlook

From the discovery of initial nanostructures to advanced construction of tunable properties at the nanolevel, nanobiosystems have emerged as nanostructures designed to perform systematic functions at the molecular level. Multilayered nanoparticles are one example of nanobiosystems that are being developed with many different materials to perform a series of functions in the biological environment. Moreover, the advancement of nanobiosystems beyond the research setting will rely on the integration of these multi-functions and the application to the biological environment.

Many advanced functions are needed for the application of autonomous nanobiosystems in a biological environment. One aspect for advanced nanobiosystem construction is self-assembly. Although complex molecules and materials will likely be constructed for the nanobiosystem, natural incorporation of these molecules will increase the ability to produce a functional nanobiosystem. For example, the layer-by-layer method has been used for self-assembly, where charged molecule interactions promote the spontaneous development of coatings and thin films on surfaces.[37] Layer-by-layer functionalization of gold nanoparticles has also been demonstrated as a means to develop a widely applicable coating process for gold nanodevices.[38] These and other methods of self-assembly are likely to be common routes in the development of multilayered nanodevices because they mimic and work together with native chemical and biological processes, thus dictating the function of these nanostructures in biological environments.

In addition to self-assembly, multifunctional nanobiosystems are needed for complex roles in biological applications, such as regenerative nanomedicine at the single-cell level. Some key functions that a nanobiosystem must be able to perform include: targeting, monitoring, external manipulation, error-checking, delivery, and degradation/stealth function. A general schematic of such multifunctional nanobiosystems as described in several publications [39–43] is shown in Figure 1. For example, doxorubicin-loaded thermo-sensitive micelles combined with ultrasound sonication were able to target and deliver drug to cancer cell xenografts in nude mice, and results indicated significant reduction in tumor volumes.[44] This study demonstrated passive targeting of the nanobiosystem via leaky tumor vasculature, delivery of drug to the tumor site via thermal release, and stealth function of the polymers micelle materials to enable sufficient circulation times for tumor uptake. Gross monitoring of micelle accumulation within the tumor site was also performed by ultrasound imaging. One specific challenge for passively targeted nanobiosystems arises when intracellular activation and cytoplasmic delivery are needed for therapeutic efficacy. This is because of the limited amount of drug or therapeutic agent that can be delivery with a nanobiosystem. Merely delivering nanoparticles in the vicinity of tumor cells through leaky vasculature does not guarantee that the nanoparticles will have the desired therapeutic properties, especially if they are taken up through endocytosis and have not been designed to tolerate low intracellular pH environments. Therefore, active targeting and

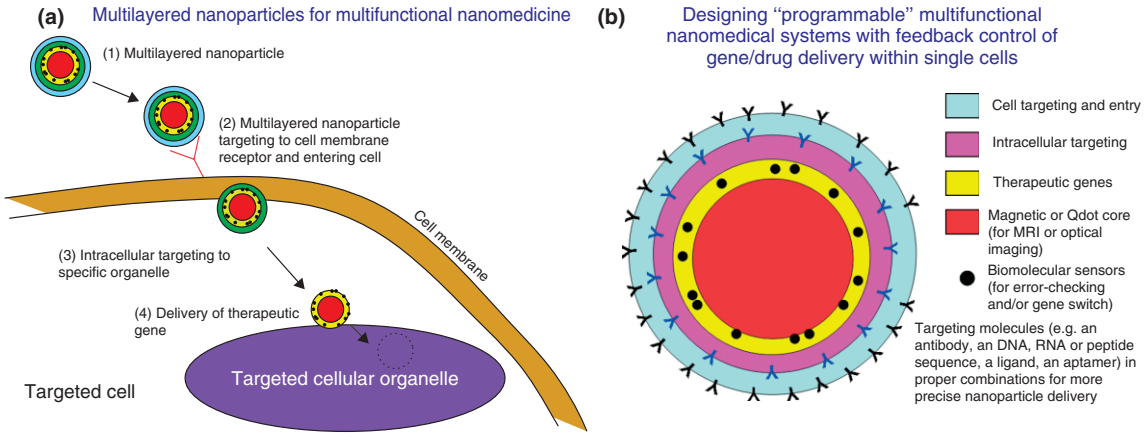

FIGURE 1 | (a) Nanobiosystems undergo a multi-step process that conceptually, and sometimes physically, require a multilayered structure, (b) Multifunctional nanobiosystems can contain specific molecules for targeting, entry, biosensing, and therapy constructed to accomplish a complex set of tasks that through de-layering and chemistry, these nanobiosystems can be considered ''programmable'' by controlling an ordered sequence of events.

