

Nanomedicine: current status and future prospects

S. Moein Moghimi,^{*,1} A. Christy Hunter,^{*} and J. Clifford Murray[†]

^{*}Molecular Targeting and Polymer Toxicology Group, School of Pharmacy, University of Brighton, Brighton, UK; and [†]Cancer Research UK, Tumour Cytokine Biology Group, Wolfson Digestive Diseases Centre, University Hospital, Nottingham, UK

ABSTRACT Applications of nanotechnology for treatment, diagnosis, monitoring, and control of biological systems has recently been referred to as “nanomedicine” by the National Institutes of Health. Research into the rational delivery and targeting of pharmaceutical, therapeutic, and diagnostic agents is at the forefront of projects in nanomedicine. These involve the identification of precise targets (cells and receptors) related to specific clinical conditions and choice of the appropriate nanocarriers to achieve the required responses while minimizing the side effects. Mononuclear phagocytes, dendritic cells, endothelial cells, and cancers (tumor cells, as well as tumor neovasculature) are key targets. Today, nanotechnology and nanoscience approaches to particle design and formulation are beginning to expand the market for many drugs and are forming the basis for a highly profitable niche within the industry, but some predicted benefits are hyped. This article will highlight rational approaches in design and surface engineering of nanoscale vehicles and entities for site-specific drug delivery and medical imaging after parenteral administration. Potential pitfalls or side effects associated with nanoparticles are also discussed.—Moghimi, S. M. Hunter, A. C., Murray, J. C. Nanomedicine: current status and future prospects. *FASEB J.* 19, 311–330 (2005)

Key Words: nanotechnology • nanosized drug delivery systems • nanoparticles • medical imaging • gene therapy • nanofibers • macrophage • endothelium • intracellular delivery • extravasation • toxicity

BACKGROUND

THE DEVELOPMENT of a wide spectrum of nanoscale technologies is beginning to change the foundations of disease diagnosis, treatment, and prevention. These technological innovations, referred to as nanomedicines by the National Institutes of Health (Bethesda, MD, USA), have the potential to turn molecular discoveries arising from genomics and proteomics into widespread benefit for patients. Nanomedicine is a large subject area and includes nanoparticles that act as biological mimetics (e.g., functionalized carbon nanotubes), “nanomachines” (e.g., those made from interchangeable DNA parts and DNA scaffolds such as octahedron and stick cube), nanofibers and polymeric

nanoconstructs as biomaterials (e.g., molecular self-assembly and nanofibers of peptides and peptide-amphiphiles for tissue engineering, shape-memory polymers as molecular switches, nanoporous membranes), and nanoscale microfabrication-based devices (e.g., silicon microchips for drug release and micromachined hollow needles and two-dimensional needle arrays from single crystal silicon), sensors and laboratory diagnostics (**Appendix 1**, page 322). Furthermore, there is a vast array of intriguing nanoscale particulate technologies capable of targeting different cells and extracellular elements in the body to deliver drugs, genetic materials, and diagnostic agents specifically to these locations. Indeed, research into the rational delivery and targeting of pharmaceutical, therapeutic, and diagnostic agents via intravenous and interstitial routes of administration with nanosized particles is at the forefront of projects in nanomedicine. This article critically evaluates key aspects of nanoparticulate design and engineering, as well as recent breakthroughs and advances in cellular and intracellular targeting with such nanoscale delivery technologies after parenteral administration, including the advantage of the nanometer scale size range, biological behavior, and safety profile. Discussion of other aspects of nanomedicine, as summarized in Appendix 1, are beyond the scope of this article.

NANOSIZED TECHNOLOGIES FOR MEDICAL IMAGING AND TARGETED DRUG DELIVERY

Nanoparticles with inherent diagnostic properties

Nanotechnology is an area of science devoted to the manipulation of atoms and molecules leading to the construction of structures in the nanometer scale size range (often 100 nm or smaller), which retain unique properties. Indeed, the physical and chemical properties of materials can significantly improve or radically change as their size is scaled down to small clusters of atoms. Small size means different arrangement and spacing for surface atoms, and these dominate the

¹ Correspondence: Molecular Targeting and Polymer Toxicology Group, School of Pharmacy, University of Brighton, Brighton BN2 4GJ, UK. E-mail: s.m.moghimi@brighton.ac.uk
doi: 10.1096/fj.04-2747rev

object's physics and chemistry (1). Colloidal gold, ironoxide crystals, and quantum dots (QDs) semiconductor nanocrystals are examples of nanoparticles, whose size is generally in the region of 1–20 nm, and have diagnostic applications in biology and medicine (**Appendix 2**, page 324). Gold nanoparticles have application as quenchers in fluorescence resonance energy transfer measurement studies (2, 3). For example, the distance-dependent optical property of gold nanoparticles has provided opportunities for evaluation of the binding of DNA-conjugated gold nanoparticles to a complementary RNA sequence (3). Iron oxide nanocrystals with superparamagnetic properties are used as contrast agents in magnetic resonance imaging (MRI), as they cause changes in the spin-spin relaxation times of neighboring water molecules, to monitor gene expression or detect pathologies such as cancer, brain inflammation, arthritis, or atherosclerotic plaques (4–11). QDs can label biological systems for detection by optical or electrical means *in vitro* and to some extent *in vivo* (12–19). The fluorescence emission wavelength (from the UV to the near-IR) of QDs can be tuned by altering the particle size, thus these nanosystems have the potential to revolutionize cell, receptor, antigen, and enzyme imaging (19–22). Indeed, a recent report demonstrated the use of QDs for tracking metastatic tumor cell extravasation (20). Their large surface area-to-volume ratio offers potential for designing multifunctional nanosystems. Undoubtedly, application of such multi-wavelength optical nanotools may eventually aid our understanding of the complex regulatory and signaling networks that govern the behavior of cells in normal and disease states.

Nanovehicles and drug carriers

In addition, there are numerous engineered constructs, assemblies, architectures, and particulate systems, whose unifying feature is the nanometer scale size range (from a few to 250 nm). These include polymeric micelles, dendrimers, polymeric and ceramic nanoparticles, protein cage architectures, viral-derived capsid nanoparticles, polyplexes, and liposomes (**Appendix 2**) (23–37). First, therapeutic and diagnostic agents can be encapsulated, covalently attached, or adsorbed on to such nanocarriers. These approaches can easily overcome drug solubility issues, particularly with the view that large proportions of new drug candidates emerging from high-throughput drug screening initiatives are water insoluble. But some carriers have a poor capacity to incorporate active compounds (e.g., dendrimers, whose size is in the order of 5–10 nm). There are alternative nanoscale approaches for solubilization of water insoluble drugs too (38). One approach is to mill the substance and then stabilize smaller particles with a coating; this forms nanocrystals in size ranges suitable for oral delivery, as well as for intravenous injection (38, 39). Thus, the reduced particle size entails high surface area and hence a strategy for faster drug release. Pharmacokinetic profiles of injectable nanocrystals may

vary from rapidly soluble in the blood to slowly dissolving. Second, by virtue of their small size and by functionalizing their surface with synthetic polymers and appropriate ligands, nanoparticulate carriers can be targeted to specific cells and locations within the body after intravenous and subcutaneous routes of injection (23–25, 31, 35, 36, 40–42). Such approaches, may enhance detection sensitivity in medical imaging, improve therapeutic effectiveness, and decrease side effects. Some of the carriers can be engineered in such a way that they can be activated by changes in the environmental pH, chemical stimuli, by the application of a rapidly oscillating magnetic field, or by application of an external heat source (24, 43–45). Such modifications offer control over particle integrity, drug delivery rates, and the location of drug release, for example within specific organelles. Some are being designed with the focus on multifunctionality; these carriers target cell receptors and delivers simultaneously drugs and biological sensors (46). Some include the incorporation of one or more nanosystems within other carriers, as in micellar encapsulation of QDs; this delineates the inherent nonspecific adsorption and aggregation of QDs in biological environments (16). In addition to these, nanoscale-based delivery strategies are beginning to make a significant impact on global pharmaceutical planning and marketing (market intelligence and life-cycle management) (23, 25).

INNER SPACE

Nanoparticles do not behave similarly; their behavior within the biological microenvironment, stability, and extracellular and cellular distribution varies with their chemical makeup, morphology, and size. These aspects are discussed with respect to intravenous and subcutaneous routes of injection.

Clearance mechanisms and opportunities for targeting

The network of blood and lymphatic vessels investing the body provides natural routes for the distribution of nutrients, clearing of unwanted materials, and delivery of therapeutic agents. Superficially, however, this network appears to provide little in the way of obvious controlled and specific access to tissues, and the science of these processes has been scant. Regardless of these limitations, nanoparticulate systems provide possibilities for access to cell populations and body compartments. When injected intravenously, particles are cleared rapidly from the circulation and predominantly by the liver (Kupffer cells) and the spleen (marginal zone and red pulp) macrophages (24). This site-specific, but passive, mode of clearance is a facet of the immune cells' primary scavenging role for particulate invaders and self-effete products. Opsonization, which is surface deposition of blood opsonic factors such as fibronectin, immunoglobulins, and complement pro-

teins, often aid particle recognition by these macrophages (24). However, size and surface characteristics of nanoparticles both play an important role in the blood opsonization processes and clearance kinetics (24, 47, 48). Larger particles (200 nm and above) are more efficient at activating the human complement system and are hence cleared faster from the blood by Kupffer cells than their smaller counterparts. This is a reflection of geometric factors and surface dynamics on the initial assembly of proteins involved in complement activation (24, 48). The binding of blood proteins and opsonins to nanoparticles differ considerably in amount and in pattern depending on surface properties such as the presence and type of functional groups and surface charge density (24, 47, 48). Thus, differential opsonization may account for differences in clearance rates and macrophage sequestration of nanoparticles. This is particularly important with the view that macrophages are heterogeneous with respect to phenotype and physiological function, even within the same tissue. Hence, a particular population of phagocytes may employ one predominant recognition mechanism. The dynamic process of protein adsorption together with deposition of a variety of opsonic factors onto the surface of nanoparticles may indicate an arrangement based on a recognition hierarchy, or cooperativity, among macrophage receptors for clearance. For example, a specific macrophage receptor might recognize the earliest changes associated with a particle surface in the blood, whereas other receptors might recognize particles at a later stage thus ensuring complete removal from the circulation. These issues have not received detailed attention but their understanding could potentially open strategies for design and surface manipulation of nanosystems that target selective macrophage subpopulations.

Small particle size also means large surface area. This may pose problems in terms of aggregation of primary nanoparticles in the biological environment, which subsequently determines the effective particle size and hence clearance kinetics. Indeed, dendrimers and QDs are well known to flocculate in biological media. Another case is interaction between certain lipid-based nanosystems and lipoproteins leading to dramatic size changes. For instance, some hydrophobic components of Cremophor EL micelles incorporate rapidly into HDL and LDL leaving the remaining components to form large lipid structures in plasma that assembles C3 convertases (49). Thus, inherent in nanoparticle design is surface manipulation and engineering. Indeed, precision surface engineering with synthetic polymers can resolve aggregation and afford control over nanoparticle interaction and fate with biological systems. For example, polydentate phosphine coating or surface modification with various hydrophilic polymers renders QDs soluble, disperse, and stable in serum (17–19, 50). There are numerous examples in the literature where the surface of nanocarriers is carefully assembled with projected “macromolecular hairs” made from poly(ethyleneglycol) or other related polymers (24). This strat-

egy suppresses macrophage recognition by an array of complex mechanisms, which collectively achieve reduced protein adsorption and surface opsonization. Here, the efficiency of the process is dependent on polymer type, their surface stability, reactivity, and physics (e.g., surface density and conformation) (24, 51, 52). Indeed, this stealth-like behavior is similar to strategies developed by pathogenic microorganisms to fend off immune detection. Suppression of opsonization events is an important first step in enhancing passive retention of nanoparticles at sites and compartments other than macrophages in contact with the blood, and simply is a reflection of long circulatory profile of such surface-manipulated nanoparticles. In this instance, particle escape from the vasculature is normally restricted to sites where the capillaries have open fenestration, as in the sinus endothelium of the liver, or when the integrity of the endothelial barrier is perturbed by inflammatory processes or by tumor growth; the latter is the result of dysregulated angiogenesis (24, 53, 54). In liver, the size of fenestrae in the sinus endothelium can be as large as 150 nm; in tumor capillaries they vary greatly but rarely exceed 300 nm. Prolonged circulation properties are ideal for slow or controlled release of therapeutic agents in the blood to treat vascular disorders. Long circulating particles may have application in vascular imaging too (e.g., detection of vascular bleeding or abnormalities) or even act as artificial nanoscale red blood cells. But surface engineering is not the only way for keeping nanoparticles away from the macrophages. Recent advances in synthetic polymer chemistry afford precise control over the architecture and polydispersity of polymers, polymer-conjugates, and block copolymers. Some of these novel materials can form sterically stabilized nanoscale self-assembling structures with macrophage-evading properties (28, 29).

If confinement to the vascular system is necessary, then splenic filtration processes must be borne in mind. Splenic filtration at interendothelial cell slits is predominant. This is particularly true for rigid or nondeformable particles whose size exceeds the width of the cell slits (200–250 nm) (24). Otherwise, opportunities are there for gaining efficient access to splenic red-pulp compartments with nanoparticles (**Fig. 1**).

