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# Development of Multifunctional Nanoparticles for Targeted Drug Delivery and Non-invasive Imaging of Therapeutic Effect

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## Abstract

Nanotechnology is a multidisciplinary scientific field undergoing explosive development. Nanometer-sized particles offer novel structural, optical and electronic properties that are not attainable with individual molecules or bulk solids. Advances in nanomedicine can be made by engineering biodegradable nanoparticles such as magnetic iron oxide nanoparticles, polymers, dendrimers and liposomes that are capable of targeted delivery of both imaging agents and anticancer drugs. This leads toward the concept and possibility of personalized medicine for the potential of early detection of cancer lesions, determination of molecular signatures of the tumor by non-invasive imaging and, most importantly, molecular targeted cancer therapy. Increasing evidence suggests that the nanoparticles, whose surface contains a targeting molecule that binds to receptors highly expressed in tumor cells, can serve as cancer image contrast agents to increase sensitivity and specificity in tumor detection. In comparison with other small molecule contrast agents, the advantage of using nanoparticles is their large surface area and the possibility of surface modifications for further conjugation or encapsulation of large amounts of therapeutic agents. Targeted nanoparticles ferry large doses of therapeutic agents into malignant cells while sparing the normal healthy cells. Such multifunctional nanodevices hold the promise of significant improvement of current clinical management of cancer patients. This review explores the development of nanoparticles for enabling and improving the targeted delivery of therapeutic agents, the potential of nanomedicine, and the development of novel and more effective diagnostic and screening techniques to extend the limits of molecular diagnostics providing point-of-care diagnosis and more personalized medicine.

### Keywords

Nanotechnology; nanomedicine; multifunctional nanoparticles; molecular diagnosis; targeted therapy; drug delivery; nanotherapeutics; tumor imaging



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## INTRODUCTION

Nanotechnology (derived from the Greek word *nano* meaning *dwarf*) is generally defined as the science and engineering of constructing and assembling objects on a scale smaller than one hundred nanometers. The potential for nanotechnology comes from its gathering of minds as a multidisciplinary field that combines chemistry, bioengineering, biology and medicine. Nanotechnology inspires the imagination, but not all visions of the future of nanotechnology are pleasant. Images of nanotechnology in movies sometimes involve nanobots that have gone awry. Many scientists take these concerns seriously and are working to insure that the fruits of nanotechnology are safe and positive.

One of the greatest values of nanotechnology will be in the development of new and effective medical diagnostics and treatments (i.e. nanomedicine) [1]. The ability to image cellular migration *in vivo* could be very useful for studying inflammation, tumors, immune response, and effects of stem cell therapy. Promoting nanotechnology for transforming diagnosis, prevention and treatment is the focus of the recently formed National Cancer Institute (NCI) *Alliance for Nanotechnology in Cancer*. According to the American Cancer Society, for the year 2007, there will be almost 1.5 million people diagnosed with cancer in the US alone. Cancer has overtaken heart disease as the leading cause of death for adults according to the Centers for Disease Control and Prevention. Obviously, a better method is essential to accurately diagnose and treat cancer earlier in its evolution from a microscopic disease to a macroscopic/metastatic disease.

Current requirements in translational oncology include: advanced technologies for tumor imaging and early detection, new and novel methods for accurate and early diagnosis and prognosis, overcoming the adverse side effects of chemotherapy drugs by targeting, and treating aggressive and lethal cancer phenotypes such as bone metastasis. Advances in these areas hold a great promise for improving the survival of cancer patients, and will lead to personalized oncology in which cancer detection, diagnosis, and therapy are tailored to each individual's tumor molecular profile and also for predictive oncology in which genetic and molecular markers are used to predict disease development, progression, and clinical outcomes. In fact, many of the major breakthroughs in medicine over the last 20 years have come not from treatment but from imaging, identification and characterization of disease processes.

Nanoparticles (NPs) show tremendous promise in noninvasive tumor imaging, early detection, and drug delivery [2], while exhibiting optical, magnetic and structural properties that are not feasible for single molecules. Because of their vast surface area, diverse surface chemistry, and unique pharmacokinetics, a major advantage of NPs over other systems is that they potentially can combine and deliver several functionalities simultaneously and specifically to a tumor. Most conventional anticancer agents do not differentiate between cancerous and normal cells, leading to systemic toxicity and adverse effects. Developing multifunctional nanotechnology naturally needs to overcome various challenges. One of these is to engineer NPs that are able to target to tumor cells or tumor environment after systemic delivery. This is achieved by conjugating (by electrostatic binding, noncovalent biotin-avidin binding, direct covalent crosslinking or nickel-based histidine tagging) NPs with a molecule or biomarker that binds to receptors found on tumor cells. Functional NPs were prepared by covalently linking them to biological molecules such as peptides, proteins, nucleic acids, or small-molecule ligands [3–5]. Superparamagnetic iron oxide NPs as a contrast agent for lymph node prostate cancer detection [6] and polymeric NPs for targeted gene delivery to tumor vasculatures [7] have been explored. New technologies using metal and semiconductor NPs are also under intense development for molecular profiling studies and multiplexed biological assays [8, 9].