receptor-mediated uptake, where the nanobiosystem binds and activates a cellular receptor for intracellular delivery, is needed to selectively increase the amount of drug delivered inside of the diseased cell. This may also allow for less total drug to be delivered overall, leading to reduced side effects and nonspecific toxicity.

Additional studies are being performed to maintain monitoring and external manipulation of targeted cells with nanobiosystems. Bright, photostable quantum dots have been coupled with targeting peptides and siRNA molecules to explore targeted gene knockdown in cancer cells and provide a means for visualizing localization of the nanobiosystem. In one study, enhanced green fluorescent protein (EGFP) siRNA was delivered to EGFP-expressing HeLa cells by peptide-targeted quantum dots. Interestingly, this group noted that quantum dots were trapped in endosomal vesicles and cationic complex-induced release of the quantum dot particles was needed to achieve EGFP knockdown.[45] Nanobiosystems with external modulation of therapeutic properties are also being developed for cancer cell removal. El-Sayed and colleagues synthesized antibody-targeted gold nanorods for photothermal removal of cancer cells. For this application, laser light in the visible range applied to the nanorod-targeted tumor site resulted in heating sufficient to kill targeted cancer cells.[46] Another way to deliver a therapeutic response on nanobiosystems is to deliver a therapeutic gene sequence tethered to a nanoparticle and expressed under the control of an upstream molecular biosensor switch. This concept has been tested on *in vitro* systems modeling the oxidative stress damage that occurs in retinopathies[47,48] using reporter gene constructs that express in response to an oxidative stress biosensor as shown in Figure 2. These studies demonstrate additional advantages of targeted nanobiosystems for monitoring of therapeutic delivery (siRNA) and selective ablation of diseased cells. Construction of such multifunctional nanobiosystems is a current challenge because of the difficulty of assessing whether the all of the biomolecules (e.g., siRNA, targeting peptides) remain functional after crosslinking or conjugation to the nanobiosystem. Current characterization techniques are rapidly advancing to address the need to study smaller structures at a more detailed level, and some of these techniques are described in Section 2.0.

There are abundant examples and formulations of nanobiosystems (Table 1). The development of nanobiosystems today is the result of nanomaterial building blocks and advanced construction of nanostructures. Current research is focused on the incorporation of multiple functions within nanobiosystems in order to apply these systems as biosensors, diagnostic, and therapeutic agents.

HOW DO WE SEE AND MEASURE NANOBIOSYSTEMS?

Nanobiosystems are Sub-optical

Since nanobiosystems are by definition very small, typically 150 nm or lower (National Institutes of

