Expert Opinion

Multifunctional nanoparticles for imaging, delivery and targeting in cancer therapy

Marian E Gindy & Robert K Prud'homme†
Princeton University, Chemical Engineering, Princeton, NJ 08544, USA

The application of nanoparticles for the delivery and targeting of pharmaceutical, therapeutic and diagnostic agents in cancer therapy has received significant attention in recent years. Nanoparticles may be constructed from a wide range of materials and used to encapsulate or solubilize chemotherapeutic agents for improved delivery *in vivo* or to provide unique optical, magnetic and electrical properties for imaging and therapy. Several functional nanoparticles have already been demonstrated, including some clinically approved liposome drug formulations and metallic imaging agents. The next generation of nanoparticle-based research is directed at the consolidation of functions into strategically engineered multifunctional systems, which may ultimately facilitate the realization of individual therapy. These multiplexed nanoparticles may be capable of identifying malignant cells by means of molecular detection, visualizing their location in the body by providing enhanced contrast in medical imaging techniques, killing diseased cells with minimal side effects through selective drug targeting, and monitoring treatment in real time. This article highlights recent progress in the design and engineering of multifunctional systems, as well as discusses the development of a new, scalable and economic method for the modular preparation of multiplex nanoparticles where functional properties can be precisely and simply tailored.

Keywords: diagnostics, drug delivery, Flash NanoPrecipitation, imaging, multifunctional, nanoparticle

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1. Introduction

Cancer is the leading cause of morbidity and mortality in the US, recently surpassing heart disease [1]. In 2007, it was estimated that ~ 10 million cases of cancer occurred globally, with nearly 1.43 million new cases and 560,000 deaths projected to occur in the US in the year 2008 [2]. Although conventional treatment options such as chemotherapy, radiation and surgery have experienced significant advances over the past few decades, they remain far from optimal.

Accordingly, the principal goal of current cancer research is to develop clinically useful technologies for improved diagnosis and treatment of the disease in patients. Achieving this goal involves a multidisciplinary approach to address critical unmet needs in established treatment regimens, including lack of early disease detection, poor drug bioavailability, nonspecific systemic drug distribution, inadequate drug concentrations reaching the tumor, and inability to monitor therapeutic responses in real time.

Nanotechnology has the potential to offer solutions to several of these obstacles [3,4]. The nanometer sizes of many materials (polymers, metals, semiconductors) offer distinct advantages for *in vivo* applications, where, in general, the increased surface area and quantum effects dictated by the nanometer scale present new optical, electronic, magnetic and structural properties not available from individual molecules or bulk solids. Nanoparticles with properties of self-assembly, stability, specificity,

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drug encapsulation and imaging contrast have already been used clinically to provide targeted cellular/tissue delivery of chemotherapeutics, to improve drug bioavailability, to sustain drug effect in target tissue and to diagnose disease [5,6].

The establishment of these first-generation nanoparticles as commercial medicines or imaging agents has prompted investigation of second-generation materials with increasing degree of complexity [7,8]. These engineered multifunctional systems are intended to integrate therapeutic and diagnostic or monitoring components in a synergistic fashion to achieve a more potent target response. For example, enabling technologies are envisioned to include:

- Imaging agents and diagnostics that allow clinicians to detect cancer in its earliest stages.
- Systems that provide real-time assessments of therapeutic efficacy.
- Targeted devices capable of bypassing biological barriers to deliver multiple therapeutic agents directly to tissues in the microenvironment that play a critical role in the growth and metastasis of cancer.
- Agents that monitor predictive molecular changes to identify precancerous cells.
- Research tools that make possible rapid identification of new targets for clinical development.

In this article, select single-function nanoparticles commonly investigated for *in vivo* drug delivery, medical imaging and diagnostics are addressed. Current examples of multifunctional systems, where two or more components are cooperatively integrated to improve their application potential, are then discussed. Finally, a new fabrication method - Flash NanoPrecipitation - developed to address key challenges associated with the economical, scalable and tunable preparation of complex multifunctional nanoparticles, is highlighted.

2. Nanoparticles in drug delivery

The utility of nanoparticles for the delivery of small molecule chemotherapeutics *in vivo* is now well established, with various formulations of doxorubicin, danorubicin and paclitaxel already commercially available (**Table 1**) [9-11]. In general, nano particles are designed to address limitations of conventional drug delivery systems, including nonspecific biodistribution and targeting, low aqueous solubility, poor bioavailability and low therapeutic indices stemming from insufficient drug concentration at disease sites.

Drug carrying nanoparticles can be synthesized from a range of materials using a variety of techniques. Nanoparticle formations by precipitation, emulsion, or lipid extrusion methods are widely used. The most commonly used materials are based on biocompatible, and preferably biodegradable, lipids (liposomes) or polymers (polymer micelles, nanoparticles, or dendrimers) [12-15]. Drugs may be dispersed in a matrix, encapsulated in a vesicle, dissolved in a hydrophilic or hydrophobic core, or attached to the surface of a nanoparticle [11,16].

2.1 Liposomes

Liposomes are the archetypal nanoparticles for delivery of chemotherapeutic compounds [12,14,17]. These self-assembled structures are composed of phospholipids in which an outer lipid bilayer surrounds a central aqueous space. Water-soluble drug compounds can be trapped in the interior of the liposome-enclosed aqueous core, and the encapsulating bilayer can be used to deliver hydrophobic drugs. Liposomal drug delivery systems have been studied extensively since the 1970s to increase the solubility and therapeutic index of chemotherapeutic agents [14,18]. In 1995, Doxil® (Ortho Biotech), a liposome-encapsulated doxorubicin formulation, became the first liposome system to gain approval by the US Food and Drug Administration (FDA) for clinical human use. It was fully approved in 2005 [19]. The therapy remains in use today and has demonstrated potent activity against a wide range of human cancers. Further examples of clinically approved liposome drug formulations are listed in **Table 1**.

Although clinically successful in some applications, liposomal drug carriers are limited by poor storage stability, rapid clearance from the bloodstream, nonspecific uptake by the mononuclear phagocytic system and rapid drug release profiles *in vivo* [14]. Furthermore, the limited volume of the lipid bilayer makes the delivery of hydrophobic drugs highly inefficient.

2.2 Polymer micelles and nanoparticles

To minimize inherent instability and degradation limitations associated with liposome formulations, polymer-based drug carriers were developed [6,11]. Compared with liposomes and surfactant micelles, polymeric systems demonstrate a series of attractive properties as pharmaceutical carriers, including high drug loading capability, prolonged circulation time stemming from higher stability *in vivo*, and slower rates of dissociation that allow retention of loaded materials for a longer period of time [13,15,20]. In addition, the delivery systems can be designed to provide either controlled release or triggered release of the therapeutic molecule [21,22].