Nanotechnology is opening new therapeutic opportunities for agents that cannot be used effectively as conventional formulations due to poor bioavailability or drug instability. The Food and Drug Administration (FDA) recently approved Abraxane™, albumin-paclitaxel (Taxol™) NPs, for the treatment of metastatic breast cancer. Abraxane uses albumin, a human protein, to deliver the chemotherapy and does not contain chemical solvents like Cremophor™ eliminating the need for premedication with steroids or antihistamines for hypersensitivity reactions caused by the solvent. This new formulation was superior to Taxol according to the overall response rate in a randomized, open-labeled trial of 460 patients. Side effects were less intense even though a 50% higher dose of Taxol was delivered [10]. NP-based novel delivery methods are also at the forefront in the development of new formulations of off-patent and soon-to-be off-patent drugs.

Several important issues must first be addressed before nanotechnology applications are realized in cancer patients [11]. Foremost, it is necessary to produce NPs that are able to selectively accumulate at the tumor after systemic delivery. To design and synthesize multi-functional NPs for simultaneous imaging and treatment, it is equally important that such particles not only generate strong signals and contrast for *in vivo* tumor imaging, but also have a chemically and biologically active surface with a variety of functional groups for conjugation of tumor targeting ligands and therapeutic agents. Nanotechnologies will extend the limits of current molecular diagnostics and permit accurate diagnosis as well as the development of personalized medicine [12]. This review focuses on the potential of nanomedicine as it specifically relates to developing novel diagnostic and screening techniques, and multifunctional NPs to enable targeted delivery of therapeutic agents.

## BIOMARKERS

Molecular biomarkers include altered or mutant genes, RNAs, proteins, lipids, carbohydrates and small metabolite molecules, and their altered expressions that are correlated with a biological behavior or a clinical outcome. Identification of cancer biomarkers is one of the most promising approaches for the detection of early-stage malignant or even premalignant lesions. The role of biomarkers in cancer detection and progression is a major effort at various laboratories aimed at the development of novel and simple approaches for early detection of human cancer [13].

Molecular profiling studies, the major contributors of cancer biomarker discoveries, are based on an association or correlation between a molecular signature and cancer behavior. One of the pioneering molecular profiling studies showed that gene expression patterns could classify tumors, yielding new insights into tumor pathology such as stage, grade, clinical course, and response to treatment [14]. Gene expression studies of cancer cell lines further revealed that the molecular signature of each tumor is a result of the combined tumoral, stromal, and inflammatory factors of the original heterogeneous tumor [15]. Correlation of gene expression patterns with clinical outcome was first reported for diffuse large B-cell lymphoma [16] and the concept of a specific molecular portrait for each patient’s tumor was later validated [17, 18]. Recent efforts on cancer molecular profiling have utilized proteomic approaches and the combination of cDNA microarrays with tissue microarrays for biomarker discovery and immunohistochemical validation [19–25]. The use of oligonucleotide-based nanoparticles containing DNA sequences complementary to message RNAs of biomarker genes, such as molecular beacon imaging provides a simple and semi-quantitative way for simultaneous detection of the expression levels of multiple biomarker genes in a single cell level [26].

In breast and prostate cancers, a deadly step is the appearance of *lethal phenotypes*, such as bone-metastatic, hormone-independent, and radiation- and chemotherapy-resistant

phenotypes. It has been hypothesized that each of these aggressive behaviors could be predicted by a defining set of biomarkers. By defining the interrelationships among these biomarkers, it may be possible to diagnose and prognosticate cancer based on a patient's molecular profile leading to personalized and predictive medicine. Estrogen and progestogen receptors, and HER2/neu represent molecular biomarkers currently used in routine clinical practice to aid treatment decisions in cases involving breast cancer [27, 28]. For prostate cancer, a number of gene and protein biomarkers have been identified including p504S, hepsin, Pim-1, protease/KLK4, prostein, EHZ 2, GSTP1, and STEAP [29, 30]. These markers appear to be excellent indicators of cancer aggressiveness, such as metastasis and androgen independence.

Genentech developed Trastuzumab (Herceptin™), which is a humanized monoclonal antibody designed to target overexpressed *erbB2* tyrosine kinase receptor found in ~ 25%–30% of breast cancers. To facilitate the screening of patients and to guide Trastuzumab treatment decisions, an immunohistochemistry assay for the expressed protein (Dako's HercepTest™, Ventana's Pathway™) and a nucleic acid-based chromogenic or fluorescence *in situ* hybridization (CISH/FISH) test (Abbott's PathVysion™) have been approved as *in vitro* diagnostics already. In addition, numerous PCR based methodologies have also been described for testing the expression of *erbB2*. Clinical response of lung cancer patients to AstraZeneca's epidermal growth factor receptor (EGFR) tyrosine kinase domain inhibitor Gefitinib (Iressa™) is associated with a small number of genetic mutations. Acting in a similar manner to Erlotinib (Tarceva™), Gefitinib selectively targets the mutant proteins in malignant cells. Currently, use of Trastuzumab and Gefitinib is rather restricted due to serious side effects.