Recent developments in molecular biology have begun to reveal the wealth of information contained within blood and lymphatic vessels, and in particular that on the luminal surface of endothelial cells. Molecular signatures related to particular vascular and lymphatic beds and types of endothelial cells have been identified, providing landmarks for circulating cells and molecules (55–59). These same signatures have now been exploited to direct therapeutic and diagnostic entities to selected pathological vessels, particularly those of cancer (19, 60, 61). This, however, requires assembly of the appropriate targeting ligands on nanocarriers and long circulating nanosystems, but the ultimate characteristics such as ligand density, spacing and conformation are dependent on ligand and parti-

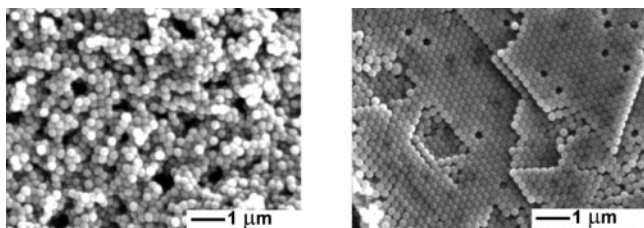


Figure 1. Scanning electron micrographs of uncoated and surface-modified polystyrene nanospheres. The left panel represent polystyrene nanospheres of 200 nm in diameter. Due to surface hydrophobicity these particles tend to aggregate. In the right panel, the surface of polystyrene nanoparticles is modified with a sufficient quantity of poloxamine 908 (a nonionic block copolymer surfactant). The thickness of the surface coating is ~ 8 nm, as measured by photon correlation spectroscopy. Coating prevents aggregation, and as a result highly ordered stacking occurs. When injected intravenously, these coated particles circulate for extended periods of time but eventually are cleared by the spleen due to filtration at the interendothelial cell slits at venous walls. In contrast, uncoated particles are cleared within minutes of intravenous injection by the hepatic Kupffer cells.

cle properties (curvature and surface reactivity). These modifications determine the extent of particle stability and aggregation *in vivo* as well as the efficiency of receptor binding and follow up events, such as the mode of particle internalization (e.g., receptor-mediated endocytosis as opposed to lipid raft-dependent macropinocytosis) and associated signaling processes. Perhaps, the predetermined chemical architecture of dendrimetric systems could be taken into advantage for optimizing ligand conformation. Nevertheless, such entities may be viewed as tiny submarines that navigate capillaries in search of signature molecules expressed by the target, a process often refereed to as active targeting. This concept is applicable for targeting of nanosystems to other accessible cells within the vasculature (e.g., blood cells and macrophages) or even to cells and scaffolding structures after extravasation from the vasculature.

Interstitial injection of nanoparticles, however, may be the preferred choice particularly if the target is a specific lymph node or a group of them located regionally (62, 63). In lymphatic capillaries, numerous endothelial cells overlap extensively at their margins and they lack adhesion mechanisms at many points. Immediately after interstitial injection many of the overlapped endothelial cells are separated and thus passageways are provided between the interstitium and lymphatic lumen through which particles are conveyed to the nodes via the afferent lymph (62). In lymph nodes, macrophages of medullar sinuses and paracortex are mainly responsible for particle capture from the lymph (62). The fate of interstitially injected nanoparticles is controlled by their size and surface characteristics (62–64). The size of the particles must be large enough to prevent their rapid leakage into the blood capillaries; particles in the range of 30 to 100 nm usually satisfy this criterion, whereas particles larger

than 100 nm move very slowly and are susceptible to clearance by interstitial macrophages. Surface characteristics can control the extent of particle aggregation at interstitial sites, hence drainage kinetics and lymph node retention may be optimized by prior surface manipulation (64). For instance, hydrophilic nanoparticles, as opposed to their hydrophobic counterparts, repulse each other and interact poorly with the ground substance of the interstitium and drain rapidly into the initial lymphatics (**Fig. 2**).

Very small particles (1–20 nm), particularly when they are long circulatory, can slowly extravasate from the vasculature into the interstitial spaces, from which they are transported to lymph nodes by way of lymphatic vessels (62). Depending on the species, particles may leave the blood pool through the permeable endothelium in lymph nodes, thus the extent of nodal vascularization and blood supply become important. Nevertheless, these modes of particle movement from the blood and interstitial sites to the lymph nodes provide intriguing opportunities for diagnostic imaging where, essentially, only an enhancement of signal over background is required; the physicochemical properties of QDs (50) and superparamagnetic iron oxide nanocrystals (8) makes them ideal for such purposes.

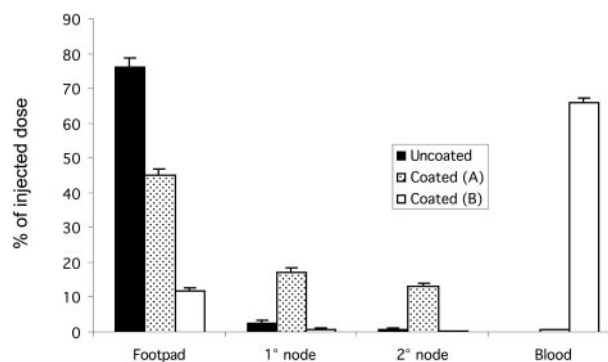


Figure 2. Modulation of lymphatic distribution of subcutaneously injected polystyrene nanoparticles (45 nm in diameter) in rats by prior surface modification with a synthetic copolymer. The results represent nanoparticle distribution at 6 h postinjection. The surface of nanoparticles was modified by adsorption of poloxamer 407. This is a block co-polymer composed of a linear hydrophobic segment (comprised of 67 propylene oxide units), which are flanked at each side by 98 units of ethylene oxide. The central segment adsorbs onto the surface of polystyrene nanoparticles, but the ethylene oxide chains extend from the particle surface. In coated-A system, ethylene oxide tails spread laterally on the nanoparticle surface and assume a “flat or mushroom-like” conformation. These particles drain rapidly from the injection site (dorsal surface of footpad), when compared with their uncoated counterparts, and subsequently are captured by macrophages in the regional lymph nodes. Here, surface coating also enhances particle retention in the 2nd node. In system (B), ethylene oxide chains are more closely packed and project outward (a brush-like conformation). These particles drain rapidly into initial lymphatics, escape clearance by lymph node macrophages, reach the systemic circulation, and remain in the blood for prolonged periods of time. For further details, see ref 64.

TARGETING

Macrophage as a target

The propensity of macrophages of the reticuloendothelial system for rapid recognition and clearance of particulate matter has provided a rational approach to macrophage-specific targeting with nanocarriers. The macrophage is a specialized host defense cell whose contribution to pathogenesis is well known. Alterations in macrophage clearance and immune effector functions contribute to common disorders such as atherosclerosis, autoimmunity, and major infections. The macrophage, therefore, is a valid pharmaceutical target and there are numerous opportunities for a focused macrophage-targeted approach (23–26). For example, although most microorganisms are killed by macrophages, many pathogenic organisms have developed means for resisting macrophage destruction following phagocytosis. In certain cases, the macrophage lysosome and/or cytoplasm is the obligate intracellular home of the microorganism, examples include *Toxoplasma gondii*, various species of *Leishmania*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes*. Passive targeting of nanoparticulate vehicles with encapsulated antimicrobial agents to infected macrophages is therefore a logical strategy for effective microbial killing (23, 24, 65–67). The endocytic pathway will direct the carrier to lysosomes where pathogens are resident. Degradation of the carrier by lysosomal enzymes releases drug into the phagosome-lysosome vesicle itself or into the cytoplasm either by diffusion or by specific transporters, depending on the physicochemical nature of the drug molecule. Approved formulations for human subjects are limited to lipid-based nanosystems (100–200 nm) with entrapped amphotericin B (Amp-B), and are recommended for treatment of visceral leishmaniasis or confirmed infections caused by specific fungal species (23, 65, 68). This mode of targeting has significantly reduced the required clinically effective quantity of Amp-B for treatment, achieving therapeutic drug concentrations in the infected macrophages. Other beneficial effects include significant reduction in nephrotoxicity, a common side effect associated with Amp-B administration, and proinflammatory cytokine release (68, 69). To further alleviate nephrotoxicity, combination strategies may be sought. One possibility for exploration is liposome emulsification, as this may further stabilize Amp-B association with the carrier. Others have used multifunctional carriers to deliver antimicrobials to macrophages (67). For example, intravenous injection of tuftsin-bearing liposomes to infected animals have not only resulted in delivery of liposome-encapsulated drugs to the macrophage phagolysosomes, but also in the nonspecific stimulation of liver and spleen macrophage functions against parasitic, fungal and bacterial infections (67). The latter effect is due to the binding of tuftsin to its receptor, which further incites macrophage antimicrobial responses.

Endocytic delivery is a route for macrophage destruction. Recently nanocarrier-mediated macrophage suicide (delivery of macrophage toxins) has proved to be a powerful approach in removing unwanted macrophages in gene therapy and other clinically relevant situations such as autoimmune blood disorders, T cell-mediated autoimmune diabetes, rheumatoid arthritis, spinal cord injury, sciatic nerve injury, and restenosis after angioplasty (70–75). Alternatively, microbial-induced macrophage apoptosis strategies may be exploited to design and surface-engineer nanoparticles with macrophage-killing properties. One example is through targeting and selective activation of Toll-like receptor 4 (76).

Macrophages and dendritic cells play critical roles in determining immunogenicity and the generation of appropriate immune responses. Systems, such as numerous polymeric and ceramic nanospheres, nanoemulsions, liposomes, protein cage architectures, and viral-derived nanoparticles act as powerful adjuvants if they are physically or covalently associated with protein antigens (33, 77–79). After endocytic uptake of nanoparticles, macrophages partially degrade the entrapped antigens and channel peptides into the MHC molecules (class I or II) for processing and presentation. Thus, there is considerable potential for nanoparticulate adjuvants for the development of new-generation vaccines made either recombinantly or from synthetic peptide antigens that are less or nonimmunogenic in their own right. Genetic immunization with nanoparticles has also received attention but the majority of attempts are based on cationic systems to allow DNA compaction (80, 81).

Recent advances in cell biology have provided a wealth of information regarding the structure, recognition properties, and signaling functions of a variety of macrophage/dendritic cells receptors, particularly those that affect immunogenicity or adjuvanticity. Harnessing these receptors as therapeutic targets may prove a better strategy for antigen delivery and targeting with particulate nanocarriers. For example, in addition to endocytic and phagocytic roles, the mannose receptor plays an important role in antigen recognition and transport within lymphoid organs (82). Antigen coupling to oxidized mannan leads to rapid recognition by the mannose receptor, followed by internalization, efficient MHC class I presentation to CD8⁺ cells, and a preferential T1 response. It is the presence of the aldehydes, and not Schiff bases, that cause rapid entry into the class I pathway (82). Conversely, after reduction processes, internalization and presentation to MHC class I molecules are diminished by 1000-fold; the outcome is class II presentation and a T2 immune response. The soluble form of mannose receptor, or recombinant probes containing the cysteine-rich domain of the mannose receptor, may also be used for antigen transport. The N-terminal cysteine-rich domain of the mannose receptor is a counter-receptor for sulfated glycoprotein cysteine-rich domain ligand expressed by subpopulations of myeloid cells in

secondary lymphoid organs (e.g., murine splenic metallophilic macrophages, subcapsular sinus macrophages in lymph nodes, follicular dendritic cells and migratory dendritic cells) (83). Indeed, the lectin activity of the cysteine-rich domain was recently exploited for targeting of acidic glycan-decorated proteins to metallophilic macrophages (83). Dectin-1 is another mannan-dependent receptor with phagocytic activity, which is expressed by macrophages, neutrophils and dendritic cells (84). This receptor recognizes β -glucans from fungi and yeasts and may potentially mediate immunostimulatory effects of β -glucans. Dectin-1 contains an immunoreceptor tyrosine-based activation motif in its cytoplasmic tail, thus ligand recognition may lead to activation of signaling pathways in myeloid cells. Dendritic cell receptors such as DEC-205 and DEC-SIGN have been implicated in antigen internalization and presentation to T cells (85–87). For example, DEC-205 can recycle and enhance antigen presentation via MHC class II-positive lysosomal compartments. An interesting approach would be to design and evaluate nanoparticles that target a combination of antigens and activators to immature dendritic cells *in vivo* for stimulating immune responses, or other appropriate agents to silence immunity. For instance, direct activation and dendritic cell migration may be achieved by specific targeting of Toll-like receptor ligands through dendritic cell specific molecules.