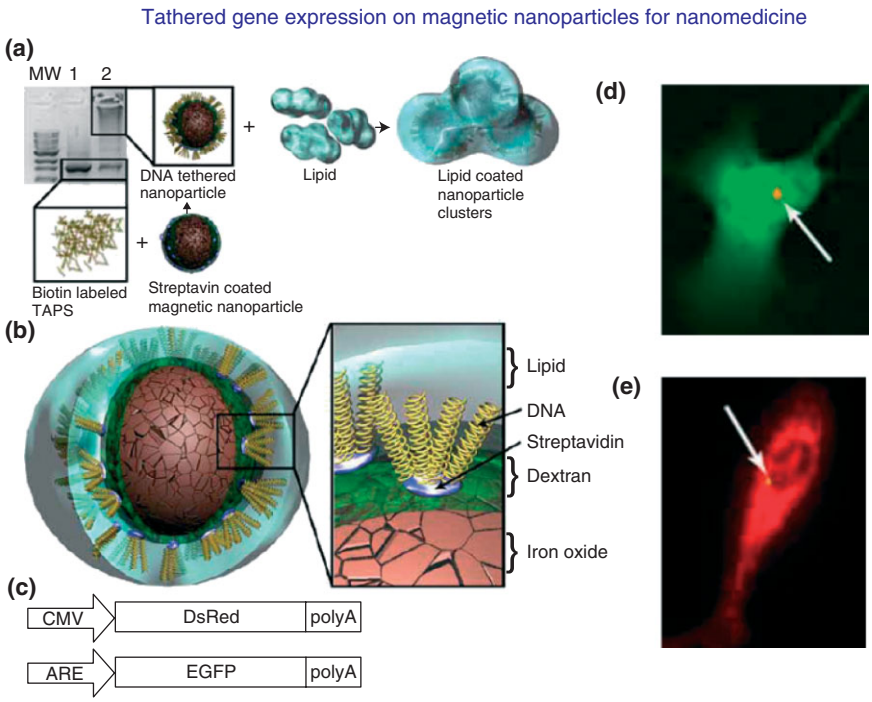

FIGURE 2 | (a) Tethered genes on nanoparticles can express genes and high efficiency, (b) Multiple layers can be built on iron oxide nanoparticle cores, (c) Reporter genes (DsRed and eGFP) can have their expression driven by a cytomegalovirus (CMV) promoter or can even be controlled by upstream antioxidant response element (ARE) molecular biosensor switches, (d) to produce eGFP reporter gene products in response to oxidative stress under control of a stress biosensor, or (e) simply express DsRed reporter gene product freely under a CMV promoter.

TABLE 1 | **Examples of Nanobiosystems and their Designed Applications**

Type of nanobiosystem	Dimensions	Materials (not including coating)	Applications	Reference
Liposomes	Spherical with diameters: 200 nm[49] 95 nm Doxil®[50] 110 nm mAb-Doxil®[50] 136, 165, 209, 275, and 318 nm[51]	Phosphatidylcholine, cholesterol, PEG-phosphatidylethanolamine[49] Distearoylphosphatidylcholine, cholesterol, PEG-distearoylphosphatidylethanolamine α-tocopherol[51]	¬TAT-peptide liposomal transfection[49] ¬ Investigation of antibody-targeted doxorubin liposomes[50] ¬ Biodistribution of liposomes with varying diameters[51] ¬ Liposomal plasmid delivery to inhibit breast cancer in mice[3]	Torchilin[49] Lukyanov[50] Awasthi[51] Chen[3]
Dendrimeric polymers	Cylindrical: 3.6 nm (G3) and 5.4 nm (G5) in diameter[9] G2 and G3 polyplex dendrimers 200–250 nm in diameter[52]	Polyamidoamine (PAMAM)[9] Polypropylenimine[52] Also polyethers, polyesters, poly(ether amides), etc.[53]	¬ Antibody-targeted dendrimers to cancer cells[8] Synthetic vector for gene delivery[52]	Esum[9] Inoue[53] Russ[52] Thomas[8]
Metal nanoparticles	Spherical with diameters: 40 nm[46] 29 nm[14] 4–10 nm[16]	Gold,[46] silver,[54] iron oxide[14] Composite metals: Iron cobalt[16]	¬ Gene delivery with magnetic field assistance[14] ¬ Dual magnetic resonance and near infrared (NIR) imaging agents[16]	Seo[16] Morishita[14] Morones[54] El-Sayed[46]
Quantum dots (QDs)	Near spherical with diameters: 2.4–4.5 nm[55] 10–15 nm with coatings[19]	Cadmium selenium (CdSe)[55] Core-shell CdSe-zinc sulfide[19]	¬ Investigate the cytotoxicity of quantum dots with different surface coatings[55] ¬ Peptide-targeted delivery of siRNA with NIR QDs[45] ¬ Antibody-targeted quantum dots for *in vivo* tumor targeting[19]	Kirchner[55] Derfus[45] Gao[19]
Carbon nanotubes (CNTs)	Cylindrical: 1 nm in diameter by 400 nm length[56] 50 nm in diameter[20]	Unmodified carbon nanotubes	¬ *In vitro* biosensor for cellular release of nitric oxide[22] ¬ Study of *in vitro* toxicity from several forms of functionalized CNTs[57] ¬ PEG functionalized CNT *in vivo* biodistribution[20]	Du[22] Sayes[56] Liu[57] Li[20]