Despite the advances made, linking biomarkers with cancer behavior and personalized treatment remain a significant challenge due to the heterogeneous nature of most tumors (especially prostate and breast cancer), containing a mixture of benign, cancerous, and stromal cells. Current molecular profiling technologies (including RT-PCR, gene chips, protein chips, electrophoresis and biomolecular mass spectrometry) are not designed to handle heterogeneous samples [31, 32]. A major limitation of all these techniques is the need to obtain patient samples using invasive or minimally invasive approaches. Bioconjugated NPs provide an essential link by which biomarkers could be functionally linked with cancer behavior. The development of novel noninvasive tumor imaging in combination with biomarker targeted imaging contrast agents has potential for early detection of human cancer and determination of the status of expression of biomarker genes for the molecular targeted therapy. In the following, the design and development of widely used NPs and their applications in cancer were discussed.

## NANOMATERIALS

Currently, a vast array of nanomaterials and nanodevices are available. Some of the most widely utilized forms of nanomaterials are quantum dots (QDs), magnetic NPs, gold NPs, carbon nanotubes, polymers, dendrimers, and liposomes. NPs with metal elements usually have optical and magnetic properties that can be used for imaging, whereas polymer- or liposome-based NPs, by themselves, do not produce imaging signals. However, various imaging contrast agents were conjugated to these NPs and results showed the feasibility of tumor imaging using these NPs. Importantly, therapeutic agents can be conjugated or encapsulated to NPs through surface modification and bioconjugation of the NPs (Fig. 1). Different nanomaterials are described in the following sections with emphasis on multifunctional particles.

## QUANTUM DOTS

QDs range from 2 to 10 nm in diameter and are made of semiconductors, the most common material being cadmium selenide capped by zinc sulfide (CdSe/ZnS). They usually consist of 10–50 atoms and possess unique optical and electronic properties [33]. QDs have 10–50 times larger molar extinction coefficients compared to organic dyes, which make them much brighter in photon-limited *in vivo* conditions. Further, QD emission wavelengths are size-tunable and hence fluoresce in different colors depending on their size after excitation with UV light. Larger particles emit light in the red end of the visible spectrum, whereas smaller particles emit in the blue range. Researchers have extended the emission wavelength into the near infrared (NIR) (650–950 nm) to take advantage of the improved tissue penetration and reduced background [34].

The potential of QDs for biomedical applications was only realized recently, despite their development for electronics and optics two decades ago. However, their application has expanded markedly in the past few years as probes for high resolution molecular imaging of cellular components and for tracking a cell's activities and movements inside the body [35–37]. *In vivo* imaging with QDs has been reported for lymph node mapping, angiogenic vessels, and cell subtype isolation. A key property for *in vivo* imaging is the unusual QD Stokes shift (300–400 nm) depending on the wavelength of the excitation light [38] and narrow emission peaks, allowing multiplexed imaging applications in which one light source is used to simultaneously excite multicolor QDs. A major advantage of using QDs over radioactive tags or organic fluorophores such as fluorescein or cyanine dyes for in vivo applications is that QDs have long blood circulation time, can fluoresce for several months *in vivo*, and are resistant to photobleaching [39]. These properties have made QDs a topic of high priority in cellular imaging and molecular profiling of pathological tissue specimen of cancer patients for diagnosis of types and stages of disease, prediction of prognosis, and directing the treatment strategy. At present, the application of QDs is restricted to *in vitro* and animal studies due to the toxicity concerns of the heavy metal, cadmium. For the future applications in human, researchers are pursuing novel methods to produce new generation of QDs with reduced amount of cadmium or without any cadmium.

## PARAMAGNETIC NANOPARTICLES

Paramagnetic NPs have a variety of applications in molecular and cellular imaging, and have been around for years as non-targeted contrasting agents for magnetic resonance imaging (MRI). They usually involve superparamagnetic iron oxide (SPIO or IO) or magnetite ($Fe_3O_4$) particles. Nanometer-sized crystals of IO are paramagnetic and possess extremely high molar relaxivity resulting in hypointensity on MRI images, creating contrast that extends to greater dimensions than the particles themselves. The bulk of the studies reported have focused on the use of nanometer-scale IO particles. Such particles may be classified as: (1) ultrasmall superparamagnetic IO particles or USPIOs (< 10–50 nm in diameter), (2) small superparamagnetic IO particles or SPIOs (50–150 nm in diameter), and (3) monocrystalline IO nanocompounds or MIONs (100–200 nm in diameter).