Endocytic loading of macrophages with nanotechnology-based contrast agents is an intriguing approach for disease detection; here the surrounding parenchyma, and not the pathology, will change in intensity. For example, after extravasation from the vasculature and lymph node arrival, intravenously injected paramagnetic iron oxide nanocrystals are sequestered by resident lymph node macrophages, and their intracellular accumulation shortens the spin relaxation process of nearby protons detectable by magnetic resonance imaging. In magnetic resonance imaging, those node regions accumulating iron oxide crystals appear dark relative to surrounding tissues. Indeed, this development has facilitated distinction between normal and tumor-bearing nodes or reactive and metastatic nodes, which is a desirable approach since a single intravenous injection provides access to a large number of lymph nodes (62). Recently, using this approach very small metastases (less than 2 mm in diameter) within normal-sized lymph nodes in patients with prostate cancer were identified (8). This is important, since microscopic tumor deposits are below the detection threshold of other advanced imaging techniques. Superparamagnetic nanoconstructs have aided visualization of vascular pathologies in arthritis (9) and atherosclerotic plaques (10, 11); this is a reflection of increased endothelial permeability and access to resident macrophages. Similarly, capture of near-infrared fluorescent type II quantum dots by lymph node macrophages has allowed sentinel lymph node mapping and visualization of deeply located lymph nodes (50).

Endothelium as a target

The concept of targeting to the blood vessels is an attractive one, particularly with the view that the endothelium plays an important role in a number of pathological processes including cancer (dysregulated angiogenesis), inflammation, oxidative stress and thrombosis. Indeed, a number of studies have demonstrated a level of control of arrest and distribution of passively targeted nanoparticles by specific endothelial cells, and these were linked to the surface properties of the carrier. For instance, early studies of polystyrene nanoparticles, designed to minimize Kupffer cell uptake, indicated exclusive arrest by the bone marrow sinus lining endothelial cells in rabbits (88). Arrest was followed by receptor-mediated internalization. Another example is the localization of intravenously injected polysorbate 80-coated nanoparticles to murine and rat blood-brain endothelial cells (89, 90). Recent studies have shown that cationic liposomes within 1 h of entering the circulation, are internalized into endosomes and lysosomes of endothelial cells in a characteristic organ- and vessel-specific manner (91). These patterns seem to bear no relationship to the morphological characteristics of the endothelium associated with a particular site, but probably reflect vessel-specific expression of receptors for which such particles, or their surface-associated blood proteins, are ligands.

But recent dramatic progress in the development of a human vascular map, in particular through the application of cell and molecular biological tools such as serial analysis of gene expression (SAGE), subtractive proteomic mapping, and *in vivo* phage display, is generating yet another level of possibilities for specific targeting of drugs and biological agents (55–59). For example, SAGE examines the spectrum of mRNA species within a cell or tissue and allows rapid comparison with other cell types or tissues. Phage, however, behaves as a nanomachine; it can be engineered to display numerous peptides on its surface and after injection one can select peptides that make the phage home to a given target. Recently, Arap et al. (56) described the first attempt at mapping the vasculature of a human patient using a phage display technique. The authors surveyed 47,160 sequences that localized to different organs within the patient, and determined that in many cases these sequences were similar to those of known ligands for endothelial cell-surface proteins, thus validating this technique for identifying novel endothelial targets in humans. This approach is now being used in a number of ways to target therapeutic agents, particularly to the vasculature of solid tumors. Examples include integrins $\alpha v \beta 3$, $\alpha v \beta 5$ and $\alpha 5 \beta 1$, which are up-regulated in angiogenic endothelial cells and play a role in the process of angiogenesis (55, 58, 92, 93). They bind with high affinity to sequences containing a characteristic RGD (Arg-Gly-Asp) motif, which seems to be central to anti-integrin approaches. Indeed, *in vivo* phage display studies by Assa-Munt et al. (94) have led to the development of a cyclic nonapeptide RGD-4C,

which avidly binds to the integrins $\alpha\beta 3$ and $\alpha\beta 5$. Coupling of RGD-4C to doxorubicin yielded a compound significantly more effective than doxorubicin alone, and with less side effects to the heart and liver, the main sites of doxorubicin toxicity (95). Zitzmann et al. (96) have shown that the homing peptide RGD-4C binds to human breast cancer cells grown as xenografts in nude mice, as well as to endothelial cells, but not to nonendothelial normal cells. Inserting RGD sequences into adenovirus surface protein has been used to affect the tropism of the viral gene therapy vectors for targeting purposes (97, 98). Other discovered binding sequences, such as the HWGF peptide motif, can bind to matrix metalloproteases-2 and -9, and dramatically enhances adenoviral tropism to the endothelial and smooth muscle cells of large blood vessels (99). The hexapeptide NGR is another homing molecule derived from in vivo phage display studies (95). This peptide specifically binds to aminopeptidase N on angiogenic endothelial cells and when coupled to the cytokine TNF α , it produces 8- to 10-fold enhancement of the therapeutic efficacy of doxorubicin and melphalan against murine tumors (100). Phage display studies have further revealed the ability of LyP-1, a cyclic nonapeptide, to recognize tumor lymphatic vessels (as well as tumor cells in certain tumors) and translocate to the nucleus (101). Although some of the described antiangiogenic attempts are promising, it appears that local destruction of endothelial cells committed to an angiogenic program will not be sufficient to destroy the tumor. A significant proportion of endothelial cells will be recruited from the bone marrow pool as well as the circulating blood, and endothelial cells lost through treatment or natural processes, will be replaced on demand (102). Therefore future therapeutic approaches must acknowledge this population. By the same token, this new population, which will ultimately differentiate into mature endothelial cells, must by definition express a unique set of cell surface markers, consistent with its progenitor role, which identifies it and facilitates targeting.

Nevertheless, several studies have now combined the specificity of endothelial molecular markers with nanoparticles. For example, as a novel anti-angiogenic strategy targeted at solid tumors, some investigators have used a synthetic analog of $\alpha\beta 3$ to target therapeutic genes complexed with cationic nanoparticles at tumor-associated endothelial cells (103). Similar approaches have now been extended for site-specific imaging with $\alpha\beta 3$ -targeted paramagnetic nanoparticles. This attempt detected and characterized early angiogenesis induced by minute solid tumors with magnetic resonance imaging (104). This is a valuable tool with which to phenotypic categorization and patient selection as well as track the effectiveness of antitumor treatment regimens. Similarly, selective targeting of peptide-coated QDs to blood and lymphatic vessels in tumors have been demonstrated (19). Another interesting approach was the ability of NGR motif-decorated lipo-

somes to specifically attack tumors by shutting down their blood supply (105).

Other important targets expressed on the endothelium for therapeutic drug delivery include cell adhesion molecules (CAM). Intercellular cell adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) are perhaps the most studied of the CAMs as targets. Antibodies against ICAM-1 and PECAM-1, which could theoretically be used to deliver therapeutic cargoes to endothelial cells are not taken up. However, anti-ICAM-1 and anti-PECAM-1 nanoparticles are readily endocytosed, by a unique but poorly understood pathway (106). Such anti-CAM nanoparticles have been shown to successfully deliver a variety of cargoes to pulmonary and cardiac endothelium in vivo (107–109). Endothelial leukocyte cell adhesion molecule-1 and vascular adhesion molecule-1, which are moderately expressed on the vascular endothelium of tumors having marked leukocytic infiltration (e.g., solid Hodgkin's tumors, breast carcinoma and non-small cell lung carcinoma), have served as targets for therapeutic interventions with nano-constructs (110).

Extravasation: targeting of solid cancers

The development of “stealth” technologies has provided opportunities for passive accumulation of intravenously injected nanoparticles (20–150 nm) in pathological sites expressing “leaky” vasculature by extravasation (24). Although, attempts have included delivery of drugs and imaging agents with different nanoscale technologies to the underlying parenchyma of injured arteries and rheumatoid arthritis, the majority of efforts are concentrated on solid tumors (23–25, 28, 29, 40, 42, 111–114).

As a result of perfusion heterogeneity, the spatial distribution of stealth nanoparticles in solid tumors is heterogeneous and unpredictable. As has been elegantly demonstrated by Jain (115) structural and functional abnormalities of blood and lymphatic vessels within solid tumors impede efficient delivery of not only systemic nanoparticles, but macromolecules. Already compromised by abnormal hydrostatic pressure gradients, compressive mechanical forces generated by tumor cell proliferation cause intratumoral vessels to compress and collapse (116). Tumor-specific cytotoxic therapy, reducing tumor cell number, may result in more efficient delivery, by decompressing these same vessels (116); however, this enhanced perfusion could provide a route for metastasis. Distribution, organization and relative levels of collagen, decorin, and hyaluronan impede the diffusion of macromolecules and nanoparticles in tumors (117). Thus, diffusion of macromolecules and nanoparticles will vary with tumor types, anatomical locations, and possibly by factors that influence extracellular matrix composition and/or structure.

In spite of these limitations, there are regulatory approved formulations of long circulating liposomes with entrapped doxorubicin for management/treat-

ment of AIDS-related Kaposi's sarcoma, refractory ovarian cancer, and metastatic breast cancer (23, 118). These formulations exhibit favorable pharmacokinetics when compared with the free drug, for example the area under the curve after a dose of 50 mg/m² doxorubicin encapsulated in stealth liposomes is ~300-fold greater than that of free doxorubicin. Clearance and volume of distribution are reduced by at least 250- and 60-fold, respectively (118). As a result of these promising pharmacokinetic profiles, stealth doxorubicin containing liposomes are currently undergoing additional early- to late-phase clinical trials.

A number of engineering issues must be considered when applying particulate stealth systems for cancer drug delivery. First, the carrier must have a high drug loading capacity and remain stable within the vasculature with minimum drug loss. This criterion is met with the current regulatory approved stealth liposome formulation of doxorubicin. Here, doxorubicin is loaded actively by an ammonium sulfate gradient (as doxorubicin sulfate) yielding highly stable liposomes with high contents of doxorubicin aggregates (119). Second, it has been widely established that the majority of extravasated particulate systems, such as liposomes, do not interact with target cancer cells (111). They are often distributed heterogeneously in perivascular clusters that do not move significantly. The process of particle extravasation must be followed by the efflux of drug from the carrier, resulting in target exposure (being tumor cells, tumor-associated macrophages, components of tumor vasculature, or extracellular mediators such as proangiogenic proteases) to drug molecules. Here, the drug must be released at a rate that maintains free drug levels in the therapeutic range. However, the rate of drug release from liposomes depends on drug type and the encapsulation method (119). For example, stealth liposomes with entrapped cisplatin (where cisplatin is loaded passively) lack antitumor activity despite accumulation in tumor interstitial sites (120); this is simply due to the poor and extremely slow release of cisplatin from extravasated liposomes. This is in contrast to effective antitumor property of the same liposomal lipid composition but with entrapped doxorubicin. It is suggested that nonspecific chemical disruption, or collapse of the liposomal pH gradient, which was initially used to load liposomes actively with doxorubicin, triggers doxorubicin release (perhaps, in both monomeric and aggregated forms) from intact vesicles in a slow manner (119). If this proposed mechanism is true, then some of the antitumor effects of liposomal doxorubicin may be ascribed to antiangiogenesis since these vesicles accumulate at tumor interstitial sites closed to proangiogenic vessels. Thus, the therapeutic potential of liposomal doxorubicin in animal models with drug-resistance tumors is worthy of exploration. Nonetheless, the most prominent feature of liposomal doxorubicin formulations has been a decrease in many of the side effects associated with free doxorubicin or doxorubicin encapsulated in macro-

phage-prone nanocarriers rather than an increase in potency (111, 118).

The issue of drug release from nanocarriers remains central to cancer chemotherapy. Therefore, application of a number of solid and polymeric nanoparticles for cancer drug delivery must be viewed cautiously since drug molecules may not be released from extravasated nanoparticles at sufficient rates. To date, the most effective approaches are described with liposomes and to some extent with polymeric micelles, although the latter constructs have a low encapsulation volume. There are a number of biochemical-based advances that can trigger drug release from accumulated liposomes at interstitial sites. One attractive approach is enzyme-mediated liposome destabilization. For example, local concentration of secretory phospholipase A2 (sPLA2) is highly elevated in inflammatory and cancer tissues (121, 122). The ester linkage in the *sn*-2 position of phospholipid is rapidly hydrolyzed by sPLA2. This leads to production of a fatty acid and a lysolipid, which have a synergistic membrane perturbing and permeabilizing effect (123). The activity of sPLA2 is much higher toward aggregated phospholipids (liposomes and micelles) than lipid monomers. sPLA2 can destabilize sterically protected liposomes bearing the substrate (121). So, by choosing an appropriate lipid composition one may control the extent of vesicle destabilization by sPLA2 (124). For example, stealth vesicles with entrapped cisplatin but composed of a nontoxic sPLA2 sensitive lipid prodrug (an ether lipid) were extremely effective in killing cancer cells in the presence of sPLA2, whereas the corresponding control formulation without sPLA2 sensitive lipids expressed insignificant cytotoxicity. Future efforts may consider combination strategies with sPLA2 sensitive and insensitive stealth liposomes, thus generating both rapid and prolonged interstitial drug release profiles in pathologies with elevated sPLA2 activity.

