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Design of Multifunctional Nanomedical Systems

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Abstract—Multifunctional nanoparticles hold great promise for drug/gene delivery and simultaneous diagnostics and therapeutics (''theragnostics'') including use of core materials that provide *in vivo* imaging and opportunities for externally modulated therapeutic interventions. Multilayered nanoparticles can act as nanomedical systems with on-board molecular programming done through the chemistry of highly specialized layers to accomplish complex and potentially decision-making tasks. The targeting process itself is a multistep process consisting of initial cell recognition through cell surface receptors, cell entry through the membrane in a manner to prevent undesired alterations of the nanomedical system, re-targeting to the appropriate sub-region of the cell where the therapeutic package can be localized, and potentially control of that therapeutic process through feedback systems using molecular biosensors. This paper describes a bionanoengineering design process in which sophisticated nanomedical platform systems can be designed for diagnosis and treatment of disease. The feasibility of most of these subsystems has been demonstrated, but the full integration of these interacting sub-components remains a challenge for the field. Specific examples of sub-components developed for specific applications are described.

Keywords—Nanomedicine, Nanoparticles, Bionanoengineering, Bionanotechnology.

INTRODUCTION

General Overview

Nanomedicine is a fundamental nanotechnology approach because it approaches medicine in a bottoms-up rather than top-down approach and performs parallel-processing medicine at the single cell level. Three paradigms of conventional medicine can be challenged by nanomedical systems. First, most conventional drug delivery systems work on the basis of chemical gradients of drugs that are delivered nonspecifically throughout the body. This results in many undesired and toxic side effects to normal bystander cells. Only recently have targeted therapies, such as immunotherapies of cancers with monoclonal antibodies, been able to reduce the undesired immune reactions enough to demonstrate the tremendous potential of these new ''targeted therapies''. Nanomedicine holds the promise of much greater localization of therapies to the diseased tissue or organ while introducing perhaps thousands of times less drug to the body which greatly reduces the possibility of side effects. Second, the use of biomolecular sensors and feedback loops to control the dose of therapeutic delivery at the single cell level represents one of the paradigm-shifting changes of nanomedicine. The therapeutic dose must be correct for a given patient as well as the optimal dose at the single cell level to avoid undesired tissue and organ damage. Third, and partially due to the new capabilities introduced by feedback control of therapy at the single cell level, the potential for regenerative nanomedicine is possible by no longer just trying to destroy diseased cells. The future goal is to either restore diseased cells to normal or at least to place them in a more benign state. The extensive destruction to tissues and organs caused by conventional therapies frequency leads to organ failure and patient death due as much to the therapy as to the disease itself. The ability to keep diseased organs functioning would reduce the need for organ transplants, always in short supply. In this paper, the basic components in the design of nanomedical systems are described.

NANOMEDICAL SYSTEM DESIGN AND CONSTRUCTION

Multifunctional nanomedical systems allow for the targeted molecular delivery of cellular therapy through

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layered construction and encoded disassembly (Fig. 1).[58] While this concept can be accomplished by numerous strategies, multilayered nanoparticles will serve as the primary example in this review. The outermost layer of the nanoparticle serves to target to a specific cell type and facilitate entry, and this layer disassembles upon completing its desired function. The intracellular targeting to a specific organelle is the next objective of this system. At this point, the nanoparticle is localized such that it can deliver its payload effectively. For example, this may consist of a therapeutic gene or signaling molecule to mediate desired pathways. While many systems published in the literature employ only a subset of the overall strategy, all systems can be thought of in terms of this more general strategy.

Nanomedical System Concepts

Nanoparticle core materials are the first step in creating a multifunctional layered nanomedical system. It is critical to consider the purpose behind selecting a certain core material for the nanoparticle system. Properties of the core material can provide essential information about nanoparticle localization and cellular effects in a biological environment. Moreover, magnetic and thermal properties can be used to modulate the behavior and location of nanoparticles post-application *in vitro* or *in vivo*.

Core particle materials provide the foundation of a therapeutic delivery system. The material for the core particle is important because it will provide unique capabilities for nanoparticle detection and manipulation. Moreover, the desired characteristics are not entirely contained within one specific core material, which has lead to a great deal of research on many different core particle materials and complex composite materials. Numerous nanoparticle formulations focus on therapeutic gene delivery applications and requirements for various diseases. Ideally, the core particle material is biocompatible in order to be evade the immune response and avoid cytotoxicity.[70] Some common groups of materials include metallic,[30,73] polymeric,[46,96] and biological[1,33,47] (also see Table 1).

An appropriate size range is required in order for a nanomedical system to be effective. The system needs to be capable of targeting, entering, and providing therapy at a single cell level. The ideal size range for the nanoparticle diameter is between 10 and 100 nm. While some particles used for cell targeting and drug delivery, such as liposomes, are larger than this, their size range is beyond that of a nanomaterial.[25] Also, existing polymeric nanostructures primarily used for drug delivery cannot meet the efficiency of a smaller nanoparticle or provide adequate functionalization of layers. Similarly, a particle needs to be large enough (greater than 10 nm in diameter) so that it is not cleared from the body too quickly. A limitation is placed on the nanomedical system's size in order to effectively meet its goal. On the smaller end of this size range, there is a greater the likelihood of evading immune cell interaction, and the nanoparticles are protected from the surrounding immune and inherent responses to a foreign material.

Detection of nanoparticles is critical for determining their effectiveness. Localization and agglomeration of the particles assists in the initial diagnosis process. It is paramount with nanomedicine to have a determination of whether or not the therapeutic target was reached, when it was reached, and whether or not a specific therapy was efficiently delivered. For instance, magnetic particles can be observed using magnetic fields and through the use of MRI.[48] In some cases, a magnetic particle system could activate a therapy when a magnetic field is applied.[85] The detection of particles can also be linked to imaging capabilities. These capabilities are reflected most often in metallic nanoparticles. This characteristic can allow imaging of the locally affected organs and tissues. Core particle materials that can provide this viewing include magnetic and semiconductor materials. Semiconductor quantum dots can assist in long term fluorescent imaging capabilities vs. traditional fluorophores.[3,20]

The degradation or removal of the material is a function of what type of core particle exists and whether it has a short- or long-term goal in the body. If degradation is desired, a material is required to be capable to breaking itself down into biodegradable parts for the cells. Removal can be considered a function of the system itself and built into its functions. Once the system has achieved its therapy and effectively removed its functional layers, a trigger can be activated that will allow the particle to be removed from the cell through a natural emission process and its contents tagged for proper and efficient removal from the body.