TABLE 1 | *continued*

Type of nanobiosystem	Dimensions	Materials (not including coating)	Applications	Reference
Nanorods	Rod shaped: 1.6 μm × 170 nm in diameter[58] 65 nm length by 11 nm width[59] Aspect ratios 2.8, 4.5[28]	Separate Au and Ni sections on Al_2O_3 nanorod[58] Gold[28,59]	¬ *In vitro* assessment of T-cell response for nanorod vaccine application[58] ¬ PEG-modified nanorods for to improve *in vivo* circulation time[59] ¬ Multiplex biosensing upon different target binding to antibodies on nanorod[28]	Salem[58] Niidome[59] Yu[28]
Nanoshells	Spherical with: 120 nm core diameter, 35-nm-thick shell[26] 120 nm core diameter, 10-nm-thick shell[27]	Silica core, gold nanoshell[26,27]	¬ Molecular imaging in live cells[26] ¬ Antibody-guided cancer cell targeting and photothermal therapy *in vitro*[27]	Loo[26] Loo[27]
RNA/DNA nanomotors	Cone shape, with molecules oriented outward; 6.8 nm in diameter at narrow end 13.8 nm in diameter at wide end[60]	RNA[34,60] DNA[32]	¬ siRNA delivery to cancer cells[34] ¬ Characterization of nanomotor function[32]	Hoeprich[60] Guo[34] Li[32]
Multilayered nanobiosystems	Spherical: 20–100 nm in diameter[44], 40 nm[46]	PEG-based micelles[44] Iron oxide-dextran nanoparticles[17] Gold nanoparticles[46]	¬ Passive tumor targeting with drug-loaded iron oxide nanoparticles *in vivo*[17] ¬ Targeted photothermal treatment of cells[46]	El-Sayed[46] Yu[17] Rapoport[44]

Health (NIH) has defined nanomedicine as the realm from 100 nm diameter and lower), they are below the normal optical limit and cannot be visualized as individual entities by light or fluorescence microscopy. They can be visualized as groups of nanobiosystems, typically by fluorescence microscopy. Many of the quantum dot nanoparticles shown in publications as apparent single fluorescent particles are actually groups of hundreds or even thousands of agglomerated particles that cannot be distinguished individually but appear as larger, optically visible objects. This includes quantum dots shown clustered inside cells, as described in Figure 4 in this review.

Physicists for several generations have been able to 'see' sub-atomic particles by their indirect effects. Other than through the use of transmission electron microscopy (TEM) and, more recently atomic force microscopy (AFM), biologists are not accustomed to dealing with 'entities' that cannot be directly measured, but inferred by indirect means, e.g., through their effects on cells or through measurements that are unique to nanobiosystems and do not exist in the natural biological world.

Nanobiosystem Characterization

Direct Visualization of Nanobiosystems by Electron Microscopy

TEM and scanning electron microscopy (SEM) allow direct visualization of nanobiosystems provided these systems are electron dense. Typically, this property is exhibited by most metals; therefore, numerous metallic nanoparticle formulations are visible by electron microscopy. The main difference for nanobiosystem characterization between TEM and SEM is that TEM provides a two-dimensional slice of the sample being analyzed, while SEM images show the surface configuration of the sample. When combined with standard image analysis techniques, TEM and SEM analyses of nanobiosystems can give size distribution analyses of those systems (at least the electron-dense portions).