NPs have been used in experimental paradigms to label and track transplanted human mesenchymal stem cells, neural stem cells, hematopoietic cells, Schwann cells, olfactory ensheathing cells, and oligodendrocyte precursors among others [40]. Several promising cellular transplantation therapies for CNS diseases and injury are currently entering human clinical trials. Activated macrophages [41] were recently tested in a Phase II clinical trial by direct injection into the spinal cord of humans with acute spinal cord injury (ProCord™; Proneuron Biotechnologies, Los Angeles, CA). As promising cellular treatments move forward, there is a critical need for noninvasive and objective methods that allow for the

identification and tracking of such cells once they have been transplanted. IO-NPs provide an excellent solution for this. An FDA-approved MRI contrast agent (Feridex™, SPIO from Bayer HealthCare) was used for labeling human cancer or stem cells [42].

Paramagnetic IO-NPs are becoming increasingly attractive for the development of target-specific MRI contrast agents. IO-NPs have unique paramagnetic properties, which generate significant susceptibility effect resulting in good contrasts at low concentrations [43–46]. IO-NPs have a long blood retention time and are generally biodegradable and considered to have low toxicity. Therefore, IO-NP is an excellent candidate for the production of imageable therapeutic nanodevices and functionalization of IO-NPs helps to achieve specific tumor targeting in addition to other desired properties. IO-NPs can be made water-soluble by coating with a hydrophilic polymer (e.g., PEG or dextran), amphiphilic copolymers or hydrophobic by coating with aliphatic surfactants or liposomes to get magnetoliposomes. Attaching PEG to NPs is a general means of preventing opsonization, reducing reticuloendothelial uptake, enhancing biocompatibility, and increasing circulation time.

Thermotherapy is defined as the ability to achieve hyperthermic temperatures of up to 42°C, which can render cancer cells more susceptible to the effects of radiation and cause apoptosis to some extent. Because SPIO-NPs undergo Brownian relaxation to generate heat by the rotation of particles influenced by an alternating field, electromagnetic fields can be used to remotely activate SPIO-NPs for cancer thermotherapy. Although there are few reports showing the efficacy of SPIO-NPs in thermotherapy of cancers [47], it is difficult to achieve the required concentrations (0.01–0.1% IO) for thermal ablation via intravenous route. Therefore, the SPIO-based thermotherapy will most likely be used in combination with chemo- and radiotherapies rather than as a stand-alone technique.

## NANOSHELLS

Nanoshells are nano-sized nanoparticles consisting of a silica core coated with a thin gold shell [48]. By fine-tuning the thickness of the core and outer shell, one can design the particles that absorb and scatter specific wavelengths of light across the visible and NIR spectrum. Typically, nanoshells that strongly absorb NIR light would be highly useful because they can create intense heat that is lethal to cells and face less interference from the tissue chromophores [49]. Nanoshells with a silica core diameter of ~120 nm and a 10 nm layer of gold shell can achieve this goal. The ability of nanoshells to scatter light is being utilized for cancer imaging, while their primary use continues to be in thermal ablation therapy. Although, focused lasers for thermotherapy were useful, simple heating cannot discriminate between tumors and healthy tissue. With nanoshell mediated approach, the energy can pass through the healthy tissue without causing harm, killing only the targeted tumor cells. The ability of nanoshells and NIR treatment were utilized to completely eliminate tumors by thermal ablation *in vivo* [50]. Also, nanoshells conjugated with antibodies were employed to specifically recognize and target breast adenocarcinoma cells overexpressing HER2 *in vitro* [48]. The antibodies were conjugated to PEG, and the antibody-PEG complex was then attached to the nanoshell surface through a sulfur-containing group located at the distal end of the PEG linker.

## CARBON NANOTUBES

Another type of nanodevice is carbon nanotubes [51], which were discovered in the late 1980s. They are composed of a distinct molecular form of carbon atoms and are 100 times stronger than steel with only one-sixth of its weight, and exhibit unusual heat and conductivity properties. It has been shown that carbon nanotubes can be used to transport DNA molecules into the cell and for thermotherapy. Single-walled carbon nanotubes (1–2 nm diameter) carrying a cargo of 15-mer DNA can be internalized by cells and accumulate

in the cytoplasm without causing cytotoxicity [52]. The cellular uptake was minimal at 4° C, while exposing the DNA-nanotube containing cells to several 10-second pulses of NIR caused endosomal rupture, triggering DNA unloading from the nanotubes and translocation into the nucleus. Normal morphology and no apparent death of the cells were observed under these conditions. Folic acid was adsorbed onto the nanotubes to allow specific binding to cancer cells overexpressing folate receptors and subsequent receptor-mediated endocytosis. Upon irradiation with NIR, only tumor cells were selectively destroyed leaving normal cells, with a low level of the receptor, unharmed. The localization and internalization of nanotubes were visualized by attached fluorescent tags [52].