Types of Core Particles

Many types of nanoparticles exist with respect to their size, shape, material, and coatings, as a few examples. The specific properties of the core materials provide distinct monitoring and therapeutic applications. For example, magnetic nanoparticles provide monitoring and localization properties based on their magnetic susceptibility. Further, some formulations of magnetic nanoparticles, namely iron oxide and dextran composites, have been FDA-approved for human clinical use as MRI contrast enhancing agents.[35] Future use of magnetic nanoparticles for *in vivo*

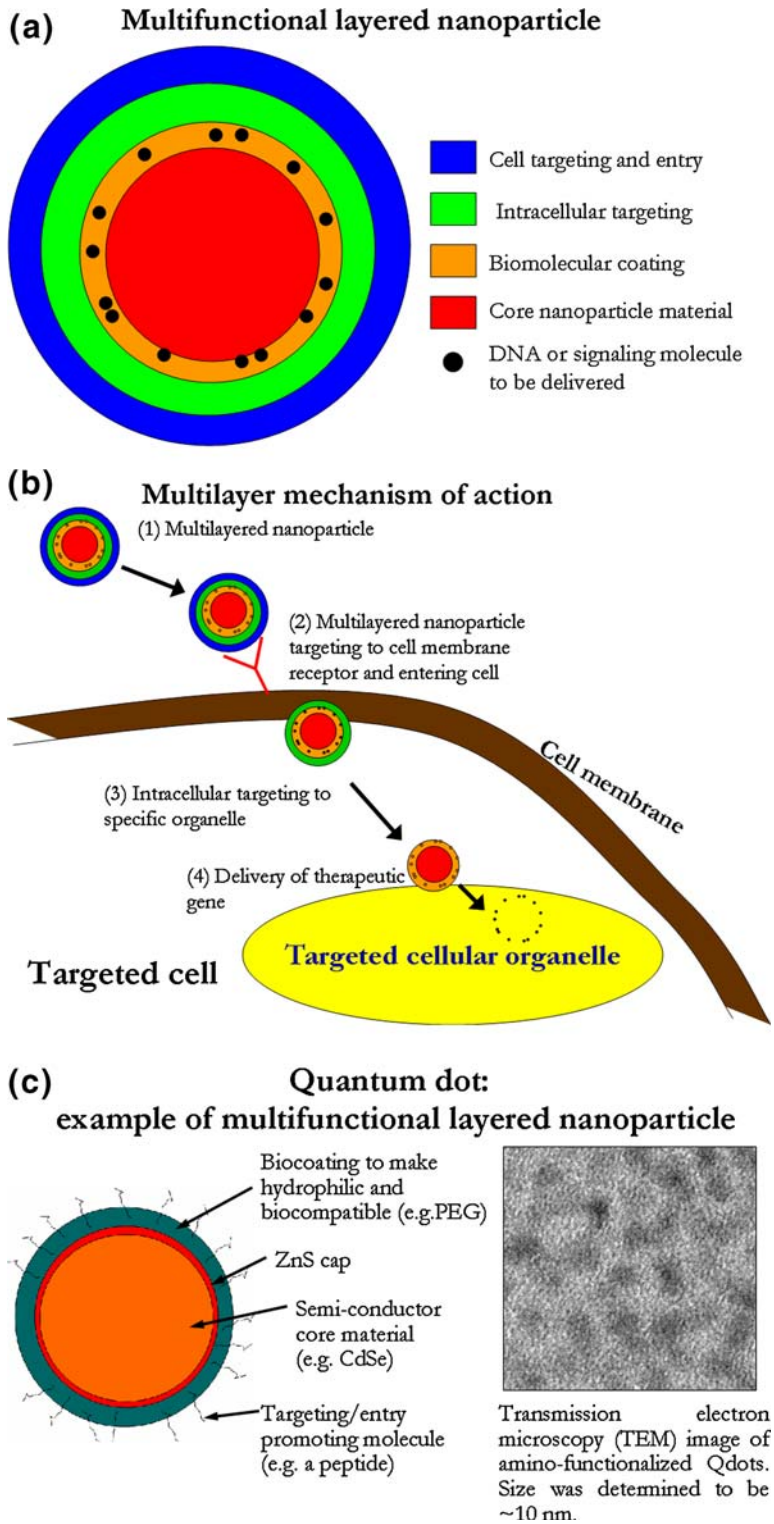

FIGURE 1. (a) Multilayered nanoparticle system containing targeting, biosensing and drug delivery molecules that are released a layer at a time. This produces a smart nanoparticle system that results in molecular programming, an ordered series of events, for drug/gene delivery. Biosensing molecules allow the feedback-controlled release of drugs, or expression of therapeutic gene sequences, at the individual cell level.[58] (b) General sequence of at least four steps for nanomedical systems (NMS) interacting with the targeted cell of interest: (1) nanoparticles in the extracellular environment, (2) NMS attachment to the cell membrane and its proper entry, (3) intracellular targeting to desired site of action, and (4) delivery of drugs or production of therapeutic genes at the desired site. (c) Example of peptide guided quantum dots where the single peptide layer performs both cell targeting and cell entry.

TABLE 1. Description of common core materials utilized for nanoparticle systems.

Type	Size	Core formulation	Layer formulation	Applications	References
Metallic	50–150 nm	Au	Polyethylene glycol (PEG); galactose-PEG	Cellular targeting to hepatocytes via galactose receptor targeting in mice	Bergen *et al.*[10]
	100 nm	Fe_3O_4	Streptavidin, DNA	DNA delivery and expression in retinal cell line *in vitro*	Prow *et al.*[74]
	50 nm	Fe_3O_4	Polyethylimine; plasmid DNA	Efficient non-viral gene delivery using pulsed magnetic field	Kamau *et al.*[48]
	10, 150 nm	FeNi	Oleic acid	Biomedical applications of nano-particles	Yin *et al.*[93]
Semiconductor	15–20 nm	CdSe	ZnS coating; poly(ethylene glycol) (PEG); RGD peptide	Cancer diagnosis and management; image guided therapy and surgery	Cai *et al.*[18]
	15–20 nm	CdSe	ZnS coating; PEG; peptide conjugation	Real time imaging of cellular pathways for specific therapies; activation of desired cellular events as probes	Rozenzhak *et al.*[78]
	4 nm	CdTe	Mercaptopropionic acid (MPA); aptamer conjugation	Screen and study targeted therapeutics; *in vivo* labeling applications	Chu *et al.*[23]
	5 nm	CdS	MPA	Improve drug delivery efficiency in target cancer cells; early cancer diagnosis; Inhibit multi-drug resistance	Li *et al.*[60]
	10–20 nm	InP	ZnS; folic acid	Deep tissue imaging; photodynamic therapy	Bharali *et al.*[15]
Polymer	197 nm	Poly(lactide-co-glycolide) (PLGA)	–	Therapeutic drug and gene delivery	Astete and Sabliov[6]
	250 nm	Poly(alkylcyanoacrylate) (PACA)	–	Drug and protein delivery	Krauel *et al.*[53]
	$<$160 nm	PEG/poly(ε-caprolactone)	–	Therapeutic DNA delivery	Jang *et al.*[46]
Ceramic	20 nm–2.5 μm	Calcium phosphate (CaP)/plasmid DNA	–	Therapeutic DNA delivery *in vitro*	Olton *et al.*[71]
	25–55 nm	Hydroxyapatite	Bovine serum albumin (BSA) matrix	Improved materials from natural polymers for biomedical applications	Nayar *et al.*[69]
	25 nm	Silica	Antisense oligonucleotides	Inhibition of cancer cells through delivery of antisense DNA	Peng *et al.*[72]
Biological	25 nm	DNA/PEG/lysine peptide	–	Efficiency to deliver DNA to ocular tissue in mice	Farjo *et al.*[33]
	175–225 nm	Chitosan-Polyethylimine copolymer	DNA	Gene delivery methods and effects *in vitro*	Jiang *et al.*[47]