There are several approaches, which describe active targeting of nanoparticles to cancer cells and related extracellular elements, but with these methods the delivery part is still passive. One interesting method was in vivo detection and imaging of tumor-associated matrix metalloproteinase-7 (matrilysin) activity with a dendrimer-based fluorogenic substrate, which served as a selective "proteolytic beacon" for matrilysin (125). Others have used active targeting in combination with stealth technology. For example, tumor-specific monoclonal antibodies, and particularly internalizing epitopes, or ligands, such as folic acid, have been attached to the distal end of poly(ethyleneglycol) chains expressed on the surface of long circulating or macrophage-evading nanocarriers (24, 40–42, 111, 119, 126, 127). Folate-targeting is an interesting approach and offers a number of advantages over monoclonal antibodies. The folate receptor expression is restricted to human ovarian, endometrial, colorectal and lung cancers; the receptor is generally absent in most normal human tissues with the exception of a few sites (41). First, folate is nonimmunogenic. Second,

folate acid-decorated nanocarriers are rapidly internalized by folate receptor-bearing cancer cells and internalization is associated with rapid receptor recycling to the plasma membrane (41). This receptor-mediated internalization approach is believed to bypass cancer cell multidrug-efflux pumps (127). But, a major problem is the extent of drug release from internalized nanocarriers. For example, in an acidic compartment such as endosome/lysosome local conditions may not favor rapid doxorubicin release from the ammonium sulfate gradient loaded doxorubicin liposomes (bearing in mind that the drug reaches acidic cellular compartments as doxorubicin sulfate salt, and the drug concentration in the liposome aqueous phase is far above the drug solubility product) (119).

Nanoparticles for cytoplasmic drug delivery

Breaching of the endosomal membrane is particularly important for priming MHC class I-restricted cytotoxic T lymphocyte responses, for survival of genetic materials against nuclease degradation in the lysosomal compartment, or for those drugs that must reach cytoplasm in sufficient quantities (as for treatment of cytoplasmic infections or reaching nuclear receptors) after endocytic delivery with nanoparticulate carriers. Here, there are advances in particle engineering too. For instance, nanoparticles made from poly(DL-lactide-co-glycolide) can escape the endo-lysosomal compartment within minutes of internalization in intact form and reach the cytoplasm (44). The mechanism of rapid escape is by selective reversal of the surface charge of nanoparticles from the anionic to the cationic state in endo-lysosomes, thus resulting in a local particle-membrane interaction with subsequent cytoplasmic release. Another impressive approach for cytoplasmic delivery of nanoparticles is their surface manipulation with short peptides known as protein transduction domains such as HIV-1 TAT protein transduction domain (TAT PTD), which is a short basic region comprising residues 48–57, or heterologous recombinant TAT-fusion peptides (128–130). The electrostatic interaction between the cationic TAT PTD and negatively charged cell-surface constituents, such as heparan sulfate proteoglycans and glycoproteins containing sialic acids, is a necessary event before internalization (131). After this ionic interaction, cellular uptake occurs by lipid raft-dependent macropinocytosis in a receptor-independent manner; this is followed by a pH drop and destabilization of integrity of the macropinosome vesicle lipid bilayer, which ultimately results in the release of TAT-cargo into the cytosol (131). This mode of entry may further suggest the avidity of TAT PTD for glycosphosphoinositol-anchored glycoproteins, which are present in lipid rafts, or binding to cholesterol membrane constituents that trigger macropinocytosis. An important feature of macropinosomes is that they do not fuse into lysosomes to degrade their contents. Although, these approaches have the potential to deliver and release drugs cytoplasmically for a sustained

therapeutic effect in conditions such as cancer and stroke, possible cytotoxicity arising from the carrier components cannot be ruled out and warrants detailed investigation (132).

A number of gene transfer approaches are also based on destabilization of internalized vesicles via the surface charge effect. For instance, a wide range of cationic particles and synthetic polycations in linear, branched, or dendrimer form have been used to condense DNA, antisense oligonucleotides, and small interfering RNAs into nanostructures amenable to cellular internalization via endocytosis (34, 133, 134). For example, poly(ethylenimine)s are believed to act as proton sponges; they buffer the low pH in the endosomes and potentially induce membrane rupture, resulting in the release of polycation/nucleic complexes into the cytoplasm (134).

Other examples include pH-sensitive microparticles (e.g., spray-dried particles made from a combination of phospholipids and the pH-sensitive polymethylacrylate (Eudragit E100) and pH-sensitive and fusogenic liposomes, which can efficiently smuggle their cargo into cytosol (43, 135). Vesicles that are pH-sensitive are assembled from a combination of unsaturated phosphatidylethanolamine and mildly acidic amphiphiles; this composition confers bilayer stability at neutral pH or above but destabilizes and becomes fusion-competent at the acidic pH of late endosomes (43). Alternatively, by mimicking the characteristics of certain toxins or viruses, cytoplasmic delivery of agents can be enhanced. This requires co-encapsulation of bacterial pore-forming toxins (e.g., listeriolysin O) or fusion peptides found in the envelope of glycoproteins of certain viruses in particulate vehicles (136). Indeed, an influenza vaccine based on the latter principle is currently available for human use (79). This vaccine is administered parenterally and is well tolerated in children, young adults and the elderly. The influenza strains chosen are dependent on the yearly recommendations of the World Health Organization. The delivery system is comprised of unilamellar vesicles of 150 nm in diameter, but intercalated into their lipid bilayer are viral components, which include neuraminidase and hemagglutinin (HA) glycoproteins. The mode of action of these virus-like liposomes (virosomes) is dependent on HA glycoproteins, the major antigens of the influenza virus. The HA is composed of two subunits, HA1 and HA2. The first subunit has high affinity for sialic acid present on the surface of antigen presenting cells thus facilitating virosome binding. The HA2 subunit is a fusion peptide and is activated at low pH (~5.0). Hence, in late endosomes, where pH is acidic, the virosome becomes fusion-competent; this process releases entrapped antigens into the cytosol for subsequent processing and presentation (79). Similarly, Wadia et al. (131) have generated a fusogenic influenza dTAT-HA2 peptide; this complex specifically enhances macropinosome escape.

TOXICITY ISSUES

Nanocarriers may overcome solubility or stability issues for the drug and minimize drug-induced side effects. But there could be significant toxicity issues associated with the nanocarriers themselves, which requires resolution. Over the past couple of years, a number of toxicology reports have demonstrated that exposure to nanotechnology derived particles pose serious risks to biological systems (137–139). For instance, exposure of human keratinocytes to insoluble single-wall carbon nanotubes (see also Appendix 1) was associated with oxidative stress and apoptosis (139). The issue of toxicity becomes even more serious for intravenously injected nanoparticles, as size partly determines tissue distribution. Thus, what is the ultimate fate of nanocarriers and their constituents in the body, and particularly those which are not bio-degradable such as functionalized carbon nanotubes and coating agents such as poly(ethyleneglycol)? Can these constituents or their degradation products exert untoward immunological and pharmacological activities? Can polymeric vectors used for gene delivery as well as other polymer-based biomaterials interfere with cellular machineries or induce altered gene expression? If so, what are the long-term consequences? Finally, to what extent can we translate cellular and immunological toxicity results observed in animal models to humans, as there are distinct intra- and interspecies variation.

Cell death and altered gene expression

Recent evidence is drawing attention to some of the above questions, but investigation in this avenue of research is scant. For example, though much has been made of the promise of cadmium selenide QDs in imaging, little is known about their metabolism and potential deleterious effects. However, cadmium selenide QDs are lethal to cells under UV irradiation, as this releases highly toxic cadmium ions (140). Some polymeric micelles depending on the nature of their monomer constituents, can induce cell death via apoptosis or necrosis, or both (141, 142). Differential gene expression has been reported in certain cells after cisplatin delivery with polymeric micelles when compared with that of free cisplatin treatment (143). Degradation products arising from poly(L-lactic acid) particles show cytotoxicity, at least, to immune cells (144), thus raising concern over their application for sustained cytosolic drug release. Certain polymeric constituents used in nanoparticle design and engineering act as inhibitors of P-glycoprotein efflux pumps expressed in polarized endothelial cells that form the exterior of the blood-brain barrier (145), and could potentially interfere with transport of a number of modulators and homeostatic mediators in the central nervous system (146). Multidrug resistant tumors express these pumps, and recent studies have demonstrated that the inhibition of such pumps by synthetic polymers is due to cell sensitization as well as ATP depletion (147). Remark-

ably, these mechanisms were correlated with low expression of ATP binding cassette genes after polymer treatment, indicating that these polymers have the ability to modulate gene expression but the molecular basis of these events remains unknown (147). During the past few years we have witnessed a surge in the development and creation of polymeric self-assemblies and nanofibers that act as scaffolds for cell attachment, proliferation, and encapsulation, which are intended to be used as synthetic replacements for biological tissues (148–150). The effect of such engineered polymeric structures on gene expression therefore is critical in tissue engineering and cell therapy, as these materials may initiate a number of unexpected effects. Conversely, cell-material interaction may identify a host of materials effects that offer new levels of control over cell behavior. Indeed, these predictions have been highlighted by recent developments in nanoliter scale synthesis of arrayed biomaterials, which are providing rapid and more insight into stem cell-material interactions (150). For example, some polymers supported high levels of stem cell differentiation into epithelial-like cells, whereas other polymers helped stem cell growth in the absence of certain growth factors.

Cell death and gene therapy

A very clear warning is evident from the poor success in human gene therapy with viruses. Although, viral vectors are extremely efficient delivery systems for nucleic acids, they can induce severe immunotoxicity as well as inadvertent gene expression changes after random integration into the host genome (151, 152). These issues have generated a surge in design and engineering of synthetic polycationic nonviral gene transfer systems. However, the polycationic nature of the gene-delivery vehicles can induce immediate or delayed cytotoxicity by mechanisms involving necrosis as well as apoptosis (153, 154). Necrosis may occur as a result of membrane destabilization or pore formation after interaction between the cationic components of the delivery system with cell surface proteoglycans and negatively charged proteins in cytoskeleton, such as actin. In the case of Jurkat T cells the apoptotic mechanism appears to be due to polycation-mediated release of Bcl-2-sensitive proteins such as cytochrome *c* from the mitochondrial intermembrane space and altered mitochondrial functions (**Fig. 3**). However, different cationic materials, and depending on their molecular weights and polydispersity, may initiate apoptosis at different times and by different mechanisms or modes. The effect of these materials on cell death may depend on cell nature, mitochondrial content and the extent mitochondrial heterogeneity. Nevertheless, cytotoxic gene-delivery systems may compromise transcription and translation processes and potentially limit protein expression. In protocols, which attempt to restore gene function, for instance in metabolic disorders, such toxicity issues take on even greater importance. In addition to these, cDNA microarray expres-

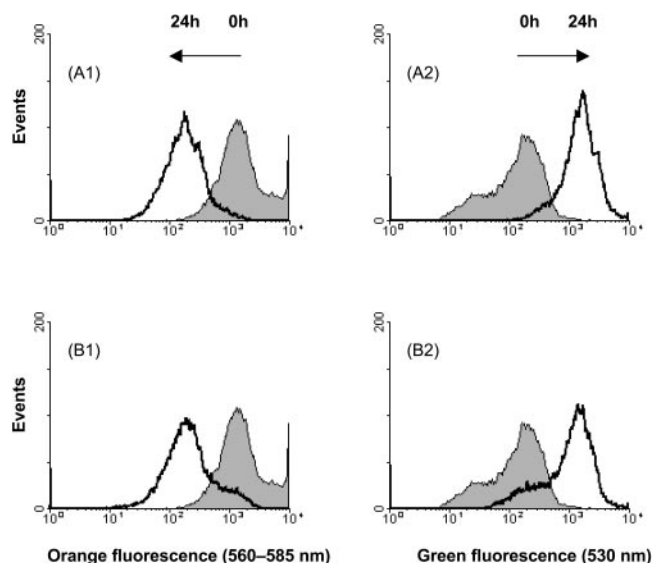


Figure 3. Changes in mitochondrial membrane potential (MMP) in Jurkat T cells after treatment with poly(ethylenimine), PEI. *A1, A2*) Cells were treated with a linear high molecular mass PEI (750 kDa) at a final concentration of 13.3 nM; *B1, B2*) cells were treated with a branched PEI (25 kDa) at a concentration of 400 nM. Loss of MMP is an early event in several types of apoptosis, and can be determined by the MMP-specific fluorescent probe JC-1. The normally high transmembrane potential of healthy cells loaded with JC-1 allows for the formation and sequestration in the mitochondrion of JC-1 aggregates, detectable by a peak in orange fluorescent (filled panels at 0 h). As MMP is lost, these aggregates dissipate into the cytoplasm as monomers and fluorescence shifts to 530 nm (green). This confirms that the loss of JC-1 aggregate is not due to an overall loss of dye from the cell. MMP loss is dramatic 24 h post-PEI treatment (indicated by changes in orange and green fluorescence). Apoptosis was further confirmed by cytochrome *c* release from mitochondria as well as caspase 3 activation. Loss of MMP presumably occurs after cytochrome *c* release and could be due to the cleavage of the 75 kDa subunit of mitochondrial respiratory complex I by activated caspases.

sion profiling studies have recently revealed marked changes in the expression of cell proliferation, differentiation and proapoptotic genes in human epithelial cells, after treatment with cationic formulations (155). This raises further concern as to whether such delivery systems could adversely influence the desired effects of the delivered genetic agents. For instance cationic carriers may exacerbate, attenuate or even mask the effects of delivered nucleic acids. Thus, gene transfer/therapy represents an important area where smart macromolecular design and engineering is critical to achieving a successful outcome in the near future and could benefit through recent advances in high-throughput approaches to polymer design and screening (156, 157). Such approaches may lead to understanding of the molecular basis of interaction between cationic polymers and mitochondrial and nuclear membrane as well as cationic polymers and BCL-2 family of proteins comprising inhibitors and inducers of apoptosis.