Among particle-forming compounds, amphiphilic polymers are of particular significance. In selective solvents, amphiphilic diblock and triblock copolymers, for example, self-associate to form micelles when the polymer concentration is at or above the critical micelle concentration (CMC) [13]. For amphiphiles consisting of hydrophilic and hydrophobic segments, the self-assembly in aqueous solutions results in the formation of nanometer-sized particles with hydrophobic core/hydrophilic shell-type structures. The core regions serve as reservoirs for hydrophobic drugs, whereas the hydrophilic exterior imparts stability to the carrier in aqueous environments [13,20,23]. Depending on the method of preparation, the drug can be chemically, electrostatically, or physically entrapped *in situ* during particle formation, or covalently bound to the polymer assemblies.

Several polymer nanoparticles are now in various states of preclinical and clinical development as carriers of chemotherapeutics [24-26]. Representative examples include

Table 1. Representative examples of marketed nanoparticle-based drug delivery and imaging contrast agents.

Commercial name	Nanoparticle carrier	Active agent	Approved indications
Drug delivery			
Doxil Caelyx	PEGylated liposome	Doxorubicin	Ovarian cancer, AIDs-related Kaposi's sarcoma, and recurrent breast cancer. Combinatorial therapy (with bortezomib) of multiple myeloma
Myocet	Liposome	Doxorubicin	Combinatorial therapy (with cyclophosphamide) of recurrent breast cancer, ovarian cancer and AIDs-related Kaposi's sarcoma
DaunoXome	Liposome	Daunorubicin	Kaposi's sarcoma
Onco TCS	Liposome	Vincristine	Relapsed aggressive non-Hodgkin's lymphoma
Abraxane	Albumin	Paclitaxel	Metastatic breast cancer
Abelcet Amphotec	Liposome	Amphotericin B	Treatment of invasive fungal infections in patients who are refractory to or intolerant of conventional amphotericin B
AmBisome	Liposome	Amphotericin B	Empiric therapy for presumed fungal infections in febrile neutropenic patients. Treatment of visceral leishmaniasis. Treatment of invasive fungal infections in patients who are refractory to or intolerant of conventional amphotericin B
Imaging contrast agents			
Lumiren Gastromark	Silica	Superparamagnetic iron oxides	Imaging of the gastrointenstinal tract and abdomen with MRI
Endoderm Feridex	Dextran	Superparamagnetic iron oxides	Detection of liver and spleen lesions associated with metastases, primary liver cancer, cysts and various benign tumors, adenomas and hyperplasia with MRI
Resovist	Carbodextran	Superparamagnetic iron oxides	Detection and characterization of especially small focal liver lesions with MRI
Sinerem	Dextran	Ultra-small paramagnetic iron oxides	Blood pool visualization and differentiation of metastatic and non-metastatic lymph nodes in patients with confirmed primary cancer who are at risk for lymph node metastases with MRI

Genexol-PM, a micellar vehicle for delivery of paclitaxel now in Phase II clinical trials, SP1049C, a carrier of doxorubicin in Phase II studies as first-line therapy in inoperable metastatic adenocarcinoma of the esophagus, XYOTAX, a poly-(L)-glutamic acid-based carrier of paclitaxel, which recently qualified for fast track designation for the treatment of poor performance status women with first-line advanced non-small cell lung cancer, and IT-101, a cyclodextrin-containing polymer carrier of camptothecin, for which Phase II clinical studies were initiated in 2008 [26,27].

3. Nanoparticles in imaging, diagnostics and therapy

Much attention has also been focused on inorganic nanoparticles in cancer treatment applications ranging from imaging, diagnostics and sensing to new therapies [28]. Owing to their sub-micrometer size, metal and semiconductor nanoparticles have unique electronic, optical and catalytic properties that vary significantly from properties of the bulk substance [29,30]. Of the wide number of materials available, nanoparticles based on gold, semiconducting metals and iron oxides are of particular interest. When applied to biological systems, they have the potential to improve imaging techniques such as X-ray imaging, computed tomography (CT), near-infrared (NIR) fluorescence imaging, positron emission spectroscopy (PET) and magnetic resonance imaging (MRI) [31-33]. Nanoparticle probes can endow imaging techniques with enhanced signal sensitivity, better spatial resolution, and the ability to relay information about biological systems at the molecular and cellular levels.

3.1 Magnetic resonance imaging

Magnetic resonance imaging is a powerful tool for the diagnosis of disease and the study of biological processes such as cancer metastasis and inflammation. Superparamagnetic iron oxide nanoparticles, in particular, are effective contrast agents that provide high sensitivity in MRI [30,33]. First introduced in the mid-1980s, they are typically composed of iron oxide-based cores (magnetite, maghemite, or other insoluble ferrites) in the size range 5 – 10 nm coated with water-soluble stabilizing layers. In the presence of a magnetic field, these magnetic

domains possess large magnetic moments that produce localized disturbances in magnetic field homogeneity. Owing to these magnetic disturbances, there exists a large susceptibility difference between the iron oxide crystals and nearby protons, which causes rapid dephasing of spins and a resultant decrease in transverse and translational relaxation times. The shortening of the transverse relaxation time, specifically, results in a darkening of the image relative to unaffected areas, corresponding to a 'negative' contrast enhancement. Compared with micrometer-sized magnetic particles and chelates of paramagnetic ions such as gadolinium diethylenetriaminopentaacetic acid (Gd-DTPA), superparamagnetic iron oxide nanoparticles are much more efficient as relaxation promoters, and their effect on the relaxivities of water is measurable even at nanomolar concentrations [34]. Commercial examples of superparamagnetic MRI contrast agents include Lumiren, silicon-coated iron oxide particles with a diameter of ~ 300 nm, Endorem and Ferixex I.V, liver-specific, dextran-coated iron oxide particles of ~ 150 nm diameter, and Sinerm, a blood pool contrast agent (**Table 1**).

3.2 Optical imaging

In vivo imaging of deep tissue (> 500 μm) by means of optical methods is of particular interest, as costs are generally lower than for competing technologies, including MRI. Nonetheless, most conventional optical imaging techniques are not suitable for imaging thick living tissue owing to light scattering by biological matter, which leads to poor spatial resolution. Photoacoustic imaging, also called optoacoustic tomography (OAT), is a new medical imaging method that uses optical illumination and ultrasonic detection to overcome this limitation [35]. In OAT, tissue is irradiated with a short laser pulse tuned to the NIR region of the spectrum, where wavelengths between 600 and 900 nm provide near transparency in living tissue. Light-absorbing components in tissue, such as hemoglobin of blood, absorb photons and transfer energy in the form of heat to the surroundings, resulting in thermoelastic expansion and generation of acoustic waves. The measured acoustic pressure is used to create a spatially varying distribution of the absorbed energy from the illuminated sample. As ultrasound waves can travel through tissue with minimal scattering and attenuation, OAT is capable of locating optically absorbing objects deep within tissue. For example, OAT has been used to detect abnormal angiogenesis in advanced tumors, where the increased blood content of the tumor acts as an endogenous contrast agent.