Delivering siRNA to target cells is highly problematic because of the instability of siRNA and low uptake efficiency. Using nanotubes as vehicles for delivery of siRNA presents great promise, and nanotubes carrying siRNA were shown to rapidly enter tumor cells and release the cargo to exert RNA interference on target gene expression [53]. After intralesional injection, siRNA was delivered into tumor cells resulting in excellent gene silencing, inhibition of cancer cell proliferation *in vitro* and suppression of tumor growth in mouse models. Nanotubes contained -CONH-$(CH_2)_6$-$NH_3^+$ functional groups to facilitate the conjugation of siRNA targeting murine telomerase reverse transcriptase, the catalytic subunit of telomerase. Telomerase inhibition is an important strategy for targeted cancer therapy because it is critical for immortalization and is expressed in a majority of malignant tumors but not in most normal somatic cells. Within 48 hours following the siRNA-nanotube treatment, the tumor cells showed senescence-like features and reduced telomerase activity.

Nanotubes were employed as carriers for imaging and therapeutic agent delivery and the biodistribution of radio-labeled nanotubes was investigated in mice by *in vivo* positron emission tomography (PET), ex vivo biodistribution and Raman spectroscopy. It was found that the nanotubes functionalized with phospholipids bearing PEG were surprisingly stable *in vivo* with long circulation times and low uptake by the reticuloendothelial system. Efficient targeting of integrin positive tumor in mice was achieved with nanotubes coated with PEG chains linked to an argine-glycine-aspartic acid (RGD) peptide [54]. In addition, nanotubes were made temperature- and pH-responsive [55]. Although recent studies revealed that administration of nanotubes did not produce apparent cytotoxic effect in mice [56], the fate of the nanotubes inside the cells and tissues has yet to be investigated thoroughly before further development for human use.

## DENDRIMERS

Dendrimers (*dendron* in Greek meaning *tree*, also called arborols and cascade molecules) are repeatedly branched polymers that are normally 2–10 nm in diameter, with approximately spherical shapes. Low-molecular weight species include monodisperse and highly symmetric dendrimers and dendrons, while the high-molecular weight species encompass dendronized polymers, hyperbranched polymers, and brush polymers (also called bottle brushes). Due to the lack of molar mass distribution, dendrimers and dendrons are macromolecules but not polymers. A dendrimer can be water-soluble when its end-group is hydrophilic (e.g., a carboxyl group). It is also possible to design a water-soluble dendrimer with internal hydrophobicity allowing it to carry a hydrophobic drug in its interior. The most commonly studied system has been the family of PAMAM (polyamidoamine) dendrimers, but the variety of building blocks is growing rapidly [57]. The polymer branches provide vast amounts of surface area to which therapeutic agents and targeting molecules could be attached. A typical dendrimer contains an ammonia core that is reacted with acrylic acid to produce a tri-acid molecule, which is then reacted with ethylenediamine to produce a tri-amine (generation 0, $G_0$, product). This tri-amine is reacted with acrylic acid to produce a

hexa-acid, which is further reacted with ethylenediamine to produce a hexa-amine ($G_1$ product). Desired generation is achieved by continuing these alternating reactions, and the starting core may also consist of sugars or other molecules to create a surface with multiple amines or multiple acids and to provide the means of attaching multiple functionalities.

A multifunctional dendrimer conjugated with fluorescein isothiocyanate for imaging, folic acid as a biomarker for targeting cancer cells overexpressing folate receptors, and paclitaxel as a chemotherapeutic drug was recently synthesized [58]. The amino groups were partially acetylated to improve solubility and prevent nonspecific targeting and the functionalities (imaging agent, biomarker and drug) were conjugated to the remaining nonacetylated primary amino groups. Fluorescein was attached using thiourea bond, while folic acid was covalently conjugated via condensation reaction between the γ-carboxyl group of the folic acid and the primary amino group of the dendrimer. Finally, paclitaxel was attached covalently through an ester bond to facilitate easy cleavage. This dendrimer conjugate acted as a pro-drug that remains inactive until the drug release from the carrier. The remaining primary amino groups were converted to -OH to prevent nonspecific targeting during delivery. Drug-free dendrimers were not cytotoxic *in vitro* and the drug-loaded dendrimers had no effect on folate receptor-negative cells. Toxicity was observed only with the intracellular delivery of paclitaxel and not merely due to its presence in the media. Drug susceptibility of folate receptor-positive cells was observed at ~100 nM for dendrimer conjugates. At 200 nM (equivalent to 800 nM of free paclitaxel), the dendrimer conjugates were toxic irrespective of folate receptors due to nonspecific binding, whereas drug-free dendrimers were nontoxic at 200 nM.