Pseudoallergy and idiosyncratic reactions

Finally, another potential pitfall associated with nano-carrier infusion into human subjects is the generation of non-IgE-mediated signs of hypersensitivity (158). These reactions are idiosyncratic and are believed to be secondary to complement activation, and presumably are a reflection of an individual's immune cell sensitivity to complement-derived mediators (158, 159). Hypersensitivity can be ameliorated by slowing the rate of infusion or by patient premedication, and often fails to appear on repeat administration of the nanocarrier. Idiosyncratic reactions occur after infusion of stealth systems, such as poly(ethylene glycol)-grafted liposomes (158, 160). Refined surface engineering may eventually eliminate such side effects, for example by better polymer design, linkage modification, controlling the conformation and packing of grafted polymers and/or by introducing complement regulatory proteins or inhibitors on to the nanoparticle surface (24, 47, 52). However, the ultimate goal is to understand the molecular mechanism of complement activation-related pseudoallergy, which operates in a small population of individuals. Future developments in immunogenomics and predictive gene-derived toxicogenomic may eventually provide new methods for assessing an individual's sensitivity to nanomedicines and hence reduce the risk of immune-mediated side effects.

THE FUTURE OF NANOMEDICINE

Nanotechnology is beginning to change the scale and methods of vascular imaging and drug delivery.^{2–7} Indeed, the NIH Roadmap's 'Nanomedicine Initiatives' envisage that nanoscale technologies will begin yielding more medical benefits within the next 10 years. This includes the development of nanoscale laboratory-based diagnostic and drug discovery platform devices such as nanoscale cantilevers for chemical force microscopes, microchip devices, nanopore sequencing, etc. (see also Appendix 1) (161). The National Cancer Institute has related programs too, with the goal of producing nanometer scale multifunctional entities that can diagnose, deliver therapeutic agents, and monitor cancer treatment progress. These include design and engineering of targeted contrast agents that improve the resolution of cancer cells to the single cell level, and nanodevices capable of addressing the biological and evolutionary diversity of the multiple cancer cells that make up a tumor within an individual. Thus, for the full in vivo potential of nanotechnology in targeted imaging and drug delivery to be realized, nanocarriers have to get smarter. Pertinent to realizing this promise is a clear understanding of both physicochemical and physiological processes. These form the basis of complex interactions inherent to the fingerprint of a nanovehicle and its microenvironment. Examples of which include carrier stability,

extracellular and intracellular drug release rates in different pathologies, interaction with biological milieu, such as opsonization, and other barriers en route to the target site, be it anatomical, physiological, immunological or biochemical, and exploitation of opportunities offered by disease states (e.g., tissue-specific receptor expression and escape routes from the vasculature). Inherently, carrier design and targeting strategies may vary in relation to the type, developmental stage, and location of the disease.

Toxicity issues are of particular concern but are often ignored. Therefore, it is essential that fundamental research be carried out to address these issues if successful efficient application of these technologies is going to be achieved. The future of nanomedicine will depend on rational design of nanotechnology materials and tools based around a detailed and thorough understanding of biological processes rather than forcing applications for some materials currently in vogue. FJ

Appendix 1 – Bionanotechnology: selected examples of nanotechnology tools and nanomachines in medical research, experimental therapeutics and diagnostics

Nanopore sequencing

This is an ultra-rapid method of sequencing based on pore nanoengineering and assembly. A small electric potential draws a charged strand of DNA through a pore of 1–2 nm in diameter in an α -hemolysin protein complex, which is inserted into a lipid bilayer separating two conductive compartments. The current and time profile is recorded and these are translated into electronic signatures to identify each base. This method can sequence more than 1000 bases per second. This technology has much potential for the detection of single nucleotide polymorphisms, and for gene diagnosis of pathogens.

Further reading:

Chen, C. M., and Peng, E. H. (2003) Nanopore sequencing of polynucleotides assisted by a rotating electric field. *Appl. Phys. Lett.* **82**, 1308–1310.

Jia, S. Z., Sun, H. Y., and Wang, Q. L. (2002) Nanopore technology and its applications. *Prog. Biochem. Biophys.* **29**, 202–205.

Cantilevers with functionalized tips

The enhanced spatial, force and chemical resolution of the atomic force microscope (AFM) and chemical force microscope can be taken into advantage for designing nanoscale diagnostic assays. The AFM probes intramolecular forces between a very fine and functionalized silicon or single-walled carbon nanotube tip, located at the end of a small cantilever beam, and a surface. The probe is attached to a piezoelectric scanner tube, which scans the probe across a selected area of the sample surface. Interamolecular and intraatomic forces between the tip and the sample cause the cantilever to deflect; cantilever deflection is then measured by a laser light reflected from the back of the cantilever to a detector. The tip can be chemically modified in order to probe a molecular structure of interest in drug discovery and measure biochemical interactions such as those between antigens and antibodies.

Further reading:

Meyer, E., Hug, H. J., and Bennewitz, R. (Eds.) (2003) *Scanning Probe Microscopy: The Lab on a Tip*. Springer-Verlag, Berlin.

Microneedles

Micromachined needles and lancets with design adjustable bevel angles, wall thickness and channel dimensions have been engineered from single crystal silicon by combination of fusion bonding, photolithography, and anisotropic plasma etching. This technology is being applied to painless drug infusion, cellular injection, and a number of diagnostic procedures (e.g., glucose monitoring).

Further reading:

Sparks, D., and Hubbard, T. (2004) Micromachined needles and lancets with design adjustable bevel angles. *J. Micromechan. Microengineering* **14**, 1230–1233.

Microchips for drug delivery

These are microfabricated devices that incorporate micrometer-scale pumps, valves, and flow channels and allow controlled release of single or multiple drugs on demand. These devices are particularly useful for long-term treatment of conditions requiring pulsatile drug release after implantation in a patient. The release mechanism is based on the electrochemical dissolution of thin anode membranes covering microreservoirs, which are filled with drugs. Thus, controlled delivery systems can be designed to release pulses of different

drugs by using different materials for the membrane. Recently, microchip devices of 1.2 cm in diameter and thickness of approximately 500 μm with 36 drug reservoirs were fabricated from poly(L-lactic acid). The drug reservoirs were covered with poly(D,L-lactic-co-glycolic acid) membranes of different molecular masses.

Further reading:

Grayson, A. C. R., Choi, I. S., Tyler, B. M., Wang, P. P., Brem, H., Cima, M. J., and Langer, R. (2003) Multi-pulse drug delivery from a resorbable polymeric microchip device. *Nat. Mat.* **2**, 767–772.

Nucleic acid lattices and scaffolds

DNA can be programmed to self-assemble into an array of remarkable nanometer-scale structures different from the double helix. Stick cube, a construct shaped like a cube formed from sticks, and truncated DNA octahedron are two examples. For instance, the cube self-assembles from DNA fragments that are designed to adhere to one another. The free ends are connected by ligases, resulting in six closed loops, one for each face of the cube. Due to the helical nature of DNA, each of these loops is twisted around the loops that flank it, thus ensuring that the cube cannot come apart. Such scaffolds and assemblies can hold biological molecules in an ordered array for x-ray crystallography. This approach could be particularly useful for those materials that do not form a regular crystalline structure on their own (e.g., certain cell receptors that function as drug targets). These architectures could also hold molecule-size electronic devices, or be used to engineer materials with precise molecular configurations. Future efforts may lead to the design of DNA devices that can replicate, and DNA machines with moving parts as nanomechanical sensors, switches and tweezers.

Further reading:

Yan, H., Zhang X., Shen, Z., and Seeman, N. C. (2002) A robust DNA mechanical device controlled by hybridization topology. *Nature* **415**, 62–65.

Shih, W. M., Quispe, J. D., and Joyce, G. F. (2004) A 1.7-kilobase single-stranded DNA that folds into a nanoscale octahedron. *Nature* **427**, 618–621.

Soukup, G. A., and Breaker, R. R. (1999) Nucleic acids molecular switches. *Trend. Biotechnol.* **17**, 469–476.

Nanofibers as biomaterials

By applying molecular self-assembly, nanofibers of various structures and chemistries can be formed. Nanofibers may be designed to present high densities of bioactive molecules such as those which promote cell adhesion and growth. For example amphiphiles that present the pentapeptide epitope IKVAV, an amino acid sequence of laminin that promotes neurite adhesion, can self-assemble in aqueous media, or when injected directly into a tissue, to form fibers with a diameter of 5–10 nm. Indeed, these scaffolds were shown to induce rapid differentiation of cells to neurons, while discouraging the development of astrocytes. This presumably suggest that synthetic materials may have the ability to modulate selective gene expression (see Toxicity section).

Another interesting approach was the design of a synthetic collagen substitute, based on a material composed of a long hydrophobic alkyl group on one end and a hydrophilic peptide on the other that self-assembles into nanocylindrical structures. These nanocylinders guided the formation of hydroxyapatite crystallites with orientations and sizes similar to those in natural bone.

Further reading:

Silva, G. A., Czeisler, C., Niece, K. L., Beniash, E., Harrington, D. A., Kessler, J. A., and Stupp, S. I. (2004) Selective differentiation of neural progenitor cells by high-epitope density nanofibers. *Science* **303**, 1352–1355.

Taton, T. A. (2001) Boning up on biology. *Nature* **412**, 491–492.

Carbon nanotubes

Carbon nanotubes belong to the family of fullerenes and consists of graphite sheets rolled up into a tubular form. These structures can be obtained either as single- (characterized by the presence of a single graphene sheet) or multi-walled (formed from several concentric graphene sheets) nanotubes. The diameter and the length of single-walled nanotubes may vary between 0.5–3.0 nm and 20–1000 nm, respectively. The corresponding dimensions for multi-walled nanotubes are 1.5–100 nm and 1–50 μm , respectively. Carbon nanotubes can be made water soluble by surface functionalization. Molecular and ionic migration through carbon nanotubes can occur, thus offering opportunities for fabrication of molecular sensors and electronic nucleic acid sequencing. Carbon nanotubes can apparently cross the cell membrane as ‘nanoneedles’ without perturbing or disrupting the membrane and localize into cytosol and mitochondria. However, the mechanisms are poorly understood.

A number of carbon nanotubes derivatives, such as tris-malonic acid derivative of the fullerene C_{60} , express superoxide dismutase mimetic properties and are protective in cell culture and animal models of

injury, including degeneration of dopaminergic neurons in Parkinson's diseases and nervous system ischemia. The mechanism of action by C₆₀ compounds appears to be through catalytic dismutation of superoxide. Furthermore, single-walled carbon nanotubes of 0.9–1.3 nm have been shown to block potassium channel subunits in a dose-dependent manner. However, not much is known with respect to *in vivo* toxicity of functionalized carbon nanotubes and their eventual intracellular fate. In the absence of detailed pharmacokinetic and toxicological studies, and their poor capacity to incorporate and release active compounds, the predicted benefits of carbon nanotubes in drug, antigen, and gene delivery remain hyped.

Further reading:

- Bianco, A. (2004) Carbon nanotubes for the delivery of therapeutic molecules. *Exp. Opin. Drug Deliv.* **1**, 57–65.
- Lopez, C. F., Nielsen, S. O., Moore, P. B., and Klein, M. L. (2004) Understanding nature's design for a nanosyringe. *Proc. Natl. Acad. Sci. USA* **101**, 4431–4434.
- Ali, S. S., Hardt, J. L., Quick, K. L., Kim-Han, J. S., Erlanger, B. F., Huang, T. T., Epstein, C. J., and Dugan, L. (2004) A biologically effective fullerene (C₆₀) derivative with superoxide dismutase mimetic properties. *Free Radical Biol. Med.* **37**, 1191–1202.
- Park, K. H., Chhowalla, M., Iqbal, Z., and Sesti, F. (2003) Single-walled carbon nanotubes are a new class of ion channel blockers. *J. Biol. Chem.* **278**, 50212–50216.

Appendix 2 – Definition of some nanoparticles with biological and medical application

Superparamagnetic iron oxide crystals

These entities are usually prepared by the alkaline co-precipitation of appropriate ratios of Fe²⁺ and Fe³⁺ salts in water in the presence of a suitable hydrophilic polymer such as dextran or poly(ethyleneglycol). This yields an iron core of 4–5 nm in diameter, which is hexagonally shaped and surrounded by dextran or poly(ethyleneglycol) molecules. These crystals possess large magnetic moments when brought into a magnetic field, thus producing a localized disturbance in magnetic field homogeneity, but the magnetic memory is lost when the field is removed. Due to such induced magnetic disturbances, there exist a large susceptibility difference between superparamagnetic iron oxide crystals and the nearby protons, causing rapid dephasing of spins and resultant decrease in T2 relaxation times with a loss of local signal intensity. But the effects of these crystals on T1 relaxation times are relatively minor, compared with the T2 effects. These crystals are therefore “negative enhancers”.