localization and hyperthermia treatment are common applications of research in progress.[2,11,83,94] Magnetic nanoparticle cores are typically synthesized from iron, cobalt, nickel, and alloys of these metals.[16,50,56,68,82,93,98] The use of these metals as core materials often requires a stabilizing agent, such as a surfactant or polymer, to reduce agglomeration and increase dispersion in various solvents and in the bloodstream. Much research has been performed on increasing the water solubility of magnetic nanoparticles in order to optimize use in biological environments.[65,82,95] Further addition of amine or carboxyl groups, generally via polymer coatings, provides a link to functionalize these magnetic nanoparticles with other biomolecules.[67]

Other core nanomaterials, specifically semiconductor nanoparticles, have the advantage of fluorescence. These particles consist of core elements of cadmium, selenium, and tellurium. To provide stability especially needed for biological environments, semiconductor particles are coated with zinc sulfide to create a particle approximately 15 nm in diameter (Fig. 1c). Moreover, a hydrophilic polyethylene glycol (PEG) coating is also placed on the particle to allow for further functionalization with amine or carboxyl groups.[29,36] Through these functional groups, molecular layers can be constructed on the core nanoparticle to provide biocompatibility, cell targeting, intracellular localization, biosensor diagnostics, and drug or gene delivery.

Another approach for the core material is to combine two different materials to form a composite. This can be achieved by simply mixing two materials, such as metal alloys, or designing a core-shell structure.[88,89] For example, gold metal shells are of particular interest to many researchers due to bioinert properties of gold and plasmon resonance effects.[7,61] For example, Loo and researchers attached a cancer targeting antibody to gold nanoshells to provide targeted delivery to cancer cells.[62] Another approach with gold surfaces is to attach certain peptides with a cysteine amino acid at the preferred site of attachment to link to gold surfaces through thiol linkages.[59] In general, simple aqueous chemistry techniques are strongly desired in order to minimize exposure to organic solvents, which can be a source for cytotoxicity and necrosis in biological environments. A core–shell structure embodies the advantages of two materials. In one approach, Wang *et al.* characterized composite iron oxide and CdSe/ZnS quantum dot shell nanoparticles based on of fluorescent and magnetic properties and demonstrated successful magnetic separation and imaging of breast cancer cells.[88] This example of composite core nanoparticle highlights the advantages of both specific materials in nanomedical applications.

Functional Layers

The construction of multilayered, multifunctional nanoparticles constitutes a form of molecular programming; for which the systematic step-by-step de-layering is accomplished. This process is completed through the use of layered disassembly, where the outermost layer disassembles first and subsequent layers follow. In this design, each chemical structure will disassemble when it encounters molecules in the cell that they are designed to detect.[58]

Molecular layers are attached around the core nanoparticle, which is typically about 20–40 nm in diameter and composed of various core materials, such as previously discussed magnetic materials or semiconductor quantum dot particles. An important concept for layered construction is that molecules may be arranged in staggered orientations or embedded within layers. Therapeutic genes or drugs can be tethered to the surface of these core particles in a manner such that these molecules are free to interact with their eventual targets. The middle functional layer(s) includes intracellular targeting molecule(s) in order to bring the nanoparticles to its intended site of action. Lastly, the outermost layer of the nanomedical system contains the cell surface targeting molecules designed to help the NP bind to the cell of interest and to try to avoid binding to other cell types.

These targeting molecules can be aptamers, antibodies, peptides, and other molecules. This latter aspect is particularly important if the nanomedical system is to provide improvement over current therapies which can cause damage to bystander, non-targeted cells. Sometimes the outermost two layers of the nanomedical system can be accomplished with a single molecule, e.g. a single peptide sequence that does not only bind to the cell surface but also pulls the nanoparticles through the cell membrane. Dual-purpose peptides are being used for other nanomedical applications.

Cell Targeting and Entry

Cellular uptake of nanoparticles has been a challenge facing researchers based on existing methodology. Previous methods relied on chemicals that effectively permeabilize the cell membrane to allow nanoparticle uptake. An example of this is Lipofectamine® (Invitrogen, Carlsbad, CA), a commonly used transfection agent, however these methods can be quite disruptive to the cell membrane and do not allow for targeting of specific cells.[76] Electroporation applies large electric pulses that temporarily disturb the phospholipid bilayer, allowing biomolecules and other entities to pass into the cell.[37] However, this method