In early stages of the disease, however, angiogenesis is not sufficient to differentiate the tumor from healthy tissue, making exogenous contrast agents necessary [36]. For this purpose, gold nanoparticles have gained interest as exogenous contrast agents in photoacoustic imaging [37]. Gold nanoparticles show narrow and intense absorption and scattering bands due to the phenomenon of plasmon resonance. This occurs at the resonance condition of the collective oscillation that the conduction electrons experience in an electromagnetic field of the appropriate wavelength. When excited at the wavelength corresponding to the surface plasmon, the photoacoustic effect generated by the accumulated gold nanoparticles allows for higher sensitivity and better image resolution. Gold nanoparticles are also under evaluation as contrast agents for other optical imaging techniques, including two-photon luminescence imaging [31] and X-ray imaging [32].

3.3 Fluorescence imaging

Imaging with inorganic fluorescent probes has raised new possibilities for ultra-sensitive and multiplexed imaging in living cells, animal models and possibly in human subjects. Semi conductor quantum dots (QDs) are good examples of such probes [38]. Quantum dots have unique optical and electronic properties, which manifest in molar extinction coefficients that are 10 – 50 times larger than those of organic dyes, making them significantly brighter in photon-limited conditions [28,39]. Furthermore, the emission wavelengths of QDs are size dependent and can be made to emit into the NIR, permitting diminished background fluorescence. Quantum dots have been used successfully *in vitro* for the dynamic investigation of cellular processes, including cell migration, differentiation and metastasis, as well as *in vivo* for the identification of distinct features in tumor models [38,39].

3.4 Photothermal therapy

Hyperthermia-based therapy is a form of cancer treatment that uses an elevated temperature to kill the tumor tissue [40]. Traditionally, this therapeutic regimen has relied on laser excitation of light-absorbing organic dyes. However, in recent years, noble metal nanostructures, in particular gold, have emerged as candidates well suited for applications in cancer phototherapy. By tuning physical properties of the nanostructures, such as particle size, shape (nanorods and nanoshells) and aggregation state, their maximum absorption can be shifted into the NIR spectral range, allowing deeper penetration into biotissues [29]. On laser irradiation, the strong surface plasmon resonance shown by these materials, coupled with high absorption coefficients, result in efficient heat generation, which can be localized at the disease site. So far, laser excitation of gold nanoparticles has been demonstrated successfully in several animal models [41].

Iron oxide nanoparticles have also been explored for photothermal therapy. In this approach, the application of a magnetic field of required strength and frequency is used to induce heating of the nanoparticles localized to the tumor site. Ivkov *et al.* [42] demonstrated that if sufficient quantities of nanoparticles are present in the tumor to maintain a temperature of > 42°C for over 30 min, tumor cells can be effectively destroyed. Although these methods have been demonstrated successfully in animal models, there is at present no therapeutic use in human subjects. A major challenge to successful clinical implementation remains the need for selective delivery of energy to cancerous cells and not to adjacent healthy cells.

4. Multiple functionality nanoparticles

The continuous progression of nanoscale drug delivery, therapeutic and imaging agents in cancer therapy suggests the potential for the development of multifunctional nanoparticles that combine several properties into a single nanoscale system. These multifunctional systems may be capable of detecting and visualizing malignant cancer cells in the body (real-time *in vivo* imaging and molecular detection), killing cancer cells with minimal side effects by sparing normal cells (targeted drug delivery or controlled drug release) and improving the monitoring of treatment effects (multimodal imaging modalities), ultimately facilitating the realization of more effective therapeutic regimens and earlier and more accurate disease detection.

4.1 Drug delivery and targeting

The primary goals of nanoparticle-mediated delivery of anticancer drugs are to endow the agent(s) comprising the therapeutic formulation with the means to overcome: i) the biological barriers that prevent delivery to the target; ii) loss of activity in the blood circulation; and iii) poor targeting selectivity [43,44]. The first generation of multifunctional nanomaterials aimed to address these delivery challenges either through optimization of the drug carrier physical properties or by conjugation of the drug carrier to biomolecular targeting ligands.

4.1.1 Clearance mechanisms

Drug delivery for efficient accumulation in the body is hampered by the rapid recognition of the carrier and subsequent kidney and/or hepatic clearance from circulation [45]. Opsonins, plasma proteins such as immunoglobulins and complements, can bind to circulating particles and remove them from the circulation within minutes through the reticuloendothelial system (RES), a group of cells (macrophages, specialized endothelial cells and fibroblasts) having the ability to sequester particles. Rapid uptake of nanoparticles by the cells of the RES reduces availability of the nanoparticles at the target site.

Many attempts have been made to avoid the RES uptake of nanoparticles and prolong their blood circulation. Experience with liposomes has demonstrated that the opsonization process can be modulated, to some extent, by physicochemical properties of the particles, including particle surface hydrophilicity, size and surface charge [46]. For example, particles with largely hydrophobic surfaces are efficiently coated with plasma components and are rapidly removed from the circulation, primarily by the liver, spleen and lungs. Imparting stealth-shielding properties to the surface of the particles can reduce recognition of the circulating particles by opsonins and limit their phagocytosis by the RES cells [12]. Among the strategies used to extend nanoparticle blood circulation, surface modification with poly(ethylene glycol) (PEG) has proved to be most effective [47,48]. PEG is a non-toxic, non-immunogenic polymer approved by the FDA for internal human use. The PEG shielding mechanism of action is thought to result from PEG steric hindrance, which stems from a loss of conformational entropy of the bound PEG chains on the approach of serum opsonins, preventing deposition on particle surfaces. The low interfacial free energy of PEG in water contributes to this repulsion. In particular, long-chain PEG surface coatings (molecular mass ≥ 5 kDa) are found to be superior to shorter PEGs at reducing nonspecific uptake of nanoparticles by the RES and binding to non-target tissue such as the liver and spleen [14,49]. Examples of clinically used liposomes with PEG surface coatings that have been reported to show enhanced circulation times *in vivo* include Doxil and Caelyx (**Table 1**).

In addition to the long circulation property, modifications to improve the target specificity of the nanoparticles based on passive and active targeting strategies have been undertaken.