Iron oxide crystals are also amenable to surface functionalization with small surface functional groups or multivalent small molecules as well as by conjugating proteins, antibodies, and oligonucleotides for active-targeting *in vivo* or for *in vitro* diagnostic procedures. Recently a number of small libraries of surface-functionalized iron oxide nanoparticles were synthesized from the parent aminated dextran caged iron oxide nanoparticles. These parent particles were first labeled with fluoresceins, thus generating particles that are both magnetic and fluorescent, then activated with N-succinimidyl 3-(2-pyridyldithio)propionate, and reacted with thiol-containing surface modifiers (see Schellenberger *et al.*, below). Fluorochrome attachment allows the screening by a wide range of high-throughput fluorescence-based screening methods as well as FACS.

Further reading:

- Schellenberger, E. A., Reynolds, F., Weissleder, R., and Josephson, L. (2004) Surface-functionalized nanoparticle library yields probes for apoptotic cells. *ChemBioChem* **5**, 275–279.
- See also references (4,5,62).

Quantum dots

These are nano-scale crystalline structures made from a variety of different compounds, such as cadmium selenide, that can transform the colour of light, and have been around since the 1980s. Quantum dots absorb white light and then re-emit it a couple of nanoseconds later at a specific wavelength. By varying the size and composition of quantum dots, the emission wavelength can be tuned from blue to near infrared. For example, 2nm quantum dots luminesce bright green, while 5nm quantum dots luminesce red. Quantum dots have greater flexibility, when compared to other fluorescent materials, and this makes them suitable for use in building nano-scale computing applications where light is used to process information. These structures offer new capabilities for multicolour optical coding in gene expression studies, high throughput screening, and *in vivo* imaging.

Further reading:

- Schmid, G. (Ed.) (2004) Nanoparticles: From Theory to Application. Wiley-VCH Verlag GmbH KGaA & Co., Weinheim.

Dendrimers

These are highly branched macromolecules with controlled near monodisperse three-dimensional architecture emanating from a central core. Polymer growth starts from a central core molecule and growth occurs in an outward direction by a series of polymerisation reactions. Hence, precise control over size can be achieved by the extent of polymerisation, starting from a few nanometers. Cavities in the core structure and folding of the branches create cages and channels. The surface groups of dendrimers are amenable to modification and can be tailored for specific applications. Therapeutic and diagnostic agents are usually attached to surface groups on dendrimers by chemical modification.

Further reading:

Tomalia, D. A., and Fréchet, J. M. J. (2002) Discovery of dendrimers and dendritic polymers: a brief historical perspective. *J. Polym. Sci. Part A: Polym. Chem.* **40**, 2719–2728.
Tomalia, D. A., and Fréchet, J. M. J. (Eds.) (2001) *Dendrimers and Other Dendritic Polymers*. John Wiley & Sons Ltd., West Sussex.
See also references (30–32).

Polymeric micelles

Micelles are formed in solution as aggregates in which the component molecules (e.g., amphiphilic AB-type or ABA-type block copolymers, where A and B are hydrophobic and hydrophilic components, respectively) are generally arranged in a spheroidal structure with hydrophobic cores shielded from the water by a mantle of hydrophilic groups. These dynamic systems, which are usually below 50 nm in diameter, are used for the systemic delivery of water-insoluble drugs. Drugs or contrast agents may be trapped physically within the hydrophobic cores or can be linked covalently to component molecules of the micelle. See references (25, 27–29,114).

Liposomes

These are closed vesicles that form on hydration of dry phospholipids above their transition temperature. Liposomes are classified into three basic types based on their size and number of bilayers. Multilamellar vesicles consist of several lipid bilayers separated from one another by aqueous spaces. These entities are heterogeneous in size, often ranging from a few hundreds to thousands of nanometers in diameter. On the other hand, both small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) consist of a single bilayer surrounding the entrapped aqueous space. SUVs are less than 100 nm in size whereas LUVs have diameters larger than 100 nm. Drug molecules can be either entrapped in the aqueous space or intercalated into the lipid bilayer of liposomes, depending on the physicochemical characteristics of the drug. The liposome surface is amenable to modification with targeting ligands and polymers.

Further reading:

New, R. R. C., Torchilin, V. P., and Weissig, V. (Eds.) (2003) *Liposomes (Practical Approach)*. Oxford University Press.
Lasic, D. D., and Papahadjopoulos, D. (Eds.) (1998) *Medical Application of Liposomes*. Elsevier Science B. V., Amsterdam.
See also references (23) and (24).

Nanospheres

These are spherical objects, ranging from tens to hundreds of nanometers in size, consisting of synthetic or natural polymers (collagen, albumin). The drug of interest is dissolved, entrapped, attached or encapsulated throughout or within the polymeric matrix. Depending on the method of preparation, the release characteristic of the incorporated drug can be controlled. As with liposomes, technology also allows precision surface modification of nanospheres with polymeric and biological materials for specific applications or targeting to the desired locations in the body. See also Figs. 1 & 2.

Further reading:

Ranade, V. V., and Hollinger, M. A. (Eds.) *Drug Delivery Systems*. CRC Press, Boca Raton, FL, 2nd edition.
See also references (24–26).

Aquasomes (carbohydrate-ceramic nanoparticles)

These are spherical 60–300 nm particles used for drug and antigen delivery. The particle core is composed of nanocrystalline calcium phosphate or ceramic diamond, and is covered by a polyhydroxyl oligomeric film. Drugs and antigens are then adsorbed on to the surface of these particles. See reference (33).

Polyplexes/Lipopolyplexes

These are assemblies, which form spontaneously between nucleic acids and polycations or cationic liposomes (or polycations conjugated to targeting ligands or hydrophilic polymers), and are used in transfection protocols. The shape, size distribution, and transfection capability of these complexes depends on their composition and charge ratio of nucleic acid to that of cationic lipid/polymer. Examples of polycations that have been used in gene transfer/therapy protocols include poly-L-lysine, linear- and branched-poly(ethylenimine), poly(amidoamine), poly- β -amino esters, and cationic cyclodextrin.

Further reading:

Curiel, D. T., and Douglas, J. T. (Eds.) (2002) Vector Targeting for Therapeutic Gene Delivery. Wiley-Liss Inc., Hoboken, New Jersey.
 Findeis, M. A. (Ed.) (2001) Nonviral Vectors for Gene Therapy. *Method. Mol. Med.* Vol. 65, Humana Press, Totowa, New Jersey.
 Kabanov, A. V., Felgner, P. L., and Seymour, L. W. (Eds.) (1998) Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial. John Wiley & Sons Ltd., Chichester, West Sussex.

REFERENCES

- Poole, C. P. (ed.) (2003) Introduction to Nanotechnology. John Wiley & Sons, Hoboken, New Jersey.
- Tyagi, S., and Kramer, F. R. (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.* **14**, 303–308
- Taton, T. A., Lu, G., and Mirkin, C. A. (2001) Two-color labeling of oligonucleotides array via size-selective scattering of nanoparticle probes. *J. Am. Chem. Soc.* **123**, 5164–5165
- Jaffer, F. A., and Weissleder, R. (2004) Seeing within—molecular imaging of the cardiovascular system. *Circ. Res.* **94**, 433–445
- Perez, J. M., Josephson, L., and Weissleder, R. (2004) Use of magnetic nanoparticles as nanosensors to probe for molecular interactions. *ChemBioChem* **5**, 261–264
- Saleh, A., Schroeter, M., Jinkmanns, C., Hartung, H. P., Modder, U., and Jander, S. (2004) In vivo MRI of brain inflammation in human ischaemic stroke. *Brain* **127**, 1670–1677
- Dousset, V., Ballarino, L., Delalande, C., Coussemacq, M., Canioni, P., Petry, K. G., and Caille, J. M. (1999) Comparison of ultrasmall particles of iron oxide (USIOP)-enhanced T2-weighted, conventional T2-weighted, and gadolinium-enhanced T1-weighted MR images in rats with experimental autoimmune encephalomyelitis. *Am. J. Neuroradiol.* **20**, 223–227
- Harisinghani, M. G., Barentsz, J., Hahn, P. F., Deserno, W. M., Tabatabaei, S., Hulsbergen van de Kaa, C., de la Rosette, J., and Weissleder, R. (2003) Noninvasive detection of clinically occult lymph-node metastases in prostate cancer. *N. Engl. J. Med.* **348**, 2491–2499
- Dardzinski, B. J., Schmithorst, V. J., Holland, S. K., Boivin, G. P., Imagawa, T., Watanabe, S., Lewis, J. M., and Hirsch, R. (2001) MR imaging of murine arthritis using ultrasmall superparamagnetic iron oxide particles. *Magn. Res. Imaging* **19**, 1209–1216
- Ruehm, S. G., Corot, C., Vogt, P., Kolb, S., and Debatin, J. F. (2001) Magnetic resonance imaging of atherosclerotic plaque with ultrasmall superparamagnetic particles of iron oxide in hyperlipidemic rabbits. *Circulation* **103**, 415–422
- Kooi, M. E., Cappendijk, V. C., Cleutjens, K. B. J. M., Kessels, A. G. H., Kitslaar, P. J. E. H. M., Borgers, M., Frederik, P. M., Daemen, M. J. A. P., and van Engelshoven, J. M. A. (2003) Accumulation of ultrasmall superparamagnetic particles of iron oxide in human atherosclerotic plaques can be detected by in vivo magnetic resonance imaging. *Circulation* **107**, 2453–2458
- Watson, A., Wu, X., and Bruchez, M. (2003) Lighting up cells with quantum dots. *Biotechniques* **34**, 296–303
- Chan, W. C., Maxwell, D. J., Gao, X., Bailey, R. E., Han, M., and Nie, S. (2002) Luminescent quantum dots for multiplexed biological detection and imaging. *Curr. Opin. Biotechnol.* **13**, 40–46
- Jaiswal, J. K., Mattoussi, H., Mauro, J. M., and Simon, S. M. (2003) Long-term multiple color imaging of live cells using quantum dot bioconjugates. *Nat. Biotechnol.* **21**, 47–51
- Larson, D. R., Zipfel, W. R., Williams, R. M., Clark, S. W., Bruchez, M. P., Wise, F. W., and Webb, W. W. (2003) Water-soluble quantum dots for multiphoton fluorescence imaging in vivo. *Science* **300**, 1434–1436
- Dubertret, B., Skourides, P., Norris, D. J., Noireaux, V., Brivanlou, A. H., and Libchaber, A. (2002) In vivo imaging of quantum dots encapsulated in phospholipid micelles. *Science* **298**, 1759–1762
- Ballou, B., Lagerholm, B. C., Ernst, L. A., Bruchez, M. P., and Waggoner, A. S. (2004) Noninvasive imaging of quantum dots in mice. *Bioconjugate Chem.* **15**, 79–86
- Gao, X., Cui, Y., Levenson, R. M., Chung, L. W. K., and Nie, S. (2004) In vivo cancer targeting and imaging with semiconductor quantum dots. *Nat. Biotechnol.* **22**, 969–976
- Akerman, M. E., Chan, W. C. W., Laakkonen, P., Bhatia, S. N., and Ruoslahti, E. (2002) Nanocrystal targeting in vivo. *Proc. Natl. Acad. Sci. USA* **99**, 12617–12621
- Voura, E. B., Jaiswal, J. K., Mattoussi, H., and Simon, S. M. (2004) Tracking metastatic tumor cell extravasation with quantum dot nanocrystals and fluorescence emission-scanning microscopy. *Nat. Med.* **10**, 993–998
- Dahan, M., Levi, S., Luccardini, C., Rostaing, P., Riveau, B., and Triller, A. (2003) Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. *Science* **302**, 442–445
- Wu, X., Liu, H., Liu, J., Haley, K. N., Treadway, J. A., Larson, J. P., Ge, N., Peale, F., and Bruchez, M. P. (2003) Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. *Nat. Biotechnol.* **21**, 41–46
- Allen, T. M., and Cullis, P. R. (2004) Drug delivery systems: entering the mainstream. *Science* **303**, 1818–1822
- Moghimi, S. M., Hunter, A. C., and Murray, J. C. (2001) Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol. Rev.* **53**, 283–318
- Sahoo, S. K., and Labhasetwar, V. (2003) Nanotech approaches to drug delivery and imaging. *Drug Discov. Today* **8**, 1112–1120
- Panyam, J., and Labhasetwar, V. (2004) Biodegradable nanoparticles for drug and gene delivery to cells and tissues. *Adv. Drug Deliv. Rev.* **55**, 329–347
- Oh, K. T., Bronich, T. K., and Kabanov, A. V. (2004) Micellar formulations for drug delivery based on mixtures of hydrophobic and hydrophilic Pluronic® block copolymers. *J. Control Release* **94**, 411–422
- Adams, M. L., Lavasanifar, A., and Kwon, G. S. (2003) Amphiphilic block copolymers for drug delivery. *J. Pharm. Sci.* **92**, 1343–1355
- Gao, Z. G., Lukyanov, A. N., Singhal, A., and Torchilin, V. P. (2002) Diacyllipid-polymer micelles as nanocarriers for poorly soluble anticancer drugs. *Nano Lett.* **2**, 979–982
- Morgan, M. T., Carnahan, M. A., Immoos, C. E., Ribeiro, A. A., Finkelstein, S., Lee, S. J., and Grinstaff, M. W. (2003) Dendritic molecular capsules for hydrophobic compounds. *J. Am. Chem. Soc.* **125**, 15485–15489
- Krämer, M., Stumbé, J. F., Grimm, G., Kaufmann, B., Krüger, U., Weber, M., and Haag, R. (2004) Dendritic polyamines:

- simple access to new materials with defined treelike structures for application in nonviral gene delivery. *ChemBioChem* **5**, 1081–1087
32. Haag, R. (2004) Supramolecular drug-delivery systems based on polymeric core-shell architectures. *Angew. Chem. Int. Ed. Engl.* **43**, 278–282
33. Kossovsky, N., Gelman, A., Rajguru, S., Nguyen, R., Sponsler, E., Hnatyszyn, H. J., Chow, K., Chung, A., Torres, M., Zemanovich, J., et al. (1996) Control of molecular polymorphisms by a structured carbohydrate/ceramic delivery vehicle – aquasomes. *J. Control Release* **39**, 383–388
34. Schmidt-Wolf, G. D., and Schmidt-Wolf, I. G. H. (2003) Non-viral and hybrid vectors in human gene therapy: an update. *Trends Mol. Med.* **9**, 67–72
35. Raja, K. S., Wang, Q., Gonzalez, M. J., Manchester, M., Johnson, J. E., and Finn, M. G. (2003) Hybrid virus-polymer materials. I. Synthesis and properties of PEG-decorated cowpea mosaic virus. *Biomacromolecules* **4**, 472–476
36. Fenske, D. B., MacLachlan, I., and Cullis, P. R. (2001) Long-circulating vectors for the systemic delivery of genes. *Curr. Opin. Mol. Therap.* **3**, 153–158
37. Verderone, G., van Craynest, N., Boussif, O., Santaella, C., Bischoff, R., Kolbe, H. V. J., and Vierling, P. (2000) Lipopoly-cationic telomers for gene transfer: synthesis and evaluation of their in vitro transfection efficiency. *J. Med. Chem.* **43**, 1367–1379
38. Rabinow, B. E. (2004) Nanosuspensions in drug delivery. *Nat. Rev. Drug Discov.* **3**, 785–796
39. Liversidge, G. G., and Cundy, K. C. (1995) Particle size reduction for improvement of oral bioavailability of hydrophobic drugs. I. Absolute oral bioavailability of nanocrystalline danazol in beagle dogs. *Int. J. Pharm.* **125**, 91–97
40. Allen, T. M. (2002) Ligand-targeted therapeutics in anticancer therapy. *Nat. Rev. Cancer* **2**, 750–763
41. Sudimack, J., and Lee, R. J. (2000) Targeted drug delivery via the folate receptor. *Adv. Drug Deliv. Rev.* **41**, 147–162
42. Torchillin, V. P., Lukyanov, A. N., Gao, Z. G., and Papahadjopoulos-Sternberg, B. (2003) Immunomicelles: targeted pharmaceutical carriers for poorly soluble drugs. *Proc. Natl. Acad. Sci. USA* **100**, 6039–6044
43. Drummond, D. C., Zignani, M., and Leroux, J. C. (2000) Current status of pH-sensitive liposomes in drug delivery. *Prog. Lipid Res.* **39**, 409–460
44. Panyam, J., Zhou, W. Z., Prabha, S., Sahoo, S. K., and Labhasetwar, V. (2002) Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. *FASEB J.* **16**, 1217–1226
45. Clark, H. A., Hoyer, M., Philbert, M. A., and Kopeiman, R. (1999) Optical nanosensors for chemical analysis inside single living cells. I. Fabrication, characterization, and methods for intracellular delivery of PEBBLE sensors. *Anal. Chem.* **71**, 4831–4836
46. Quintana, A., Raczka, E., Piehler, L., Lee, I., Myc, A., Majoros, I., Patri, A. K., Thoma, T., Mule, J., and Baker, J. R. (2002) Design and function of a dendrimer-based therapeutic nano-device targeted to tumour cells through the folate receptor. *Pharm. Res.* **19**, 1310–1316
47. Moghimi, S. M., and Szebeni, J. (2003) Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog. Lipid Res.* **42**, 463–478
48. Moghimi, S. M., and Hunter, A. C. (2001) Recognition by macrophages and liver cells of opsonized phospholipid vesicles and phospholipid headgroups. *Pharm. Res.* **18**, 1–8
49. Szebeni, J., Alving, C. R., Savay, S., Barenholz, Y., Prie, A., Danino, D., and Talmon, Y. (2001) Formation of complement-activating particles in aqueous solutions of Taxol: possible role in hypersensitivity reactions. *Int. Immunopharmacol.* **1**, 721–735
50. Kim, S., Lim, Y. T., Soltesz, E. G., De Grand, A. M., Lee, J., Nakayama, A., Parker, J. A., Mihaljevic, T., Laurence, R. G., Dor, D. M., et al. (2004) Near-infrared fluorescent type II quantum dots for sentinel lymph node mapping. *Nat. Biotechnol.* **22**, 93–97
51. Moghimi, S. M. (2002) Chemical camouflage of nanospheres with a poorly reactive surface: towards development of stealth and target-specific nanocarriers. *Biochim. Biophys. Acta* **1590**, 131–139
52. Gbadamosi, J. K., Hunter, A. C., and Moghimi, S. M. (2002) PEGylation of microspheres generates a heterogeneous population of particles with differential surface characteristics and biological performance. *FEBS Lett.* **532**, 338–344
53. Braet, F., Dezanger, R., Baekeland, M., Crabbe, E., van der Smissen, P., and Wisse, E. (1995) Structure and dynamics of the fenestra-associated cytoskeleton of rat-liver sinusoidal endothelial cells. *Hepatology* **21**, 180–189
54. Munn, L. L. (2003) Aberrant vascular architecture in tumours and its importance in drug-based therapies. *Drug Discov. Today* **8**, 396–403
55. St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B., et al. (2000) Genes expressed in human tumor endothelium. *Science* **289**, 1197–1202
56. Arap, W., Kolonin, M. G., Trepel, M., Lahdenranta, J., Cardo-Vila, M., Giordano, R. J., Mintz, P. J., Ardel, P. U., Yao, V. J., Vidal, C. I., et al. (2002) Steps toward mapping the human vasculature by phage display. *Nat. Med.* **8**, 121–127
57. Chi, J. T., Chang, H. Y., Haraldsen, G., Jahnsen, F. L., Troyanskaya, O. G., Chang, D. S., Wang, Z., Rockson, S., van de Rijn, M., Botstein, D., et al. (2003) Endothelial cell diversity revealed by global expression profiling. *Proc. Natl. Acad. Sci. USA* **100**, 10623–10628
58. Pasqualini, R., Arap, W., and McDonald, D. M. (2002) Probing the structural and molecular diversity of tumour vasculature. *Trend. Mol. Med.* **8**, 563–571
59. Oh, P., Li, Y., Yu, J., Durr, E., Krasinka, K. M., Carver, L. A., Testa, J. E., and Schnitzer, J. E. (2004) Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature (London)* **429**, 629–635
60. Ruoslahti, E. (2002) Drug targeting to specific vascular sites. *Drug Discov. Today* **7**, 1138–1143
61. Murray, J. C., and Moghimi, S. M. (2003) Endothelial cells as therapeutic targets in cancer: new biology and novel delivery systems. *Crit. Rev. Ther. Drug Carrier Syst.* **20**, 139–152
62. Moghimi, S. M., and Bonnemain, B. (1999) Subcutaneous and intravenous delivery of diagnostic agents to the lymphatic system: applications in lymphoscintigraphy and indirect lymphography. *Adv. Drug Deliv. Rev.* **37**, 295–312
63. Phillips, W. T., Klipper, R., and Goins, B. (2000) Novel method of greatly enhanced delivery of liposomes to lymph nodes. *J. Pharmacol. Exp. Ther.* **295**, 309–313
64. Moghimi, S. M. (2003) Modulation of lymphatic distribution of subcutaneously injected poloxamer 407-coated nanospheres: the effect of the ethylene oxide chain configuration. *FEBS Lett.* **540**, 241–244
65. Veerareddy, P. R., and Vobalabonia, V. (2004) Lipid-based formulas of amphotericin B. *Drugs Today* **40**, 133–145
66. Lavasanifar, A., Samuel, J., Sattari, S., and Kwon, G. S. (2002) Block copolymer micelles for the encapsulation and delivery of amphotericin B. *Pharm. Res.* **19**, 418–422
67. Agrawal, A. K., and Gupta, C. M. (2000) Tuftsin-bearing liposomes in treatment of macrophage-based infections. *Adv. Drug Deliv. Rev.* **41**, 135–146
68. Miller, C. B., Waller, E. K., Klingemann, H. G., Dignani, M. C., Anaisie, E. J., Cagnoni, P. J., McSweeney, P., Fleck, P. R., Fruchtmann, S. M., McGuirk, J., et al. (2004) Lipid formulations of amphotericin B preserve and stabilize renal functions in HSCT recipients. *Bone Marrow Transplant.* **33**, 543–548
69. Shadkhan, Y., Keisari, Y., and Segal, E. (2004) Cytokines in mice treated with amphotericin B-intralipid. *Med. Mycol.* **42**, 123–128
70. Barrera, P., van Lent, P. L. E. M., van Bloois, L., van Rooijen, N., Malefijt, M. C. D., van de Putte, L. B. A., Storm, G., and van den Berg, W. B. (2000) Synovial macrophage depletion with clodronate-containing liposomes in rheumatoid arthritis. *Arthritis Rheumat.* **43**, 1951–1959
71. Liu, T., van Rooijen, N., and Tracy, D. J. (2000) Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury. *Pain* **86**, 25–32
72. Wu, G. S., Korsgren, O., Zhang, J. G., Song, Z. S., van Rooijen, N., and Tibell, A. (2000) Pig islet xenograft rejection is markedly delayed in macrophage-depleted mice: a study in streptozotocin diabetic animals. *Xenotransplantation* **7**, 214–220
73. Danenberg, H. D., Fishbein, I., Epstein, H., Waltenberger, J., Moerman, E., Monkkonen, J., Gao, X. C., Gathi, I., Reich, R.,