Indirect Measures of Size and Charge by 'Zeta-sizing' Technologies

While individual nanobiosystems are below the optical limit and cannot be individually imaged, it does not mean that they do not still interact with light waves. Dynamic light scattering (DLS) is a standard technique used to provide size distributions of nanobiosystems. Interactions of nanobiosystems with cells and tissues are largely governed by the zeta potential of both the cell and the nanobiosystems. Zeta potential is the net electrical charge of the nanobiosystems or cell seen at a distance. Biological cells usually have a net zeta potential of approximately -30 mV because of the presence of many negatively charged molecules (e.g., sialic acid molecules) on the cell surface.[61] For a nanobiosystems to get close enough to the cell surface to interact with the cell through cell surface receptors, it must have a zeta potential in the range of -5 to -20 mV. This zeta potential can, and frequently does, change as it goes from one pH or ionic strength fluid compartment.

Topographical Analysis of Nanobiosystems by AFM

AFM attempts to use nanoengineered cantilevers to interact with cell surfaces. This is not difficult to do if cells are fixed and the measurements can be performed in an air environment. But these measurements become much more challenging if performed in a natural 'water' (or more precisely an isotonic buffer) environment for living cells. The so-called bio-AFM remains challenging but has progressed considerable in recent years. If the nanobiosystems have a magnetic component, such as the presence of a core nanoparticle made of ferric oxide, then a more recent form of this technology known as magnetic force microscopy (MFM) can be used. MFM has the advantage that it uses the magnetic properties of the nanobiosystems that are not present in most natural biological materials.[62] Hence it gives a distinct signal free from background in a way analogous to the advantages of fluorescence microscopy over standard light microscopy.

What is Actually on the Surface of Nanobiosystems?

One of the most important things to know when designing a nanobiosystems is whether or not a molecule added to the system, such as a targeting molecule or a stealth layer (e.g., PEG) is present. In multilayered nanosystems, this technology can be used to see if a new layer is actually where it should be. A relatively recent nanometrology tool known as XPS is used to bombard the surface layers of nanobiosystems to release inner core electrons that have a distinct pattern of energies. XPS analysis can determine whether specific molecules are attached to the nanoparticle as shown in Figure 3.[42]

Detection of Nanobiosystems in Biological Environments

Taking Advantage of Nanobiosystems with Metallic Properties

Another application of TEM is imaging of thin sections of cells and tissues to investigate nanobiosystem interactions. Recently, new forms of TEM can

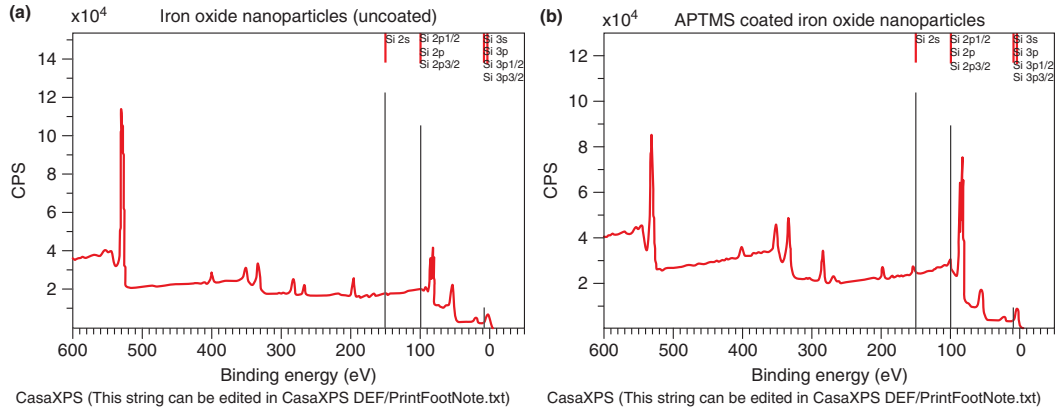

FIGURE 3 | (a) X-ray photoelectron spectroscopy (XPS) analysis of uncoated iron oxide nanoparticles, (b) XPS analysis of APTMS (3-aminopropyltrimethoxysilane) polymer silica coated iron oxide nanoparticles shows presence of silicon atoms in this coating layer.

produce images similar to 3D confocal microscopy images but at the nanolevel.[63] One challenge associated with TEM is that living system must traditionally be chemically fixed prior to TEM analysis to render them capable of surviving the near vacuum conditions under which most TEM instruments must operate. Some attempts at visualizing 'frozen' or even 'live' cells or tissue under high-speed conditions that allow them to visualize in a state nearer to living (non-fixed), but those conditions themselves are hardly equivalent to studying the natural interactions of nanobiosystems and living cells.