Methotrexate-carrying dendrimers that could recognize cells expressing folate receptors were also used to demonstrate successful *in vivo*-targeted drug delivery to cancer cells [59]. These dendrimers also carried fluorescein as a tracking agent in addition to the drug and the biomarker. Targeted dendrimers were highly effective compared to free methotrexate alone in delaying the growth of epithelial cancer xenografts in mice.

## LIPOSOMES

Liposomes (derived from Greek words *lipid* meaning *fat* and *soma* meaning *body*) are spherical vesicles composed of a lipid bi-layer membrane, resembling tiny cells with a cell membrane but nothing in the core. Liposomes, usually but not by definition, contain a core of aqueous solution while lipid spheres that contain no aqueous material are called micelles. Reverse micelles, on the other hand, can be made to encompass an aqueous environment [60]. Liposomes have been used to encapsulate and deliver chemotherapeutics for more than 3 decades. During the 1990s, they were extensively researched as potential vectors for gene therapy, and with the advent of nanotechnology, liposome research has regained considerable momentum.

Liposomes (90–150 nm) are slightly bigger than conventional NPs (≤ 100 nm), but a significant portion of biomedical nanotechnology research involves liposomes due to their unique properties. By manipulating their formulation using lipids of different fatty acid chain lengths, liposomes can be constructed to be temperature- or pH-sensitive to permit controlled release of their contents [61]. Temperature-sensitive liposomes can release the drug contents in tens of seconds at clinically attainable hyperthermia (39–42°C). Use of local hyperthermia on liposomes loaded with doxorubicin resulted in complete regressions of human tumor xenografts in all the mice studied [62].

Liposomal drug delivery has several proven benefits as liposomes are composed of the components of natural human cell membranes. Liposomes may circulate in the bloodstream for extended periods as compared to a non-liposomal drug resulting in an extended treatment

effect. Another interesting property of liposomes is their natural ability to target cancer and accumulate at the site of a tumor or infection delivering higher drug concentrations to the target. The endothelial wall of healthy human blood vessels is encapsulated by endothelial cells that are bound together by tight junctions that prevent any large particle in the blood from leaking out of the vessel. Tumor vessels do not contain the same level of seal between cells and are diagnostically leaky. Therefore, liposomes of certain sizes (< 400 nm) can rapidly enter tumor sites from the blood. Liposome carriers are also believed to play a role in reducing the harmful effects of certain drugs on healthy tissues, resulting in an improved safety profile. Some liposome NPs are already on the market: Doxil™ (Doxorubicin hydrochloride in liposome of Ortho Biotech, Bridgewater, NJ, USA) for ovarian cancer, DaunoXome™ (Daunorubicin citrate in liposome of Diatos, Paris, France) for advanced AIDS-related Kaposi's sarcoma, and AmBisomeTM (Amphotericin B in liposome of Gilead Sciences, Foster City, CA, USA) for fungal infections.

Further efforts are already underway to allow liposomes to avoid uptake by the reticuloendothelial system. These are constructed with a PEG coating and are referred to as *stealth liposomes*. They are designed to contain biological species (e.g., monoclonal antibodies, vitamins or specific antigens) conjugated as a ligand to enable binding via a specific expression on the targeted drug delivery site.

## DEVELOPMENT OF MULTIFUNCTIONAL NANOMATERIALS

Beyond clever pharmacokinetic manipulations to achieve novel delivery vehicles, a more complex goal of nanotherapeutics is to devise agents that selectively target tumor areas, provide imaging capabilities, and deliver specific therapy with minimal untoward effects elsewhere. NPs offer a wide range of surface functional groups allowing conjugation to multiple diagnostic and therapeutic agents. Multi-functional nanostructures could be used for simultaneous targeting, imaging and treatment, a major goal in cancer research and development (Table 1). However, progress has been slow and promising multifunctional platforms are still at an early or proof-of-concept stage using cultured cancer cells and are not immediately relevant to *in vivo* imaging and treatment of solid tumors [81–85].

Several studies indicated that NPs of 10–100 nm size were accumulated preferentially at tumor sites through *enhanced permeability and retention* effect [86], which arise from: (a) production of vascular endothelial growth factors (VEGFs) by tumors to promote angiogenesis of tumor endothelial cells with mature vascular structures, and (b) lack of an effective lymphatic drainage system in tumors. These factors may cause tumor-associated neovasculatures to be highly permeable, allowing the leakage of circulating macromolecules and NPs into the tumor interstitium. However, there could be numerous challenges ahead. NPs may successfully reach the tumors either through the retention effect or by using specific biomarkers, but their ability to penetrate the tumor mass may be impaired by barriers created by abnormal tumor physiology, viz., physically compromised vasculature, abnormal extracellular matrix (ECM), and high interstitial fluid pressure. Normal microvasculature (~8–10 μm in diameter) is uniformly structured, while the tumor vasculature is highly variable (20–100 μm) and chaotic. In addition, the blood flow is erratic (rapid in some parts and no flow through other parts) in tumor vessels that may be leaky along one side but not along the other side while others may not be leaky at all. Proliferating cancer cells can cause intratumoral vessels to compress and collapse [87]. Diffusion of nutrients and chemotherapeutics throughout the tumor is very inefficient due to the tumor properties [88] and the same is true with the diffusion of NPs. In addition, there are extravascular barriers to delivery, whereby NPs can extravasate but cannot penetrate through the tumor ECM. After i.v. administration, liposomes (90 nm in diameter) were able to extravasate the tumor vasculature but remained away from the blood vessel in human colon