4.1.2 Passive targeting

Passive targeting refers to the accumulation of drug or drug carrier at a site owing to physicochemical or pharmacological factors [45,50]. Uptake of nanoparticles by the Kupffer cells of the liver, for example, represents an excellent example of passive targeting. In limited cases, however, unique structural changes associated with the vascular physiology of particular pathologies could also be exploited to passively target long circulating nanoparticles to diseased tissue. For example, normal tissues possess tight, continuous vessel walls, with most pore sizes of the order of 10 – 50 nm [51]. Long-circulating particles within this size range slowly extravasate from the vasculature into the interstitial spaces to be transported by lymphatic vessels to lymph nodes (**Figure 1A**) [52]. The escape of particles greater than this size from the circulation is restricted to sites where the capillaries have open fenestrations, such as typically found in the sinus endothelium of the liver and vascular endothelium of lymph nodes, where the size of fenestrae can be as large as 150 nm [45]. In contrast to healthy vasculature, the integrity of the vasculature endothelial barrier of certain pathologies is perturbed. Examples include disease associated with inflammatory processes (rheumatoid arthritis, infarction and infections) and cancerous tumors, which have discontinuous capillary walls with pores that can vary in size between 100 and 400 nm [45,53].

Passive targeting to these diseases can thus be achieved by tuning the size of long-circulating particles within this size range. When specifically applied to cancerous tumors, this phenomenon is termed the enhanced permeation and retention (EPR) effect. First described by Maeda [54], the EPR effect relies on; i) the capillary endothelium in malignant tissue being more disordered and more permeable than capillary endothelium in normal tissues; and ii) the lack of lymphatic drainage in the tumor bed. Long-circulating particles with the appropriate particle size thus have more frequent opportunities to encounter 'leaky' tumor capillaries, extravasate and accumulate into the tumor tissue (**Figure 1B**).

Numerous studies have confirmed passive accumulation of intravenously injected long-circulating particles in experimental

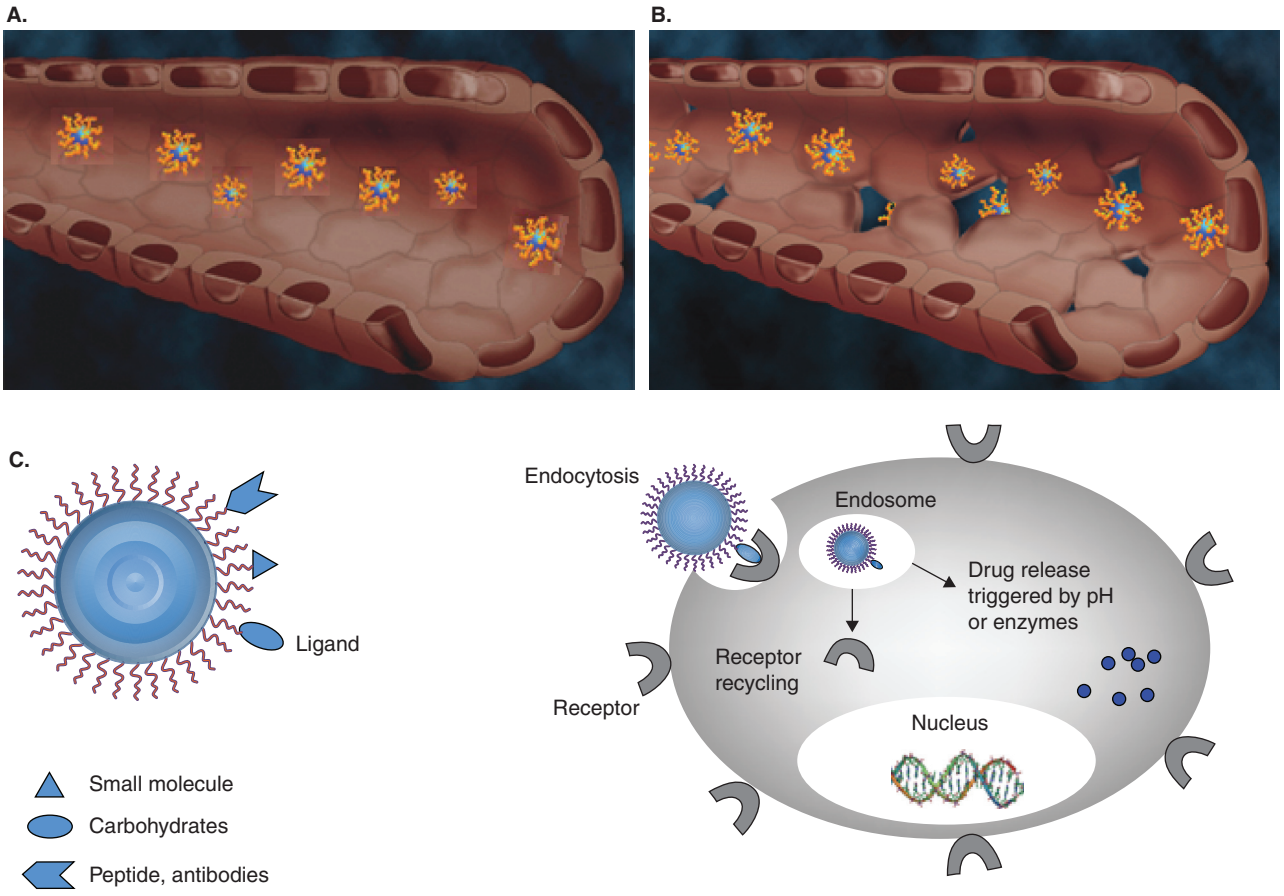

Figure 1. Tumor targeted delivery of nanoparticles by passive and active targeting mechanisms. In the passive mode, extravasation of long-circulating nanoparticles occurs by diffusion or convection. **A.** Normal tissues having tight, continuous vessel walls restrict passage of particles > 50 nm in diameter. **B.** Alternatively, similarly-sized nanoparticles can extravasate through leaky angiogenic vasculature and accumulate preferentially into tumor interstitium by the enhanced permeation and retention effect. **C.** Once in the tumor, active targeting of cell surface receptors by tumor-specific ligands on the nanoparticle surfaces triggers internalization of the nanoparticles into the cell. Drawings courtesy of W. Saad, PhD Thesis, Department of Chemical Engineering, Princeton University, 2006.

and clinical cases of solid tumors [45,55-57]. Nonetheless, the efficiency of particle delivery is often found to vary from one study to another. Experiments using liposomes of different mean size suggest that the threshold vesicle size for extravasation into tumors is ~ 400 nm [58], but other studies have shown that particles with diameters < 200 nm are more effective [58-60]. The observed range in efficacy has been attributed to the extent of particle extravasation, which depends strongly on the porosity of the angiogenic tumor vessel [53,58]. Consequently, techniques by which the size of the drug delivery carrier can be precisely tuned to match the vasculature pore size of a particular disease are required to maximize localization of the carrier at the selected target.

4.1.3 Active targeting

Beyond passive targeting through the EPR effect, delivery of particles to smaller solid tumors and metastatic cells can potentially be achieved by active targeting, whereby particle surfaces are modified with moieties directed at cell surface markers unique to these cell types (**Figure 1C**). Recent advances in cell biology have yielded a more detailed understanding of the processes involved in drug delivery at cellular and subcellular levels and have led to the identification of a variety of disease-specific ligand–receptor pairs [19,61].