Another important technique that takes advantages of difference between natural biological systems and at least partially synthetic nanobiosystems is the interaction of light waves with these metallic surfaces. Nanobiosystems with gold nanoparticle cores have made particular advantage of these properties. If light hits these metallic surfaces at the proper wavelength and angle, energy is transferred to metallic electrons which flow in the surface layers, a phenomenon known as surface plasmon resonance (SPR). This leads to a decrease in the reflected light at given angles from objects containing gold core nanobiosystems during SPR conditions.

Visualizing Nanobiosystems in vivo: NIR and MRI

Visualization of nanobiosystems *in vivo* represents a number of challenges even to visualize fairly large groups of nanobiosystems. Peptide-guided quantum dot nanobiosystems targeting human cells in nude mice can be visualized in tissue sections[64] as shown in Figure 4. However, even these visualizations showing excellent homing of these nanobiosystems to tumors *in vivo* involve thousands of agglomerated peptide-guided quantum dots per cell.

Visualizing nanobiosystems within living animals or humans is much more challenging. The scattering of light by surrounding tissue can confound the visualization of nanobiosystems from this very large background. One way to get around this problem is to wavelength shift the nanobiosystems signal through the use of an attached fluorescence molecule that has good penetration through the tissue both of its exciting wavelength and its fluorescence emission spectrum. The best region of the spectrum for accomplishing this is at the NIR. NIR probes have been used in a variety of systems to visualize nanobiosystems (e.g., targeted to tumors beneath the skin in nude mice) *in vivo*. The limits of depth of these NIR systems are still typically only a few millimeters. Intra-body cavities can also be explored by NIR-labeled nanobiosystems using endoscopy techniques capable of capturing NIR fluorescence.[65]

For more penetrating *in vivo* visualizations a technique already used in human patients is the use of nanobiosystems containing MRI contrast agents. Particularly as concerns about potential toxicity of gadolinium contrast agents grow, there will be increased use of biodegradable iron oxide core nanobiosystems which can serve as targeted MRI contrast agents.

INTERACTIONS BETWEEN NANO- AND BIOLOGICAL SYSTEMS

Biocompatibility

A critical issue for the application of nanobiosystems is biocompatibility. Moreover, this concept is relevant for nanobiosystems for *in vitro*, *ex vivo*, and *in vivo* applications for accurate interpretation of nanobiosystem function. Biocompatibility, as described in the

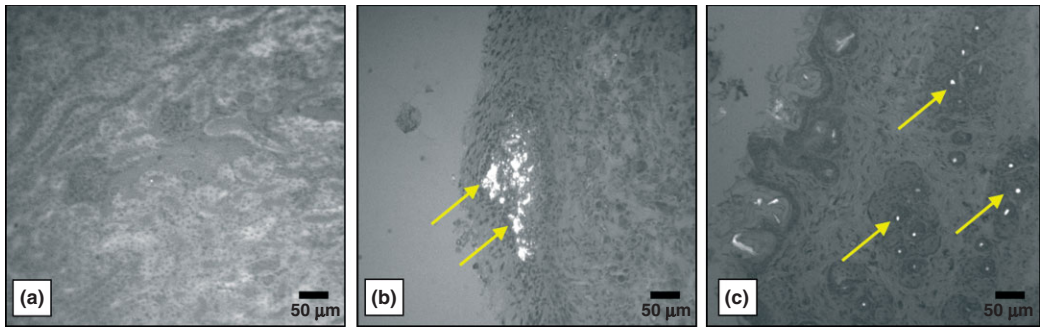

FIGURE 4 | The single bright spots in tumor cells within this histological section of SKBr3 human breast cancer cells excised from a xenograft from a nude mouse after necropsy, are thousands of agglomerated peptide-guided quantum dots nanoparticles that were injected into the tail vein of the animals and targeted to the tumor site through its vasculature.