adenocarcinoma xenografts in mice [89]. These liposomes formed perivascular clusters following extravasation and did not move significantly in tumor interstitial space. After intratumoral injection, adenovirus NPs (100 nm) transfected the tumor cells only along the needle track and did not diffuse readily across the ECM [90]. Therapeutic efficacy of NPs may be limited if they are unable to efficiently penetrate the tumor.

Fibrillar collagen also restricted the distribution of replication-deficient herpes simplex virus particles and QD-encoded silica spheres (both ~ 150 nm) [91]. However, dextran particles (40 nm) and IgG molecules were able to penetrate into the collagen-rich regions and distribute relatively uniformly within the tumor, indicating that this collagen exclusion was particle-dependent. Administering collagenase with virus particles into the tumors resulted in a greater particle distribution and improved therapeutic effect [91]. A *normalization* hypothesis was recently proposed that calls for administration of anti-angiogenic agents to remodel and normalize the existing tumor vasculature [92]. The goal is to transiently *normalize* the abnormal structure and function of tumor vasculature to restore efficient blood flow within the tumor, decrease the high interstitial fluid pressure characteristic of tumors, and improve the delivery of therapeutics. Delivery and efficacy of NPs would be improved by manipulating tumor vasculatures. Molecules that have been shown to be successful at normalization include Herceptin, which is a monoclonal antibody specific to human HER-2 to treat metastatic breast cancer [93], and Avastin (Bevacizumab), which is a monoclonal antibody specific to human VEGF [94].

The strategies for generating multifunctionalities share common approaches irrespective of the nature of NPs and involve encapsulation, covalent conjugation, or noncovalent adsorption of various moieties to allow the NPs to recognize or locate the tumor, deliver a load or kill the tumor cells, and permit visualization or imaging. Synergistic effects could be achieved by conjugating different peptides or by loading with multidrug regimens. More complicated schemes could be devised with the use of heat-labile or protease-susceptible tethers to engineer the *smart* NPs for targeted drug release. DNA with heat-labile hydrogen bonding between complementary strands may serve as a heat-labile linker. Protease-susceptible linkers could be the substrates for tumor-specific or tumor environment-specific enzymes. Tumor-specific processes and environments may be exploited to trigger the release of therapeutic agent by enzymatic activation of NPs via bonds that are sensitive to degradation under certain conditions (e.g., abnormal pH and oxygen levels, unique biomarkers, ECM remodeling and proteolytic enzymes overexpressed in tumors). A strategy for SPIO self-assembly was recently developed by designing biotin and neutravidin-coated IO-NPs that are inhibited from self-assembly by PEG chains anchoring the NPs via matrix MMP-2 cleavable peptide substrates [95]. NPs could only self-assemble upon proteolytic removal of surface PEG through MMP-2 cleavage of the peptides. MMP-2 is a tumor-specific protease correlated with cancer invasion and metastasis, and this strategy permitted magnetic resonance imaging (MRI) of protease-producing tumor cells with enhanced image contrast. Although these are the general strategies, inducing the NPs to actually perform *in vivo* as predicted by theory and addressing the biocompatibility, biostability, and biodistribution issues require extensive research.

Optical imaging is highly sensitive, but its applications *in vivo* are hampered by a limited penetration depth in the tissue and the lack of anatomic resolution and spatial information. Although NIR wavelengths improve penetration depth and 3-D fluorescence tomography provides spatial information [96], other imaging modalities such as MRI are much better for tomography and 3-D imaging. MRI offers exceptional tissue contrast and spatial resolution and has been widely used in the clinical setting. Thus, there has been considerable interest in developing dual-modality contrast agents for combined optical and MR imaging. By reacting SPIO with the cyanine based NIR fluorescent dye Cy5.5 (available from GE

Healthcare Life Sciences), dual magneto-optical probes were developed to bind to apoptotic cells [97]. Similar probes have been used to obtain anatomic and molecular information in living organisms [98]. These probes are prepared by conjugation of peptides to cross-linked IO amine (amino-CLIO), either by a disulfide linkage or a thioether linker, followed by the attachment of the dye Cy5 or Cy7. Fluorescence quenching of the attached fluorochrome occurs by interaction with the IO core, and by electronic coupling among the dye chromophores (self-quenching). This class of dual-modality probes provides the basis for *smart* or *intelligent* NPs, capable of pinpointing their position through their magnetic properties, while providing information on their environment by optical imaging.