Monoclonal antibodies (mAbs) have been the preferred class of targeting molecules for the last few decades, since Warenius *et al.* [62] first described their utility for directly targeting tumor cells in 1981. At present, there are several examples of FDA-approved anticancer antibodies in clinical practice. The mAb Herceptin (trastuzumab), a target for HER-2-overexpressing breast cancer cells, was approved by the FDA in 1998 [63]. Other FDA-approved mAbs include Erbitux, an epidermal growth factor receptor for the treatment of colorectal cancer, Rituxan (rituximab), a CD20-positive B cell for the treatment of non-Hodgkin's lymphoma, and Avastin (bevacizumab), a vascular epidermal growth factor for the treatment of metastatic colorectal, non-small lung and breast cancers [64].

Although antibodies were initially developed as independent therapies, their combination with chemotoxic drugs has proved a promising clinical strategy in oncology. Several recent clinical studies have demonstrated statistically significant improvements in efficacy of antibody and chemotherapeutic combinations compared with each drug used in isolation [65]. For example, Herceptin was shown to have synergistic antitumor activity when used in combination with paclitaxel and doxorubicin. It is now used for the treatment of patients with metastatic breast cancer whose tumors overexpress HER-2 and who have not received previous chemotherapy for their metastatic disease [66]. Similarly, the humanized anti-CD33 antibody–calicheamicin conjugate, marketed under the trade name Mylotarg, is approved for treatment of CD33-positive acute myeloid leukemia in first-relapse patients who are not candidates for cytotoxic chemotherapy. Other marketed antibody–drug conjugates include Zevalin and Bexxar, which use anti-CD20 antibodies to target radioisotopes to cancer cells [26].

In an analogous fashion, targeting of drug-loaded liposomes or polymeric nanoparticles using monoclonal antibodies is expected to yield substantial therapeutic benefits. Unlike drug–antibody conjugates, which deliver few molecules of drug per antibody molecule, relatively few ligand molecules per nanoparticle are required to deliver selectively high payloads of drugs to target cells by means of receptor-mediated internalizations. For example, trastuzumab-targeted liposomes loaded with doxorubicin have demonstrated greater antitumor activity than free drug, free antibody, or drug loaded in non-targeted liposomes in several tumor xenograft models [67]. Today, several antibody/antibody fragment-based therapeutic formulations are in preclinical and clinical trials, including several targeted nanoparticle formulations [43,65]. Examples include MBP-426, a liposomal formulation of the platinum-based drug oxaliplatin, SFT-53, a liposome vehicle containing a plasmid coding for the tumor suppressor p53, and CALAA-01, a polymer–siRNA composite nanoparticle [27]. These systems all target the transferrin receptor, which is upregulated in several types of cancer [68].

More recently, the discovery of new peptide targeting domains has permitted the development of synthetic molecules for active targeting. The polypeptide nature of these targeting ligands permits tailored optimization of ligand behavior by means of adjustment of the peptide sequence or conformation. In addition, peptides are relatively stable compared with mAbs and are also less likely to be immunogenic [69]. Early examples of peptide ligands include the arginine-glycine-aspartic acid (RGD) sequence as a target for $\alpha_v\beta_3$ integrin, leutinizing-hormone releasing-hormone (LHRH) and somatostatin [70]. A review of recent developments in the targeted delivery of nanoparticulate systems was presented by Moghimi *et al.* [45].

Small molecule ligands have also shown significant advantages as a class of targeting molecules. One of the most extensively studied molecules is folic acid (folate). Folic acid, a vitamin essential for *de novo* nucleotide synthesis, is taken up by cells by means of receptor-mediated endocytosis using membrane-associated folate receptors. Folate receptors are expressed only on certain epithelial cells in humans, but are differentially overexpressed in cells of cancers with epithelial origin. Folate targeting has been used successfully for tumor-specific drug delivery in cancers of the breast, ovary, brain and lung [71].

4.2 Drug delivery, imaging and targeting

Multifunctional nanoparticles not only afford the opportunity for targeted drug delivery, but may also facilitate verification and quantification for image-monitored therapeutic action, offering new clinical approaches to many diseases. Thus, the development of multifunctional nanoparticles which incorporate diagnostic and therapeutic properties, as well as specific targeting capability, is a continuous topic of research.

Early efforts toward multifunctional nanoparticles for integrated drug delivery and imaging focused on combining polymeric drug carriers with organic fluorescent dyes for particle visualization. Fluorescent drug particles were prepared by binding water-soluble fluorophores to the surfaces of preformed polymer nanoparticles or by chemically tethering a fluorescent dye to the hydrophobic terminus of an amphiphilic block copolymer and then permitting the polymer to self-assemble [72,73]. Organic dyes and fluorophores, however, require direct visualization and so are generally practical only for *in vitro* applications [74].

Particles having a metallic core that adds contrast to images acquired by MRI or CT, for example, are more suitable for *in vivo* biomedical applications [32,33]. However, as synthesized, inorganic nanoparticles are incompatible with biological environments owing to surface hydrophobicity or toxicity limitations. Consequently, several coating strategies have been developed to enhance their biocompatibility, improve their aqueous stability and provide chemical handles for further reactions with biomolecules [47]. Modification methods have included the adsorption of small-molecule ligands or stabilizers on the colloid surfaces [75], layer-by-layer deposition of polyelectrolyte chains [76], surface-initiated polymerization of high-density polymer brushes [77], or block copolymer deposition on colloid surfaces [78]. By extension, ligands (optionally together with drugs) can be covalently attached to the coating material [79,80]. For example, multifunctional superparamagnetic nanoparticles bearing covalently-bound drugs and targeting ligands have been reported for selective drug delivery to regional lymph nodes and for imaging of prostate cancers [33,81].

Bioconjugated inorganic nanoparticles can also act as intrinsic therapeutic and imaging agents. For example, plasmon-resonant gold nanorods have been examined as bifunctional nanoparticles for molecular imaging and photoactivated therapy. Nanorods can be imaged with single-particle sensitivity by two-photon luminescence (TPL) when excited by femtosecond-pulsed laser irradiation, and have been monitored *in vivo* while passing through blood vessels at sub-picomolar concentrations [82]. TPL imaging has also been used to characterize the targeted delivery of ligand-functionalized nanoparticles to tumor cells [83].

4.3 Multimodal imaging

Imaging techniques such as MRI, PET and CT are vital in the diagnosis of various diseases. Each imaging modality has its own merits and disadvantages, and a single technique does not possess all the required capabilities for comprehensive imaging. Therefore, multimodal imaging methods are quickly becoming important tools for state-of-the-art biomedical research. Multimodal contrast agents could aid in improving the diagnosis and treatment of diseases in their earliest stages by providing far more comprehensive data to clinicians.