field of tissue engineering, refers to the ability of a material to perform its designed function without detrimental to the host environment.[66,67] Comparing this concept to nanobiosystem development, several paradigms will be considered. First, materials may elicit toxicity in the biological environment not only because of their chemical formulation but also because of their nanostructure properties. Also, nanobiosystems are being developed for inducing cytotoxic responses in diseased cells and pathogens;[46,68] however, nonspecific cytotoxic effects of the nanobiosystem or its components are not desired. Examples will be provided to highlight recent advancements related to nanomaterial toxicity (nanotoxicity) as well as nanobiosystem interaction with specific cellular pathways and functions. 'Biocoatings' essentially coat nanomaterials, which are generally hydrophobic, with hydrophilic molecules that allow these nanomaterials to disperse in an aqueous environment since virtually all biological cells exist in water environments. These biocoatings can also produce 'biocompatibility' with cells and tissues. But these biocoatings can detach or be degraded from the nanomaterials exposing nanostructures than might be non-biocompatible or even nanotoxic.

Nanotoxicity

Nanotoxicity, or cytotoxicity of nanostructures, is a prevalent topic and much research is being performed in this area. Two common themes in nanotoxicity are: (1) discovering mechanisms of known nanostructure toxicity and (2) determining the level of nanotoxicity for novel nanostructures. For example, carbon nanotubes and CdSe quantum dots have been the subject of toxicological scrutiny because of their material formulations. However, several groups have reported that with biocompatible surface coatings, such as phenyl-SO_3H for carbon nanotubes[56] and PEG-silica for quantum dots,[55,69] these nanostructures are well tolerated by cells *in vitro*. But these biocoatings can also come off the quantum dots and expose more cytotoxic nanomaterials. On the other hand, nanostructures that are generally thought to be biocompatible, such as magnetic nanoparticles,[70] have been shown to elicit cytotoxic response in certain cell types under controlled environments.[71] Overall, the importance of nanotoxicity research is that these studies must be performed for nanobiosystems and have extreme importance in higher-risk applications, such as stem cell labeling and *in vivo* applications.[72,73] Nanotoxicity is a new and emerging field and mechanisms of nanotoxicity are currently not well-understood.

Perturbation of Cellular Function

Numerous examples of nanobiosystems are being developed to interact with specific cellular pathways and functions. However, in some cases, undesirable perturbations to cellular function occur in the host biological environment. In the cellular environment, these perturbations may occur on many levels, which can be broadly divided into three levels of oxidative stress: enzymatic response (low), inflammation response (medium), and cytotoxicity (high).[74] For example, Unfried et al.[75] demonstrated that carbon nanoparticles activated Akt signaling pathway and generate in pro-inflammatory response in lung cells. More pronounced signs of cytotoxicity, such as intracellular reactive oxygen species generation and phosphatidyl serine expression, are readily detected by cellular staining and apoptosis assays.[76,77] For example, Lovric et al.[77] reported that a breast cancer cell line (MCF-7) generated nuclear reactive oxygen species, shown by intense nuclear fluorescence after staining with dihydroethidium, after exposure to quantum dots. Further, Berry and colleagues performed an assay for the early-stage apoptosis marker, annexin-V, in addition to clathrin immunofluorescence to determine if dextran-iron oxide nanoparticles

induced apoptosis upon intracellular uptake.[78] In conclusion, several biomarkers are used to detect perturbations in cellular function induced by nanostructures. These cellular assays are an important screening method to predict large-scale toxicity prior to *in vivo* animal studies. Measuring cellular perturbation in response to nanobiosystems is a critical link to *in vivo* studies because the nanobiosystem will interact with many different cell types in the body. One of the main challenges for cellular assays is that the numerous methods to detect cytotoxicity or cellular stress lead to discrepancies in results. These conflicts will likely be resolved by performing a series of tests on numerous formulations of the nanobiosystem or nanomaterials of interest.