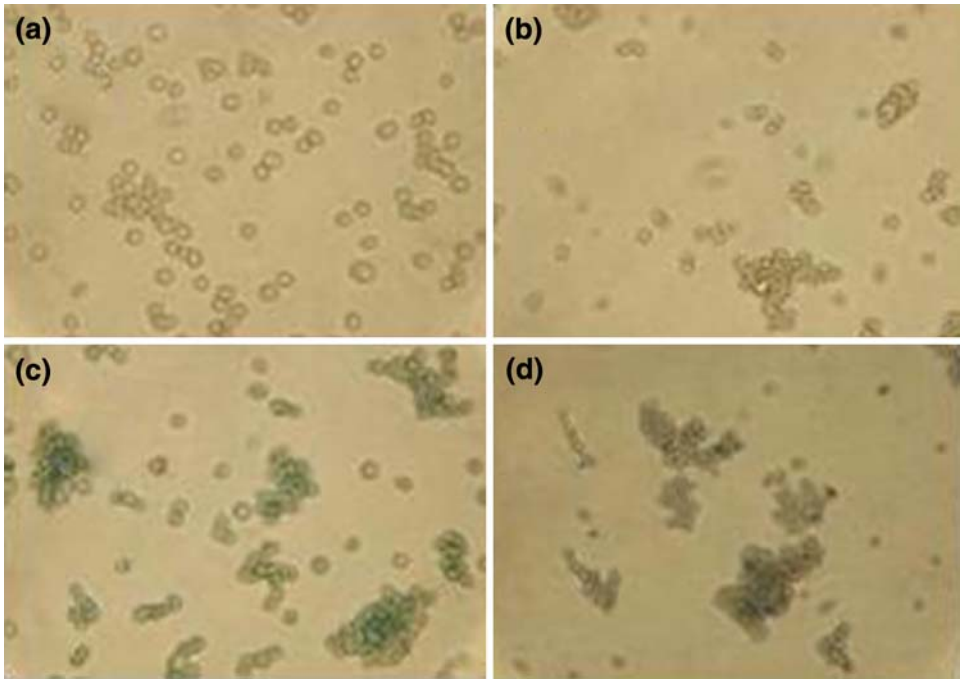

FIGURE 2. Light microscopy images of fixed cells after Prussian blue staining at 200× total magnification (a) MCF-7 cells not exposed to magnetic nanoparticles, (b) MOLT-4 cells not exposed to magnetic nanoparticles, (c) MCF-7 cells exposed to 0.5 mg/mL magnetic nanoparticles for 24 h, (d) MOLT-4 cells exposed to 0.5 mg/mL magnetic nanoparticles for 24 h.

has also been observed to sometimes induce apoptosis as well as increasing necrosis rates of the cell population.[9] One novel approach is optoinjection, where the cell membrane is transiently permeabilized to allow objects (ions, small molecules, dextrans, plasmids, proteins, and semiconductor nanocrystals) to cross the cell membrane by means of diffusion.[24] The present focus for nanoparticle delivery is the use of biomolecules to allow cells to naturally uptake molecules. This mechanism utilizes existing receptors and molecules on the cell surface to accurately and efficiently allow cellular targeting and uptake to occur.[31,66]

The current direction of most nanoparticle construction emphasizes receptor mediated uptake. For example, when nanoparticles were placed in growth media exposed to living cells for various time periods, limited nanoparticle uptake of amino-functionalized magnetic nanoparticles was observed at high concentrations by Prussian blue staining for intracellular ferric iron (Fig. 2).[76] In addition, it was not possible to detect intracellular quantum dots from passive non-specific endocytosis mechanisms. These results indicate a clear need for specific nanoparticle targeting and entry strategies.

Active targeting with nanoparticles can be achieved with the use of biomolecules such as aptamers, antibodies, and peptides. These targeting molecules aid the localization to specific cells and intracellular delivery necessary to achieve the nanomedical system's goal. Aptamers have been utilized to target cell populations due to their high affinity and selectivity properties.[23,32,43] For example, Chu and researchers used RNA aptamer-labeled quantum dots to target the prostate specific membrane antigen on two cancer cell lines in tissue phantom matrices.[23] Moreover, disease diagnosis has the potential to be improved with aptamer-targeted iron oxide nanoparticles to enhance existing magnetic resonance imaging (MRI) technology.[92] Detailed research has examined the advantages of aptamers as a targeting molecule with coordination of a fluorescence based energy transfer (FRET) system that effectively targeted cancer cells and delivered a therapeutic gene.[8] This research is an example of one of the initial steps in creating a nanomedical system.

Antibodies have been used to target specific receptors on cells; in particular, this technology has been utilized for targeting diseased cells, typically based on high expression of a particular receptor.[19,49,51,83] By attaching the non-binding Fc portion of the antibody to the nanoparticle, researchers have shown that antibody-targeted nanoparticles can target cells of interest.[45,84] One common theme with antibody-targeted nanoparticles is cancer cell targeting of protein surface receptors, such as HER2[84,94]; however, some groups are taking this approach one step further to provide therapy or selective ablation to the targeted cell types.[27,30,45] These therapeutic approaches with nanoparticle-mediated delivery mirror monoclonal

antibody targeting of chemotherapy drugs that is emerging in the clinical market today.

Furthermore, a specific peptide sequence can also be used to both target a specific cell type or cell membrane receptor and facilitate nanoparticle entry. Advantages of peptide molecules include their small size, ease of synthesis, high affinity, and intrinsically nontoxic properties.[77] Much nanoparticle delivery work has been done with cell penetrating peptides, such as the TAT peptide[12,26]; however, the direction of current peptide-nanoparticle research is focused on peptides designed for both specific cell types and protein receptor molecules. For example, peptide ligands for G-protein coupling receptors have been attached to quantum dots and used for both whole cell and single molecule imaging.[99] In addition, some peptide molecules have the ability to bind and activate cellular pathways; Vu and researchers demonstrated this concept with a neuronal peptide coupled to quantum dots for the purpose of cell targeting and neuronal differentiation in PC12 cells.[87]

Peptide Guided Quantum Dots

The translation of a previously studied targeting peptide to a nanoparticle system has been performed in our lab, and this study is an example of targeted nanoparticle delivery in cancer cells both the *in vitro* and *in vivo* environment. In this example, the peptide sequence, LTVSPWY, was attached to nanoparticles based on its previous success of induction of oligonucleotide uptake in SkBr3 human breast cancer cell line.[80] This targeting has been successfully completed by conjugation of the peptide, LTVSPWY, to quantum dot nanoparticles vs. a control breast cancer cell line (Fig. 3). These images illustrate the benefits of the both the dual functionality of the peptide targeting layer, with the nanoparticles reaching the edge of the cell and entering, as well as the fluorescent properties of the nanoparticle.

After successful *in vitro* work, this peptide was transferred to an *in vivo* athymic mouse containing a human tumor xenograft. Animals were injected with human SkBr3 breast cancer cells and tumors were allowed to form. Most animals with SkBr3 cellular injections were able to produce sufficient tumor masses with good vascularization. Quantum dots with the conjugated peptide, LTVSPWY, were injected into the tail veins of the animals. For the positive control samples (peritumoral injection), there was only one tumor that had adequate mass that could be sectioned and placed on a slide for imaging. This sample was effectively targeted and imaged (Fig. 4b) which showed the ability to image the targeted quantum dots *in vivo*. For the experimental tail vein injections, five of seven animals produced adequate tumors that were effectively targeted (Fig. 4c). The *in vivo* imaging was completed by scanning the tumor and organ sections with an inverted fluorescent microscope. The images shown in Fig. 7 illustrate the comparison between a negative control organ (kidney, Fig. 4a) and a positive peritumoral injection (Fig. 4b). The bottom panels show the experimental samples that were completed with tail vein injections (Fig. 4c). The positive control