Most commonly, magnetic nanoparticles serve as a core platform for the addition of other functional imaging moieties. For example, MRI-optical dual-mode probes composed of a fluorescent dye-doped silica core surrounded by magnetic nanoparticles can macroscopically detect neuroblastoma cancer cells by means of MRI along with subcellular information through fluorescence imaging [84]. Magnetic nanoparticles can also be coupled to radionuclides to construct MRI-PET dual-modal probes [85]. Such probes can accurately detect cancerous cells in lymph nodes, which are critical for assessing cancer metastasis. Systems using magnetic nanoparticles modified with fluorescent probes and biomolecules can also monitor gene expression and other markers in cell therapeutic studies. Each component of such multimodal probes complements the other modalities, and their synergistic materials properties can ultimately provide more accurate information both *in vitro* and *in vivo* [86].

5. Complex multifunctional nanoparticles

The successful clinical translation of any diagnostic or therapeutic multifunctional nanosystem requires control over many distinct parameters, resulting in a large number of potential variables for optimization. These factors can include component compositions, particle surface hydrophilcity, surface charge, particle size, density of targeting ligands, and multiple combinations thereof. Simple multifunctional systems, such as those described above, are limited in their capacity to fulfil these requirements. For example, the preparation of multifunctional nanoparticles by means of conjugation of labile drug molecules to the surfaces of inorganic nanostructures precludes protection of the molecules from the biological milieu and reduces further the capacity to deliver a large payload of the therapeutic. Beyond these design limitations, conjugation methods rely on unique synthesis strategies tailored for the linkage of the individual elements. The necessity of customizing synthetic protocols makes development lengthy and economically burdensome, especially for complexes with therapeutic potential, and further challenges facile incorporation of newly discovered materials into already existing constructs.

More promising and versatile is the strategy of combining several functional building blocks into a carrier matrix. For example, self-assembly methods for the preparation of multifunctional carriers, whereby hydrophobic solutes and inorganic nanostructures are *simultaneously* encapsulated within a delivery vector, are now under development [8]. In contrast to chemical conjugation routes, self-assembly methods permit the integration of materials of various properties based on non-specific thermodynamic driving forces, such as electrostatic or non-covalent interactions [87]. For this purpose, polymer nanoparticles, formed from the self-assembly of amphiphilic block copolymers in selective solvents, serve as particularly useful scaffolds (**Figure 2**). Multifunctional nanoparticles prepared by means of self-assembly routes may be designed to demonstrate simultaneously: i) prolonged circulation in the blood; ii) encapsulation and protection of a wide variety of therapeutic agents at high payload; iii) combinatorial optimization of carrier physical properties to capitalize on both passive and active targeting mechanisms; iv) responsiveness to local stimuli for controlled drug release; and v) the ability to bear multiple contrast/reporter moieties for imaging/observation by means of a variety of multimodal imaging techniques.

In practice, the reproducible and tunable preparation of complex multifunctional nanoparticles through self-assembly processes is quite challenging. So far, few examples have been reported in the literature. Nasongkla *et al.* [88] have described the preparation of poly(ethylene glycol)-*b*-poly(D,L-lactide) nanoparticles encapsulating the chemotherapeutic doxorubicin and superparamagnetic iron oxide nanoparticles. Similarly, Yang *et al.* [89] reported the preparation of poly(D,L-lactide-*co*-glycolide) nanoparticles encapsulating the same materials. However, the preparative techniques used, namely solvent evaporation and emulsification, suffer from several disadvantages, including the use of stabilizing surfactants and numerous purification steps to prepare uniformly sized particles [88,89]. Also, the loading capacity of hydrophobic solutes is generally limited by compound solubility within the particle core [90]. Finally, these preparative processes do not allow for independent specification of component compositions and furthermore do not ensure uniform distribution of actives within nanoparticle interiors. Thus, there remains a need for new assembly techniques that allow enough structural flexibility to be tuned for specific applications. Also, to be commercially practical, the techniques must be implemented at relatively low cost.

6. Flash NanoPrecipitation – a modular technology for preparation of complex multifunctional nanoparticles

In the authors' laboratory, a new technology has been developed for the preparation of multifunctional nanoparticles with the capacity for precise control over nanoparticle composition, size and surface chemistry [91,92]. The technology, termed Flash NanoPrecipitation (FNP), is a rapid solvent displacement precipitation that uses amphiphilic diblock copolymers to direct the self-assembly of nanoparticles. The particle size, uniformity of size and complex multifunctionality are controlled by rapid assembly kinetics, rather than slow equilibrium thermodynamics. Particle formation by FNP involves two

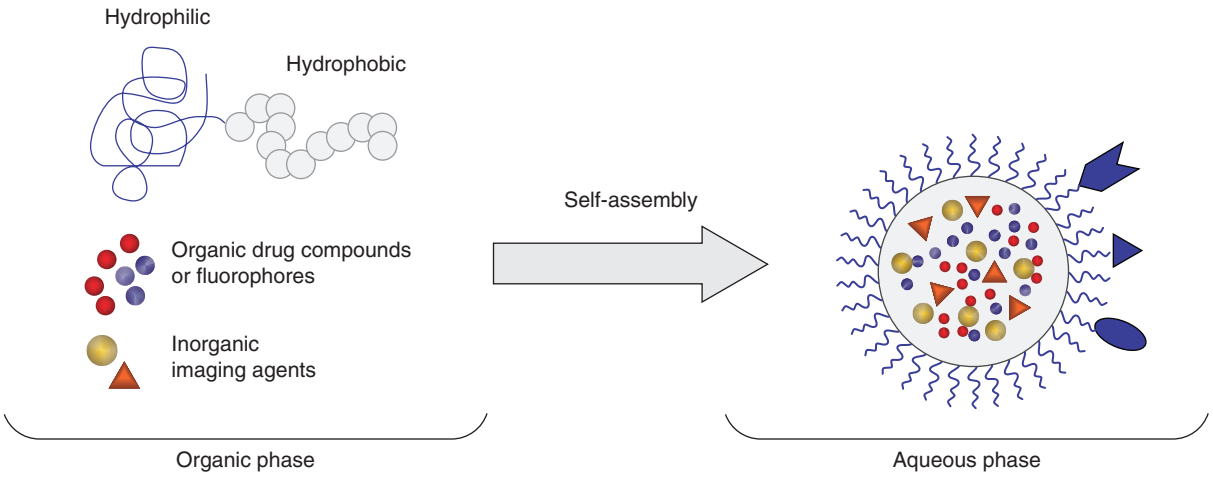

Figure 2. Schematic of multifunctional nanoparticle formation by block copolymer-directed self-assembly [92,95]**.** The modular strategy permits the preparation of carriers that combine: i) the ability to carry one or more therapeutic agents; ii) image signal amplification by way of coencapsulated contrast agents; iii) physical targeting of hyper-vascularized pathologies by means of particle size control; and iv) molecular targeting through one or more conjugated targeting ligands, which may be added post-assembly [98].

primary steps: i) dissolution of the carrier/stabilizer (amphiphilic copolymer) and organic active(s) (hydrophobic drugs) into a water-miscible solvent; and ii) rapid mixing of the solution with an antisolvent (usually water) to create high supersaturation of the hydrophobic components.