Dual function probes were also developed by linking QDs with $Fe_2O_3$ and FePt [99, 100] and by entrapping gadolinium (Gd) on the QD surface using polymer-conjugated lipids [101, 102]. But it is not clear whether such *hetero* nanostructures would be useful for *in vivo* medical imaging. A urokinase plasminogen activator receptor (uPAR) targeted IO-NPs using an amino-terminal fragment (ATF, consists of the first 135 amino acids) of urokinase plasminogen activator (uPA) protein has been developed in our laboratory [103]. These ATF-IO conjugates serve as tumor targeting and imaging probes as well as drug delivery vehicles. These NPs were labeled with Cy5.5 for dual-modality optical and MR imaging, and ATF-IO NPs bind to and are internalized by breast cancer cells *in vitro*. Systemic delivery of the ATF-IO or Cy5.5-ATF-IO leads to the accumulation of NPs in mouse mammary tumors in subcutaneous, and lung and intra-peritoneal metastases, producing strong imaging signals [Yang, L., unpublished data]. Furthermore, the methods to incorporate hydrophobic chemotherapy drugs into the amphiphilic copolymer layer coat of the IO-NPs have been developed, which enable simultaneous targeting, imaging and drug delivery [Yang, L. unpublished data].

With the advent of efficient anticancer agents and delivery mechanisms comes the challenge of elucidating useful biomarkers to monitor patients receiving these therapies [104]. Development of noninvasive biomarkers of disease response and relapse is a crucial objective in achieving real-time imaging of therapeutic effect.

## OUTLOOK

Nanotechnology has become an enabling technology for personalized medicine in which cancer detection, diagnosis, and therapy are tailored to each individual’s tumor molecular profile and for predictive oncology in which genetic/molecular markers are used to predict disease development, progression, and clinical outcomes. Current cancer treatment options (surgery, radiation, and chemotherapy) are highly invasive and are often accompanied by side effects and toxicity to healthy cells. The promises of nanotechnology in cancer research lie in the potential to overcome these drawbacks.

There are many promising research directions that require concerted effort for success. Foremost is the design and development of NPs with mono-, dual- or multiple functions, allowing detection, diagnosis, imaging, transport and controlled release of cargo, and cell destruction. Greater efficacy of lower doses of drugs and destruction of solely the cancer cells could be achieved by selective targeting of unique surface signatures of tumor cells. Rational design of NPs requires the knowledge of tumor-specific receptors that would allow endocytosis of NPs, tumor-specific biomarkers that facilitate identification of cancers, and tumor-specific homing proteins and enzymes that can permit selective uptake into cells or accumulation in tumor microenvironments. Advances in cancer biology are critically essential for the advancement in nanotechnology.

Secondly, NP molecular profiling or nanotyping for clinical oncology is important to predict cancer behavior, clinical outcome, and treatment response to allow individualized therapy.

Nanotyping of a panel of tumor markers will allow more accurate correlations than a single tumor marker in defining the aggressive phenotypes of cancer as well as determining the treatment response of early stage disease.

Finally, investigating NP distribution, metabolism, excretion, pharmacodynamics, and potential long-term toxicity *in vivo* is essential to monitor effects in patient population and to evaluate personnel involved in manufacturing and disposal. All the directions discussed above are very important in developing NPs for clinical applications.

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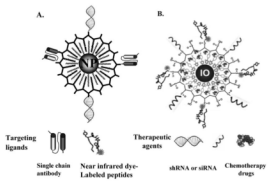


**Fig. 1.**

Multifunctional nanoparticles (NPs) depicting various functionalities. Tumor-specific antibodies, peptides, shRNA or siRNA, aptamers or other small molecules serve as biomarkers. Dye molecules, attached either to the biomarker or the nanoparticle, serve as imaging agents. Chemotherapy drugs loaded or attached on to the polymer core of the nanoparticle serve as therapeutic agents.

**Table 1**

Therapeutic Nanoparticles for Drug Delivery

| Nanoparticle | Size (nm) | Therapeutic agents used | Applications |
|---|---|---|---|
| Metallic particles (e.g., Iron oxide, Quantum dots) | < 50 nm | Anticancer agents, DNA, Proteins | Cancer therapy [63–65] |
| Liposomes | 50–100 nm | Anticancer agents, DNA, Proteins | Cancer therapy [66–68], HIV therapy [69], Vaccine delivery [70] |
| Dendrimers | < 10 nm | Anticancer, antibacterial and antiviral agents, DNA, high molecular weight drug compounds | Cancer therapy [59,71,72], Antibacterial therapy [73,74], HIV therapy [75, 76] |
| Polymeric biodegradable particles | 10–1000 nm | Plasmid DNA, Peptides, Proteins, Low molecular weight drug compounds | Brain tumor therapy [77, 78], Vaccine adjuvant [79], Diabetes therapy [80] |