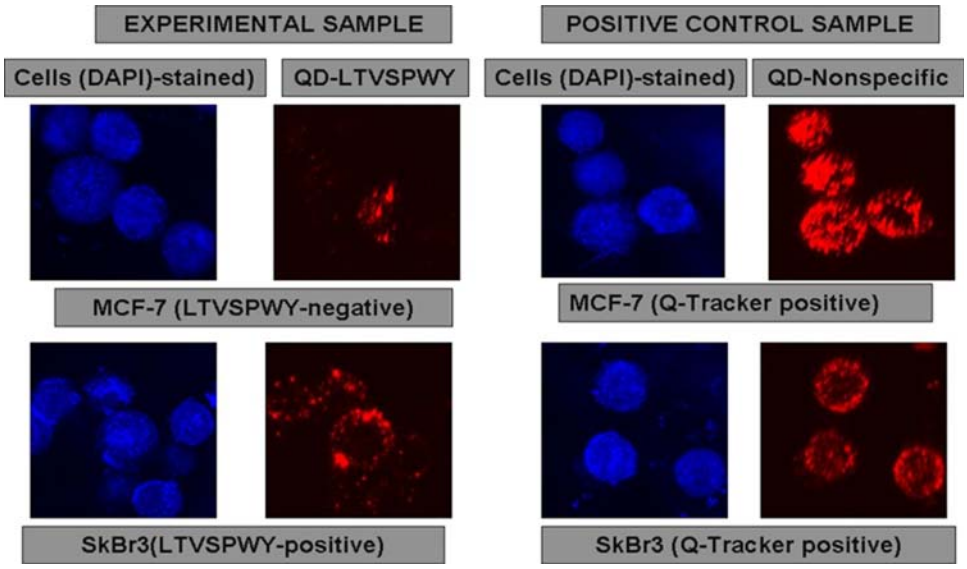

FIGURE 3. ***In vitro*** **peptide Qdots (peptide-targeted Qdot nanoparticles labeling with SkBr3 breast cancer experimental cell line and MCF-7 breast cancer control cell line. The QTracker tool consists of the Qdot nanoparticle with a nonspecific peptide molecule attached to the surface. This technology allows a positive control to be realized. The LTVSPWY peptide specific Qdots were successful at targeting and entering the SkBr3 cells.[42]**

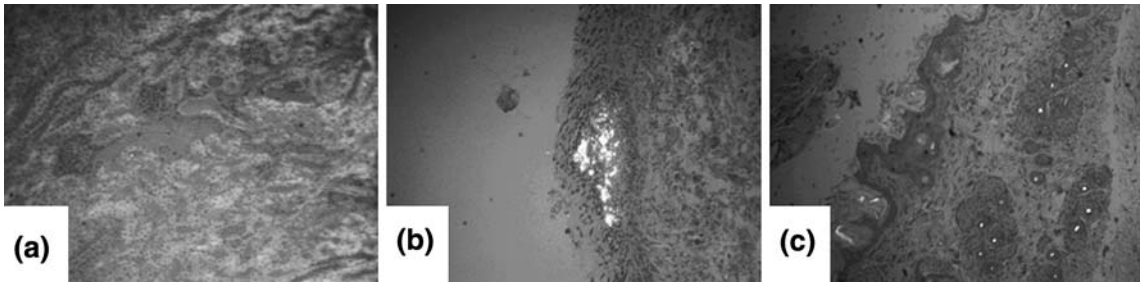

FIGURE 4. Fluorescent microscopy images of *in vivo* tissue sections. Panel a: Image of control kidney tissue, this sample did not experience any quantum dots. Panel b: Image of tumor tissue from a peritumoral injection. Panel c: Image of tumor tissue from a tail vein injection.[42]

was injected directly into the tumor tissue. This is illustrated in Fig. 4b as the nanoparticles are near the edge of the tissue and significantly agglomerated. This image is compared to Fig. 4c, where the nanoparticles were injected into the tail vein and successfully targeted and reached the tumor tissue. The quantum dots in this image are dramatically incorporated into the tumor tissue compared to the peritumoral injection image.[42]

DIAGNOSTIC AND THERAPEUTIC DELIVERY

Challenges of Rare-Cell Targeting In Vivo

Even if the complex, multi-step process of cell targeting and drug delivery is successful, a remaining important problem exists, particularly for the case of regenerative nanomedicine. The goal is not to simply kill the diseased cell but rather try to keep it alive but change its behavior, for example, through alteration of its gene expression profile. This task is much more complex than simple killing and requires precise dosing of drugs or genes at a single cell level. Controlling the number of nanomedical systems that successfully target and deliver drugs to an individual cell is an extremely difficult, perhaps impossible, task due to the inherent rare-cell targeting problem.[57]

In Situ Manufacture of Therapeutic Genes for Nanomedicine

An exciting alternative approach is to not deliver a drug but rather a gene manufacturing template to the cell. Transcription of therapeutic gene sequences occurs under the control of an upstream molecular biosensor in a feedback control guided process as is conceptually shown in Fig. 5 and described in some of our earlier work.[74] The advantage of this approach is that the cell always receives the correct therapeutic dosage regardless of how many nanoparticles reach their target. The implementation of this process has been completed using fluorescent reporter gene constructs manufactured *in situ* within living cells using upstream molecular biosensors and responding to feedback control mechanisms for biosensing of reactive oxygen species (ROS) molecules within the cells. In this work,[74] it has been demonstrated that genes tethered to ferric oxide nanoparticles can transcribe copies of genes inside living cells. For visualization purposes we transcribed eGFP and DsRed reporter genes tethered to ferric oxide nanoparticles which were used to transfect cells. Results were visualized using confocal microscopy (Fig. 5).

CYTOTOXICITY

Types of Cytotoxicity Assays

In order to validate the potential of therapeutic gene delivery with nanoparticles, *in vitro* cytotoxicity and nanoparticle effects have been examined to a large extent. Some researchers choose to compare cellular viability, proliferation, morphology and adhesion properties of cells that have been exposed to nanoparticles to control cells, and these results are generally mixed.[14,38,40] More direct cytotoxicity strategies that fluorescently label specific cellular stress biomarkers, such as ROS and lactate dehydrogenase release, show that cells tolerate magnetic nanoparticles coated with transfection agents and plasmid DNA at some concentrations.

Cytotoxicity is a primary concern with nanomedicine. While short-term cytotoxicity generally rules out highly toxic nanomaterial formulations, long-term cytotoxicity has not been fully investigated in both *in vitro* and *in vivo* environments. The lack of long-term cytotoxicity data is likely linked with challenges of nanoparticle monitoring, especially *in vivo*, because small amounts of nanoparticles are very difficult to detect. It is expected that long-term nanoparticle toxicity will develop as more sensitive detection strategies evolve, and both aspects are critical for developing successful nanomedical therapeutic delivery systems.