The mechanism of nanoparticle assembly by FNP is illustrated in **Figure 3A**. The process relies on the interplay of multiple timescales, namely: i) time for homogeneous mixing of streams (τ_{mix}); ii) time for nucleation and growth of solutes (τ_{ng}); and iii) time for self-assembly of the amphiphilic block copolymer stabilizer (τ_{sa}). When τ_{mix} is sufficiently fast, such that a selective change in solvent quality is very rapidly induced, the hydrophobic components in the mixture become supersaturated. High supersaturation drives rapid nucleation and growth of the solute in the diffusion-limited regime [93]. By balancing the nucleation and growth times of the actives with the block copolymer assembly time ($\tau_{mix} << \tau_{sa} \cong \tau_{ng}$), it is possible to arrest growth by adsorption of polymer chains on growing particle surfaces, resulting in sub-micrometer particle sizes and narrow particle size distributions.

As particle formation is extremely rapid, mixing must be very fast. Millisecond (*ca.* 10 ms) mixing has been demonstrated by Liu *et al.* for the multiple inlet vortex mixer (MIVM) geometry shown in **Figure 3B** [94]. In the MIVM, solutions of solvent and antisolvent mix in a small volume chamber, where momentum from each stream contributes independently to drive micromixing in the cell. Changes in the flow rate of individual streams, or concentration of reactants in streams, yield relatively minor changes in the performance of the MIVM, making it a highly flexible mixing device.

As particles prepared by FNP assemble spontaneously from solution by simultaneous desolvation of components, the method can also be used to generate complex multicomponent nanoparticles [92]. For example, organic solutes and colloids can be *coencapsulated* into a single multifunctional nanoparticle if the processes they undergo on solvent shifting occur within comparable timescales; $\tau_{mix} < \tau_{sa} \sim \tau_{ng} \sim \tau_{agg}$, where τ_{agg} is the time for inorganic colloid aggregation (**Figure 3A**). In contrast to slow, equilibrium precipitation processes that lead to unequal incorporation of individual components depending on their solubilities, the high levels of supersaturation attained in FNP result in spinodal decomposition and diffusion-limited aggregation of components [93], permitting *quantitative* incorporation of all actives. Consequently, the FNP process permits independent and *a priori* manipulation of nanoparticle composition dictated solely by the composition of feed streams to the mixer.

The preparation of complex multifunctional nanoparticles by FNP was established by Gindy *et al.* [92], demonstrating that therapeutic, imaging probes may be readily synthesized by this process, in a single step, without the need for conjugation chemistry. Particles with precisely tunable sizes between 75 and 275 nm, narrow particle size distributions, high active loadings and long-term stability under physiological conditions were produced. Analogous multifunctional nanoparticles for more advanced pharmaceutical applications have also been developed. For example, the preparation of novel nanoparticles coencapsulating photosensitizer drugs and upconverting phosphor nanocrystals for use in photon-activated photodynamic cancer therapy under NIR illumination was reported recently [95,96]. Similarly, FNP was used to generate multimodal, size-tunable, photostable fluorescent nanoparticles for combined drug delivery and biological imaging [97].

FNP also provides an optimal platform for selective *in vivo* nanoparticle delivery through a synergistic combination of passive and active targeting strategies. The demonstrated capacity for precise control over nanoparticle size, tuned to the

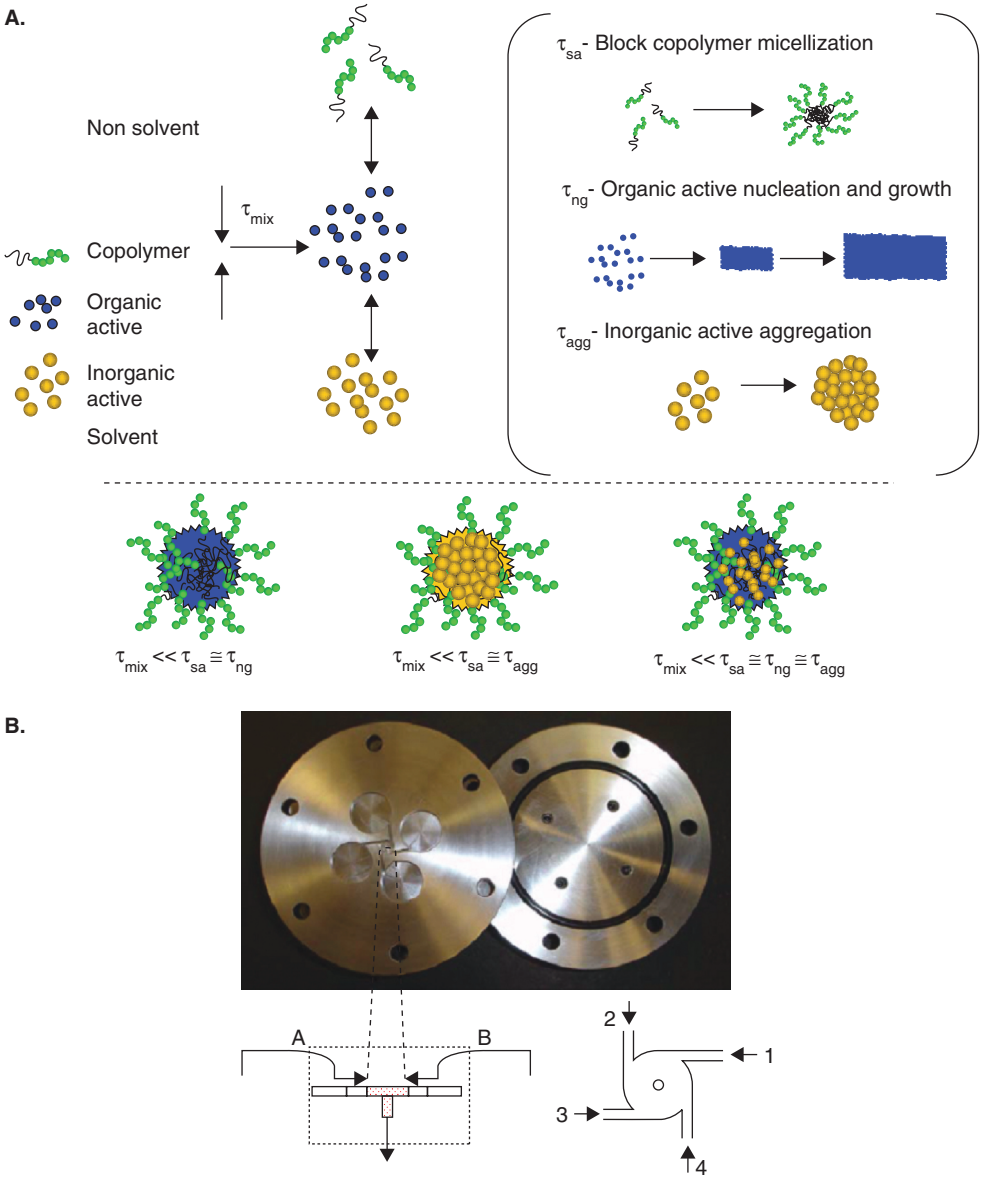

Figure 3. A. Schematic representation of parameters influencing the preparation of multifunctional nanoparticles encapsulating a combination of organic solutes and inorganic nanostructures prepared by the Flash NanoPrecipitation technology [92,100]. B. Photograph and schematic illustration of a multi-inlet vortex mixer developed for FNP [94].