Nanobiosystem Interactions *in vivo*

One of the ultimate goals for nanobiosystems is their application *in vivo*. Whether the purpose is for enhancing diagnostic sensitivity, targeted drug delivery, or another aim, the nanobiosystem must overcome several unique challenges. First, evasion of inflammation and immune response is critical; otherwise, the nanobiosystem will be removed from circulation prior to performing its task. Current research focuses on minimizing these adverse effects through controlling size, generally between 10 and 100 nm, and selection of stealth materials to improve circulation time.[57,59] Another unique challenge for nanobiosystems is understanding biodistribution and metabolic effects. Nanobiosystems, which are one the order of one-billionth the volume of a single cell, must be followed and detected over the entire body. Nanobiosystem size and detection sensitivity are the two main challenges;[7] however, metabolic pathways unique to nanomaterials are another focus area being investigated.[16] The last major concern for *in vivo* application is targeting nanobiosystems to cells and tissues of interest. Even after the nanobiosystem has shown efficacy *in vitro*, the final challenge is getting the nanobiosystem to the targeted cell or tissue.[19] Many studies today are focusing on combined physical and molecular targeting, such as applying a magnetic field near a tumor site for magnetic nanoparticle accumulation.[79] Overall, the interaction between nano- and biological systems culminates with the *in vivo* application of nanobiosystems because this environment presents unique challenges emphasizing the need for investigation in animal models and human clinical trials.

CONCLUSION

The general direction of nanobiosystems is to produce multifunctional systems containing targeting (both cellular and intracellular), cell-entry, biosensing, and drug/gene delivery molecules in an integrated package. This strategy permits the use of multiple specific-purpose molecules to be brought together in a coordinated way and in a controlled sequence of functions. The power of *in vivo* imaging can also be included in this package through the use of tagging molecules or core nanomaterials. These additional factors can be distinctly non-biological and have properties that highly distinguish themselves from biological tissue to provide for greatly improved contrast. An example is the use of iron oxide superparamagnetic materials which serve not only as MRI contrast agents but also vehicles for manipulation by magnetic fields to improve drug delivery or to allow for localized heating. Composite nanomaterials and modified shape designs (e.g., nanorods) may improve circulation time *in vivo* and improve uptake by targeted cells.

The ability to simultaneously provide *in vivo* diagnostics and therapeutics ('theragnostics') in a single nanobiosystem will allow for more sophisticated medical therapies and permit more sophisticated appraisals of therapeutic efficacy, such as *in vivo* MRI measures of tumor shrinkage. The future of nanobiosystems research will be to engineer systems such that they provide a means for swapping in or out targeting and therapeutic molecules for treatment of a wide variety of diseases. Such sophisticated nanodelivery systems should allow for not only improved targeting but also the use of far less overall drug, thereby reducing damage to bystander normal cells and tissue. Overall, nanobiosystems are being developed to perform efficient, multifunctional tasks in the biological environment, and their successful application will greatly impact the fields of biology and medicine.

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FURTHER READING

A popular source of information on nanomedical systems is offered as a series of 21 online lectures presented as either Breeze (PowerPoint) visual presentations with linked oral presentations, or as Podcasts. These are found at the National Science Foundation's NanoHUB website at http://www.nanohub.org/courses/nanomedicine
A description of a synthesis process for production of water-soluble nanoparticle for nanomedicine is given at: http://docs.lib.purdue.edu/cgi/viewcontent.cgi?article=1021&context=nanoposter
A good review of multifunctional magnetic nanoparticles is given by:
McCarthy JR, Weissleder R. Multifunctional magnetic nanoparticles for targeted imaging and therapy. *Adv Drug Deliv Rev* 60:1241–1251, 2008.