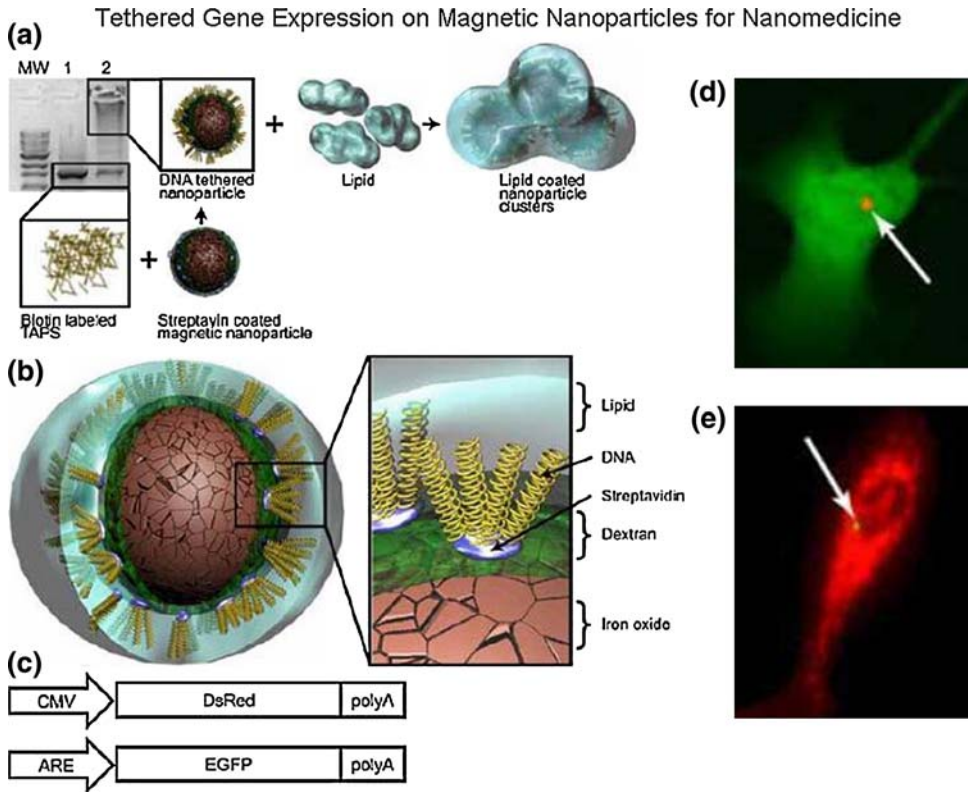

FIGURE 5. Construction and anatomy of magnetic nanoparticles. (a) Conjugation of biotin-labeled transcriptionally active PCR products (TAP) DNA to streptavidin-coated magnetic nanoparticles (MNP). A 0.8% agarose gel stained with ethidium bromide was used to visualize DNA and DNA tethered nanoparticles. The leftmost lane are molecular weight markers, from 1 to 10 kb (MW). Lane 1 contains only 5′ biotin-tagged TAP DNA (Black rectangular outline). Lane 2 is a solution containing DNA from Lane 1 combined with streptavidin-coated magnetic nanoparticles. The black rectangular outline in Lane 2 highlights 5′ TAP tethered magnetic nanoparticles. (b) Schematic of the construction of the MNP. The layered anatomy of a lipid coated DNA tethered nanoparticle. (c) Schematics of the two DNA constructs used to assess transfection and ARE activity. Lipid-coated nanocrystal transfected human retinal epithelium cells. Cells were cultured with lipid-coated nanocrystals tethered to either EGFP (green in (d)) or DsRed (red in (e)) for 48 h or 10 days, respectively. Confocal microscopy was used to visualize nanocrystals and tethered fluorescent gene expression. The nanocrystals are marked by white arrows. Adapted from our previously published work.[74]

Nanocytotoxicity is of concern when developing nanomedical systems. It is important to also understand that nanoscale materials may behave differently than their traditional bulk properties.[70] The nanoscale technology is of concern based on the interactions present on the molecular level at this size range. For instance, the metal cadmium has previously shown to be toxic, however its presence in the core of the quantum dot nanoparticle has presented mixed reviews. Leaching of cadmium ions has been observed based on the application of the unmodified core material to cells *in vitro*.[52] This was shown to cause cytotoxic effects; however these effects were eliminated (or perhaps delayed) with the application of a surface coating. For nanoparticles, there are many formulations that have to be evaluated for cytotoxicity. The differences in core material, size, surface chemistry, and biocoatings are all critical for how the cell responds. This diverse array of characteristics presents a challenge to determine the cytotoxic properties of nanoparticles. It is important to evaluate cytotoxicity using various approaches that include: staining cells with vital dyes, monitoring cell function, measuring cellular stress markers, and monitoring apoptosis events. Cytotoxicity is a critical research area because it will govern whether or not the nanomedical system will ever be successfully applied *in vivo*. Without a clear understanding of nanoparticle cytotoxicity *in vitro*, experiments in less controlled environments, such as *in vivo*, will be difficult to interpret.

Chemical stains, such as trypan blue and propidium iodide that selectively enter dead cells have been typically used as a supplementary assay to support other cytotoxicity assay results.[4,13,17,26,55,64,75,97] Functional approaches for evaluating cytotoxicity monitor vital cell functions, such as adhesion and proliferation. The presence of uncoated iron oxide nanoparticles in the growth media have been shown to decrease cellular adhesion of fibroblast cell lines, while starch and insulin coated iron oxide NPs have not significantly affected

fibroblast adhesion.[38,39,41] Similarly, cells exposed to both uncoated and dextran-stabilized iron oxide nanoparticles at a concentration of 0.05 mg/mL nanoparticles demonstrated a decrease in proliferation; however, albumin-coated nanoparticles resulted in increased cellular proliferation at the same concentration.[14] While cell proliferation and adhesions are important functions to evaluate, this type of data may not be affected by more subtle changes in cell activity due to cytotoxicity.