physiopathology of a particular cancer, for example, permits maximum accumulation at the desired site by means of the EPR effect. Similarly, appropriate selection of targeting ligand and control over nanoparticle functionalization can lead to enhanced cellular uptake of the localized particles. Using the FNP technology, Gindy *et al.* [98] showed that surface functionalized nanoparticles may be synthesized and subsequently conjugated to a targeting macromolecule by a facile two-step process. Conjugation was performed with preservation of the ligand conformation, whereas the density of targeting ligands on the nanoparticle surface was directly tunable by the ratios of functionalized to unfunctionalized block copolymer in the feed stream to the mixer. Thus, FNP has been shown to be capable of producing complex, multifunctional nanoparticles in a controlled and scalable manner, with minimal processing steps.

7. Expert opinion and conclusion

The past quarter century has seen outstanding progress in the fundamental understanding of cancer biology. Nanotechnology, led by the development of nanoparticle-based drug delivery systems and imaging contrast agents, has had an important role in translating these advances to the clinic. Particles in the nanometer size range have demonstrated attributes of prolonged

circulation *in vivo*, high drug loading capacity and controlled drug release.

Conceptually, the integration of multiple functionalities into strategically engineered nanosystems is the logical next step, and a capability worth setting as a practical goal. The most obvious combination of functionalities is drug delivery and targeting. Ideally, a drug carrier should be able to accumulate specifically in the required organ or tissue and subsequently penetrate target cells to deliver its therapeutic payload. This selective direction is important in maximizing the concentration of drug at the disease site while minimizing the total dose administered. Targeted nanoparticles have shown some exciting results in clinical studies, demonstrating their potential as therapeutic carriers. In particular, the combination of drug delivery and *passive* targeting has been widely established for liposomal formulations, where for particles in the nanometer size range, preferential organ or tissue accumulation is achieved by means of the enhanced permeation and retention effect.

Nanoparticle targeting by means of a ligand-mediated, or *active*, mechanism has also been pursued. However, the ultimate clinical value of targeting based on ligand recognition remains an area of active debate. The cost of antibody targeting may be prohibitive on a commercial scale. Targeting with smaller (and less costly) peptide ligands may be economically more attractive, but if receptors are not unique to cancer cells and are instead overexpressed, then the much larger mass of tissue in the body may clear the nanoparticles despite the cancer cells having a higher concentration of receptors. This problem is one of mass-action kinetics. These questions cannot be answered *in vitro*, and are even masked in small animal tumor models where the tumor may be a significant fraction of the weight of the animal.

Multifunctional nanoparticles that provide a synergistic combination of passive and active targeting mechanisms may be well suited to addressing these limitations. Bartlett and co-workers [99] recently established that attachment of antibody targeting ligands to long-circulating particles increases cellular internalization without affecting the overall tumor uptake. Thus, physiological properties of the nanoparticles (size and surface properties) were the primary determinant of their pharmacokinetics and biodistribution, whereas the presence of targeting ligands resulted only in an enhancement of intracellular uptake. Such considerations are of critical importance in the intelligent design of carriers capable of providing an optimal platform for *in vivo* nanoparticle delivery.

The combination of drug delivery and imaging functionalities has garnered similar biomedical interest. As all chemotherapeutics are toxic, the ability to quantitatively determine their fate *in vivo* would aid research and clinical practice dramatically. Nonetheless, imaging and delivery *in vivo* is highly challenging, as it requires non-invasive 'readouts' such as MRI, X-ray, or PET imaging. Demonstrations of nanoparticles with the dual capabilities of delivery and imaging by these modalities are few – and this area is still at the cutting edge of our capability. By extension, nanoparticle contrast probes functionalized with molecularly targeted recognition agents might provide information on the presence, relative abundance, and distribution of cancer signatures and markers associated with the tumor microenvironment.

7.1 Future perspective

The complexity of multifunctional, biologically active nanoparticle formulations offers countless interesting possibilities to enable important new therapeutic outcomes. There is great anticipation that these new nanoparticle systems will be able to revolutionize the diagnosis and treatment of human disease. As more clinical data become available and optimal material and physical properties are elucidated further, nanoparticle-based therapeutics and diagnostic agents will continue to improve. This evolution will undoubtedly lead to newer, more sophisticated multifunctional nanoparticles eventually reaching the clinic.

Yet, at present, most research is being done at the scientific stage, where questions are at the level of 'what is it possible to do?' As these questions are addressed at the level of fundamental biology and science, the next challenge will be to design processes that make possible the production of multifunctional nanosystems economically and on a large scale. New fabrication techniques, such as the Flash NanoPrecipitation process detailed in this article, will be instrumental in surmounting this challenge. The technology is unique in being able to generate nanoparticles with tunable sizes in the biologically relevant range of 50 – 500 nm, at high active loadings and material yield. Equally important is the fact that the process is scalable. Research studies can be conducted with as little as 3 mg of active, but the results scale to production runs of 1000 kg/day, as exemplified by BASF in the generation of β-carotene nanoparticles [100,101]. Nanoparticles generated by FNP can be lyophilized and reconstituted without aggregation, and are flexible enough to allow for the development of an almost unlimited number of applications, ranging from controlled delivery and sustained release of drug cocktails to formulation within porous microparticle composites for aerosol drug delivery [102]. These attributes represent great potential for the design and development of new materials with tailor-made properties.

As a physicist colleague at the Princeton Plasma Physics Laboratory is fond of saying, 'We can do things well, we can do them quickly, or we can do them cheaply. You can pick any two'. For the time being, we shall leave out the 'quickly'.

Declaration of interest

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Affiliation

Marian E Gindy[1,2] & Robert K Prud'homme[†,1]
[†]Author for correspondence
[1]Princeton University,
Chemical Engineering,
Princeton, NJ 08544, USA
Tel: +1 609 258 4577; Fax: +1 609 258 0211;
E-mail: prudhomm@Princeton.EDU
[2]Merck & Co., Inc.
Sumneytown and West Point Pikes
Mailstop WP78-302
West Point, PA 19486, USA