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay is used to investigate cellular enzymatic activity and is the most common method used to evaluate the effects of NPs. With respect to iron oxide nanoparticle exposure, several groups have reported no significant change in MTT reduction with exposure to uncoated magnetite,[44] poly-L-lysine coated magnetite,[4,90] or dextran-coated magnetite NPs at concentrations under 25 μg iron per mL. Moreover, Hussain *et al.* tested a concentration range from 0 to 250 μg iron per mL with rat liver cells and found no reduction in cell viability until the highest dose, 250 μg iron per mL. These results are important because they indicate a range of NP dosages that cells tolerate as well as compounds that can mask the toxicity effects of iron oxide. The use of the MTT assay has been common within cytotoxicity research of quantum dots.[28,63,64,79] Ryman-Rasmussen examined both the viability of the cells exposed to poly(ethylene glycol) (PEG) coated quantum dots and PEG coated amine and carboxyl functionalized quantum dots. It was determined that the functionalized coatings caused significant cytotoxicity vs. the PEG-only coated quantum dots. This illustrates how important a small change in the chemistry of the nanoparticle can affect a cellular response. While the MTT assay provides useful information, there are several inconsistencies with this assay and its use. There is large variability in toxicity concentrations when comparing different studies as determined by MTT assay results. For example, Gupta *et al.* reported uncoated iron oxide NPs caused a 20% decrease in fibroblast cell viability at a concentration of 50 μg/mL,[41] in contrast to other higher toxicity levels for uncoated magnetite NPs.[38,44] Furthermore, reports of significant changes in MTT reduction activity are not meaningful without comparing to proper controls, because it is not possible to identify the cause of change in cell function.

Two cytotoxicity markers that have commonly been evaluated are lactate dehydrogenase (LDH) release and ROS formation. Studies based on these markers have indicated cytotoxicity at lower levels of nanoparticles than data obtained using the MTT assay in some cases. For example, LDH release by rat liver cells was not significantly increased for uncoated iron oxide nanoparticles up to 250 μg/mL[44]; however, iron oxide nanoparticles coated with transfection agents have demonstrated cytotoxicity to different degrees. Two transfection agents, polyethyleneimine and protamine sulfate, were used to coat magnetite nanoparticles synthesized for a specialized transfection method, called magnetofection. The polyethyleneimine-coated magnetite NPs showed an increase in LDH release in human endothelial cells over standard transfection methods; however, LDH release was decreased to approximately the same value as standard transfection methods by increasing the mass amount of DNA loaded on the nanoparticle.[54] Also, ROS generation has been observed with quantum dots in breast cancer cells and neuroblastoma cells.[21,22,64] Lovric *et al.* and Chan *et al.* have illustrated the need for a coating around the core materials of cadmium, selenium, and tellurium. The presence of a biocoating has been shown to eliminate (or perhaps mask for some period of time) this apoptotic marker vs. the uncoated nanoparticle.[21] This small difference between nanoparticles translated into a significant difference in cellular response. The use of dihydroethidium has been used to evaluate the ROS in current research.[64] The attachment of a nonspecific peptide to a quantum dot allowed cellular uptake to occur as well as detection of ROS presence vs. control cells (Fig. 6). This is an example of the importance of interactions between cellular biochemistry and nanomedical systems. ROS are generated normally by the cell's metabolism. If their presence is increased, there is a great risk of cellular organelles and macromolecules such as lipids, proteins, and DNA may occur.[86] Measurement and detection of ROS is one way to assess cellular stress that may not be evident in other common assays. It is important to understand how a cell is affected by the presence of nanoparticles, particularly quantum dots. Figure 6 illustrates the presence of ROS with MCF-7 cells that have been exposed to quantum dots. The concern of increased ROS production can lead to organelle damage and necrosis. ROS were induced in a positive control with hydrogen peroxide. The dihydroethidium signal is brighter than Fig. 6a control and there is a greater presence of patches of stain inside the cells. The patches are seen both in the Figs. 6b and 6c. Figure 6c represents cells exposed to quantum dots. This image indicates the heightened presence of ROS when cells are exposed to quantum dots and the potential for cellular stress with exposure to this type of nanoparticle.

Cell Apoptosis in Response to Nanoparticle Exposure

Regarding nanoparticle research, there has been a limited focus on apoptosis even though this is a critical concern for the cellular environment and effects of nanotechnology. For iron oxide nanoparticles, several

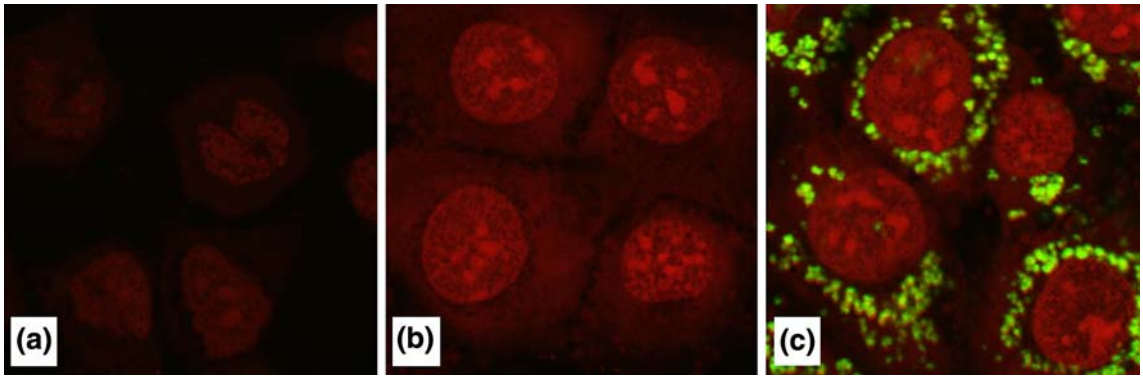

FIGURE 6. Production of reactive oxygen species (ROS) in MCF-7 breast cancer cells exposed to Qdots. Dihydroethidium is represented by the red, and Qdots are represented with green. (a) Control, MCF-7 cells plus dihydroethidium. (b) Positive control, ROS induced with H_2O_2, plus dihydroethidium. (d) Experimental, QTracker® plus dihydroethidium. The cells were exposed to the QTracker® Qdots for 24 h prior to application of dihydroethidium. The image illustrates the presence of dihydroethidium and therefore ROS in the nucleus. The cells are stressed by the presence of Qdots vs. control.

groups observed apoptotic cell populations by propidium iodide (PI) staining and observed a characteristic hypodiploid DNA peak with flow cytometry.[81,91] In addition, some researchers coupled PI staining with annexin-V binding to apoptotic markers on the extracellular membrane.[4,6,13,34] Using the Annexin-V binding assay, Berry *et al.* found an apoptotic cell population of approximately 10% upon exposing fibroblasts to 50 μg/mL uncoated and dextran stabilized iron oxide NPs for 24–48 h. In addition, Berry and researchers observed less than 5% necrotic cells in all cell populations, which is generally considered to be good viability.[13] For semiconductor nanoparticles, the core material is of concern regarding apoptotic and necrotic induction. Results have indicated the same poly(ethylene) coated quantum dots to have minimal apoptosis and necrosis in lung fibroblasts while eliciting an increased amount of apoptosis and necrosis in skin fibroblasts at equivalent doses.[97] This again indicates the importance of measuring the cytotoxicity of this technology as well as how an environmental impact within an *in vivo* system can affect the cytotoxic properties of the particle.

TUNEL Apoptosis Assays of Potential Nanoparticle Cytotoxicity

One example of apoptosis detection can be performed with the TUNEL assay, in which late-stage apoptotic cells are detected by labeled DNA strand breaks. While this assay only constitutes one measure of cytotoxicity, results showed very low amounts of induced apoptosis as seen in Fig. 7. A number of other single cell assays for early apoptosis (e.g. Annexin V) and cell viability by dye exclusion (e.g. trypan blue, or propidium iodide), can be used as simple measures for apoptosis detection. More sophisticated tests of distributions in the gene expression profile and metabolic function of the cell can be accomplished with gene or protein arrays, the subject of other studies in our laboratory. Here, one commonly used single cell assay of late apoptosis (TUNEL assay) is presented as a reminder that all nanomedical systems must evaluate the potential nanotoxicity of not only the on-board drug or gene, but also the potential toxicity of the nanodelivery vehicle itself.

DISCUSSION

Current research indicates that multifunctional medical devices can now be constructed at the nanoscale. Much of the design of these nanomedical devices can be guided by biomimetic studies of nanostructures. This has been performed through the construction of multilayered nanostructures, where layer has a unique function. Initially, core materials must be carefully selected based on their potential for diagnostic and therapeutic applications. Specifically, core material properties that enhance disease detection capabilities are clinically advantageous. Cytotoxicity of the core material and subsequent biocoatings is critical to evaluate due to the uncertainty of their stability in biological environments. In addition, functional layers and delivery methods are also critical for the many functions of nanomedical systems. In order to achieve cellular delivery, various biomolecules have been used to guide nanostructures to diseased cells. Peptides have displayed important biomimetic properties by being able to interact with specific native proteins. However, this is only the first step in design and function of the nanomedical system.

Diagnostic applications are the first clinical consideration for nanomedical systems because these

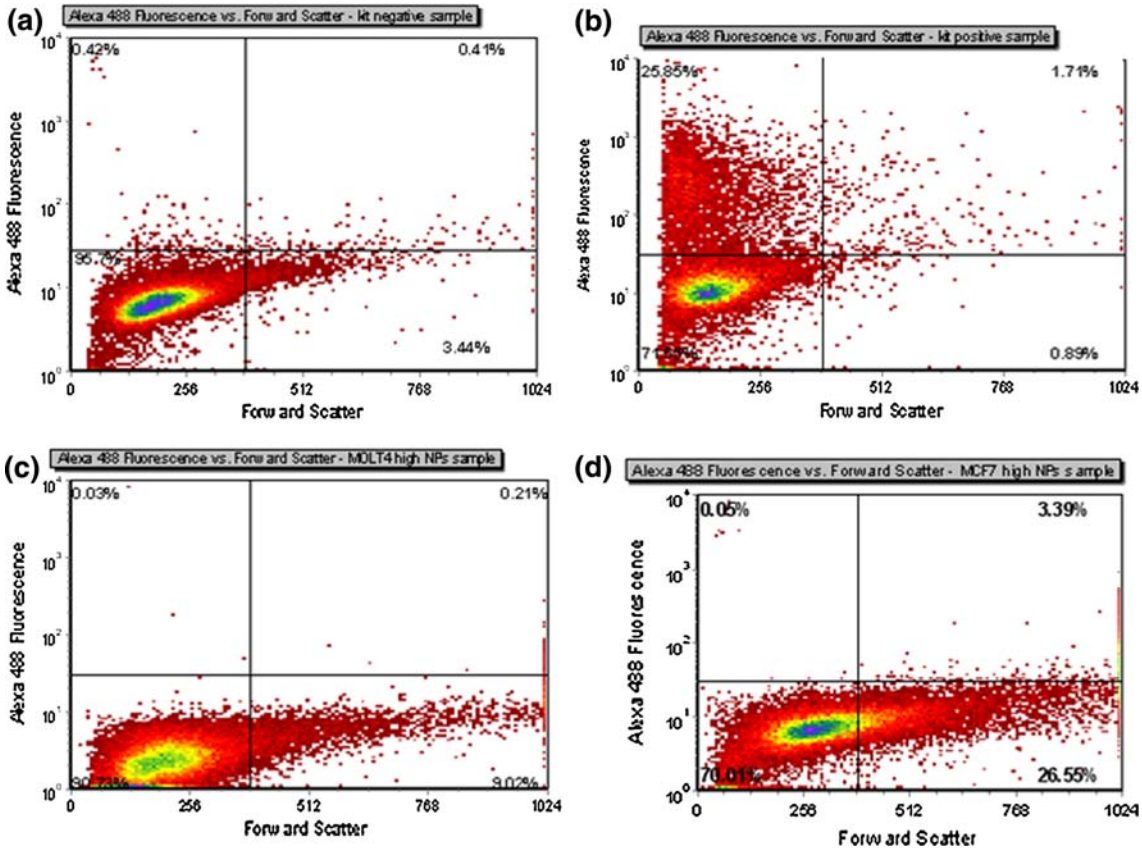

FIGURE 7. TUNEL assay results showing no observed cytotoxicity of magnetic nanoparticles on MOLT-4 and MCF-7 cells: (a) TUNEL-negative lymphoblastic cells (provided with assay), (b) TUNEL-positive lymphoblastic cells (provided with assay), (c) MOLT-4 cells exposed to 0.5 mg/mL magnetic NPs, and (d) MCF-7 cells exposed to 0.5 mg/mL magnetic NPs.

systems can be utilized in *ex vivo* environments. For example, nanomedical systems can be applied to tissue biopsies for enhanced disease diagnosis. Further, therapeutic applications require additional biomolecules and error-checking methods to effectively treat disease. These systems are being investigated for treatment and repair of diseased cells in a variety of settings. A critical characteristic in many diseases is the challenge of detecting and treating each diseased cell. Nanomedical devices attempt to more effectively target and deliver therapy to these rare cells *in vivo*. One research direction for diseased cell therapy is the *in situ* expression of therapeutic genes for cellular repair.

Overall, the development of nanomedical systems is strongly linked with fundamental engineering design. By constructing the nanomedical system from the core particle to a multilayered functional device, stepwise evaluation is conducted throughout the research process. Moreover, the transition from existing medical therapy to nanomedical approaches will be much improved due to the deliberate design and meticulous analysis. The potential to introduce fundamental engineering concepts and approaches into medicine will make the discipline of nanomedicine a new partnership of engineers and clinicians.

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