- and Golomb, G. (2003) Systemic depletion of macrophages by liposomal bisphosphonates reduces neointimal formation following balloon-injury in rat carotid artery. *J. Cardiovasc. Pharmacol.* **42**, 671–679
74. Wolf, G., Worgell, S., van Rooijen, N., and Crystal, R. G. (1997) Enhancement of in vivo adenovirus-mediated gene transfer and expression by prior depletion of tissue macrophages in the target organ. *J. Virol.* **71**, 624–629
75. Alves-Rosa, F., Stanganelli, C., Cabrera, J., van Rooijen, N., Palermo, M. S., and Isturiz, M. A. (2000) Treatment with liposome-encapsulated clodronate as a new strategic approach in the management of immune thrombocytopenic purpura in a mouse model. *Blood* **96**, 2834–2840
76. Hsu, L.-C., Park, J. M., Zhang, K., Luo, J.-L., Maeda, S., Kaufman, R. J., Eckmann, L., Guiney, D. G., and Karin, M. (2004) The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. *Nature (London)* **428**, 341–345
77. Singh, M., and O'Hagan, D. (1999) Advances in vaccine adjuvants. *Nat. Biotechnol.* **17**, 1075–1081
78. Dileo, J., Banerjee, R., Whitmore, M., Nayak, J., Falo, L., and Huang, L. (2003) LPD mediated antigen delivery to antigen presenting cells results in enhanced anti-tumor immune responses. *Mol. Ther.* **7**, 640–648
79. Mischler, R., and Metcalfe, I. C. (2002) InfleraV a trivalent virosome subunit influenza vaccine: production. *Vaccine* **20**, B17–B23
80. Cui, Z. R., and Mumper, R. J. (2003) Microparticles and nanoparticles as delivery systems for DNA vaccines. *Crit. Rev. Ther. Drug Carrier Syst.* **20**, 103–137
81. Salem, A. K., Searson, P. C., and Leong, K. W. (2003) Multifunctional nanorods for gene delivery. *Nat. Mat.* **2**, 668–671
82. Apostolopoulos, V., Pietersz, G. A., Gordon, S., Martinez-Pomares, L., and McKenzie, I. F. C. (2000) Aldehyde-mannan antigen complexes target the MHC class I antigen-presentation pathway. *Eur. J. Immunol.* **30**, 1714–1723
83. Taylor, P. R., Zamze, S., Stillion, R. J., Wong, S. Y. C., Gordon, S., and Martinez-Pomares, L. (2004) Development of a specific system for targeting protein to metallophilic macrophages. *Proc. Natl. Acad. Sci. USA* **101**, 1963–1968
84. Reid, D. M., Montoya, M., Taylor, P. R., Borrow, P., Gordon, S., Brown, G. D., and Wong, S. Y. C. (2004) Expression of the beta-glucan receptor, dectin-1, on murine leukocytes in situ correlates with its function in pathogen recognition and reveals potential roles in leukocyte interactions. *J. Leukoc. Biol.* **76**, 86–94
85. Figdor, C. G., de Vries, I. J. M., Lesterhuis, W. J., and Melief, C. J. M. (2004) Dendritic cell immunotherapy: mapping the way. *Nat. Med.* **10**, 475–480
86. Gogolak, P., Rehti, B., Hajas, G., and Rajnavolgyi, E. (2003) Targeting dendritic cells for priming cellular immune responses. *J. Mol. Recog.* **16**, 299–317
87. Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M. C., and Steinman, R. M. (2002) Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8⁺ T cell tolerance. *J. Exp. Med.* **196**, 1627–1638
88. Porter, C. J. H., Moghimi, S. M., Illum, L., and Davis, S. S. (1992) The polyoxyethylene/polyoxypropylene block co-polymer poloxamer-407 selectively redirects intravenously injected microspheres to sinusoidal endothelial cells of rabbit bone marrow. *FEBS Lett.* **305**, 62–66
89. Kreuter, J., Ramge, P., Petrov, V., Hamm, S., Gelperina, S. E., Engelhardt, B., Alyautdin, R., von Briesen, H., and Begley, D. J. (2003) Direct evidence that polysorbate-80-coated poly(butyl-cyanoacrylate) nanoparticles deliver drugs to the CNS via specific mechanisms requiring prior binding of drugs to the nanoparticles. *Pharm. Res.* **20**, 409–416
90. Steiniger, S. C. J., Kreuter, J., Khalansky, A. S., Skidan, I. N., Bobruskin, A. I., Smirnova, Z. S., Severin, S. E., Uhi, R., Kock, M., Geiger, K. D., et al. (2004) Chemotherapy of glioblastoma in rats using doxorubicin-loaded nanoparticles. *Int. J. Cancer* **109**, 759–767
91. McLean, J. W., Fox, E. A., Baluk, P., Bolton, P. B., Haskell, A., Pearlman, R., Thurston, G., Umemoto, E. Y., and McDonald, D. M. (1997) Organ-specific endothelial uptake of cationic liposome-DNA complexes in mice. *Am. J. Physiol.* **273**, H387–H404
92. Eliceiri, B. P., and Cheresch, D. A. (1999) The role of alpha v integrins during angiogenesis: insights into potential mechanisms of action and clinical development. *J. Clin. Invest.* **103**, 1227–1230
93. Kim, S., Bell, K., Mousa, S. A., and Varner, J. A. (2000) A regulation of angiogenesis in vivo by ligation of integrin $\alpha 5 \beta 1$ with the central cell-binding domain of fibronectin. *Am. J. Pathol.* **156**, 1345–1362
94. Assa-Munt, N., Jia, X., Laakkonen, P., and Ruoslahti, E. (2001) Solution structures and integrin binding activities of an RGD peptide with two isomers. *Biochemistry* **40**, 2373–2378
95. Arap, W., Pasqualini, R., and Ruoslahti, E. (1998) Cancer treatment by targeted drug delivery to tumour vasculature in a mouse model. *Science* **279**, 377–380
96. Zitzmann, S., Ehemann, V., and Schwab, M. (2002) Arginine-glycine-aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells in vivo. *Cancer Res.* **62**, 5139–5143
97. Wickham, T. J. (2000) Targeting adenovirus. *Gene Ther.* **7**, 110–114
98. Haviv, Y. S., Blackwell, J. L., Kanerva, A., Nagi, P., Krasnykh, V., Dmitriev, I., Wang, M. H., Naito, S., Lei, X. S., Hemminki, A., et al. (2002) Adenoviral gene therapy for renal cancer requires retargeting to alternative cellular receptors. *Cancer Res.* **62**, 4273–4281
99. Turunen, M. P., Puhakka, H. L., Koponen, J. K., Hiltunen, M. O., Rutanen, J., Leppanen, O., Turunen, A. M., Narvanen, A., Newby, A. C., Baker, A. H., et al. (2002) Peptide re-targeted adenovirus encoding a tissue inhibitor of metalloproteinase-1 decreases restenosis after intravascular gene transfer. *Mol. Ther.* **6**, 306–312
100. Curnis, F., Sacchi, A., and Corti, A. (2000) Improving chemotherapeutic drug penetration in tumours by vascular targeting and barrier alteration. *J. Clin. Invest.* **110**, 475–482
101. Laakkonen, P., Porkka, K., Hoffman, A. J. A., and Ruoslahti, E. (2002) Tumor-homing peptide with a targeting specificity related to lymphatic vessel. *Nat. Med.* **8**, 751–755
102. Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., Chadburn, A., Heissig, B., Marks, W., Witte, L., et al. (2001) Impaired recruitment of bone-marrow-derived endothelial and haematopoietic precursor cells blocks tumour angiogenesis and growth. *Nat. Med.* **7**, 1194–1201
103. Hood, J. D., Bednarski, M., Frausto, R., Guccione, S., Reisfeld, R. A., Xiang, R., and Cheresch, D. A. (2002) Tumor regression by targeted gene delivery to the neovasculature. *Science* **296**, 2404–2407
104. Winter, P. M., Caruthers, S. D., Kassner, A., Harris, T. D., Chinen, L. K., Allen, J. S., Lacy, E. K., Zhang, H. Y., Robertson, J. D., Wickline, S. A., et al. (2003) Molecular imaging of angiogenesis in nascent vx-2 rabbit tumours using a novel alpha(v)beta(3)-targeted nanoparticle and 1.5 tesla magnetic resonance imaging. *Cancer Res.* **63**, 5838–5843
105. Pastorino, F., Brignole, C., Marimpietri, D., Cilli, M., Gambini, C., Ribatti, D., Longhi, R., Allen, T. M., Corti, A., and Ponzoni, M. (2003) Vascular damage and anti-angiogenic effects of tumor vessel-targeted liposomal chemotherapy. *Cancer Res.* **63**, 7400–7409
106. Muro, S., Cui, X., Gajewski, C., Murciano, J.-C., Muzykantov, V. R., and Koval, M. (2003) Slow intracellular trafficking of catalase nanoparticles targeted to ICAM-1 protects endothelial cells from oxidative stress. *Am. J. Cell Physiol.* **285**, C1339–C1347
107. Muzykantov, V. R., Christofidou-Solomidou, M., Balyasnikova, I. V., Harshaw, D., Schultz, L., Fisher, A. B., and Albelda, S. M. (1999) Streptavidin facilitates internalization and pulmonary targeting of an anti-endothelial cell antibody (platelet-endothelial cell adhesion molecule-1): a strategy for vascular immunotargeting of drugs. *Proc. Natl. Acad. Sci. USA* **96**, 2379–2384
108. Christofidou-Solomidou, M., Pietra, G. G., Solomides, C. C., Arguiri, E., Harshaw, D., Fitzgerald, G. A., Albelda, S. M., and Muzykantov, V. R. (2000) Immunotargeting of glucose oxidase to endothelium in vivo causes oxidative vascular injury in the lungs. *Am. J. Physiol.* **278**, L794–L805
109. Scherpereel, A., Wiewrodt, R., Christofidou-Solomidou, M., Gervais, R., Murciano, J. C., Albelda, S. M., and Muzykantov, V. R. (2001) Cell-selective intracellular delivery of a foreign

- enzyme to endothelium in vivo using vascular immunotargeting. *FASEB J.* **15**, 416–426
110. Ran, S., Gao, B., Duffy, S., Watkins, L., Rote, N., and Thorpe, P. E. (1998) Infarction of solid Hodgkin's tumors in mice by antibody-directed targeting of tissue factor to tumour vasculature. *Cancer Res.* **58**, 4646–4653
111. Drummond, D. C., Meyer, O., Hong, K., Kirpotin, D. B., and Papahadjopoulos, D. (1999) Optimizing liposomes for delivery of chemotherapeutic agents to solid tumours. *Pharmacol. Rev.* **51**, 691–743
112. Uwatoku, T., Shimokawa, H., Abe, K., Matsumoto, Y., Hattori, T., Oi, K., Matsuda, T., Kataoka, K., and Takeshita, A. (2003) Application of nanoparticle technology for the prevention of restenosis after balloon injury in rats. *Circ. Res.* **92**, E62–E69
113. Metselaar, J. M., van der Berg, W. B., Holthuysen, A. E. M., Wauben, M. H. M., Storm, G., and van Lent, P. L. (2004) Liposomal targeting of glucocorticoids to synovial lining cells strongly increases therapeutic benefit in collagen type II arthritis. *Ann. Rheum. Dis.* **63**, 348–353
114. Lukyanov, A. N., Gao, Z. G., and Torchilin, V. P. (2003) Micelles from polyethylene glycol/phosphatidylethanolamine conjugates for tumor drug delivery. *J. Control Release* **91**, 97–102
115. Jain, R. K. (2001) Delivery of molecular medicine to solid tumors: lessons from in vivo imaging of gene expression and function. *J. Control Release* **74**, 7–25
116. Padera, T. P., Stoll, B. R., Tooredman, J. B., Capen, D., di Tomaso, E., and Jain, R. K. (2004) Cancer cells compress intratumour vessels. *Nature (London)* **427**, 695
117. Pluen, A., Boucher, Y., Ramanujan, S., McKee, T. D., Gohongi, T., di Tomaso, E., Brown, E. B., Izumi, Y., Campbell, R. B., Berk, D. A., et al. (2001) Role of tumor-host interactions in interstitial diffusion of macromolecules: cranial vs. subcutaneous tumors. *Proc. Natl. Acad. Sci. USA* **98**, 4628–4633
118. Gabizon, A., Shmeeda, H., and Barenholz, Y. (2003) Pharmacokinetics of pegylated liposomal doxorubicin—review of animal and human studies. *Clin. Pharmacokinetics* **42**, 419–436
119. Barenholz, Y. (2001) Liposome application: problems and prospects. *Curr. Opin. Colloid Interface Sci.* **6**, 66–77
120. Banadak, S., Goren, D., Horowitz, A., Tzemach, D., and Gabizon, A. (1999) Pharmacological studies of cisplatin encapsulated in long-circulating liposomes in mouse tumor models. *Anticancer Drugs* **10**, 911–920
121. Davidson, J., Jørgensen, K., Andresen, T. L., and Mouritsen, O. G. (2003) Secreted phospholipase A₂ as a new enzymatic trigger mechanism for localised liposomal drug release and absorption in diseased tissue. *Biochim. Biophys. Acta* **1609**, 95–101
122. Jørgensen, K., Davidsen, J., and Mouritsen, O. G. (2002) Biophysical mechanisms of phospholipase A₂ activation and their use in liposome-based drug delivery. *FEBS Lett.* **531**, 23–27
123. Davidson, J., Mouritsen, O. G., and Jørgensen, K. (2002) Synergistic permeability enhancing effect of lysophospholipids and fatty acids on lipid membranes. *Biochim. Biophys. Acta* **1564**, 256–262
124. Andresen, T. L., Davidsen, J., Begtrup, M., Mouritsen, O. G., and Jørgensen, K. (2004) Enzymatic release of antitumor ether lipids by specific phospholipase A₂ activation of liposome-forming prodrugs. *J. Med. Chem.* **47**, 1694–1703
125. McIntyre, J. O., Fingleton, B., Wells, K. S., Piston, D. W., Lynch, C. C., Gautam, S., and Matrisian, L. M. (2004) Development of a novel fluorogenic proteolytic beacon for in vivo detection and imaging of tumour-associated matrix metalloproteinase-7 activity. *Biochem. J.* **377**, 617–628
126. Sapra, P., and Allen, T. M. (2002) Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs. *Cancer Res.* **62**, 7190–7194
127. Goren, D., Horwitz, A. T., Tzemach, D., Tarshish, M., Zalipsky, S., and Gabizon, A. (2000) Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump. *Clin. Cancer Res.* **6**, 1949–1957
128. Mann, D. A., and Frankel, A. D. (1991) Endocytosis and targeting of exogenous HIV-1 Tat protein. *EMBO J.* **10**, 1733–1739
129. Torchilin, V. P., Levchenko, T. S., Rammohan, R., Volodina, N., Papahadjopoulos-Sternberg, B., and D'Souza, G. G. M. (2003) Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposome-DNA complexes. *Proc. Natl. Acad. Sci. USA* **100**, 1972–1977
130. Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V., and Lebleu, B. (2003) Cell-penetrating peptides—a reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* **278**, 585–590
131. Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* **10**, 310–315
132. Panyam, J., and Labhasetwar, V. (2003) Sustained cytoplasmic delivery of drugs with intracellular receptors using biodegradable nanoparticles. *Mol. Pharmaceut.* **1**, 77–84
133. Hirko, A., Tang, F., and Hughes, J. A. (2003) Cationic lipid vectors for plasmid DNA delivery. *Curr. Med. Chem.* **10**, 1185–1193
134. Kichler, A. (2004) Gene transfer with modified polyethylenimines. *J. Gene Med.* **6**, S3–S10
135. Haining, W. N., Anderson, D. G., Little, S. R., von Berwelt-Baildon, M. S., Cardoso, A. A., Alves, P., Kosmatopoulos, K., Nadler, L. M., Langer, R., and Kohane, D. S. (2004) pH-triggered microparticles for peptide vaccination. *J. Immunol.* **173**, 2578–2585
136. Mandal, M., Kawamura, K. S., Wherry, E. J., Ahmed, R., and Lee, K.-D. (2004) Cytosolic delivery of viral nucleoprotein by listeriolysin O-liposomes induces enhanced specific cytotoxic T lymphocyte response and protective immunity. *Mol. Pharmaceut.* **1**, 2–8
137. Colvin, V. L. (2003) The potential environmental impact of engineered nanomaterials. *Nat. Biotechnol.* **21**, 1166–1170
138. Hunter, A. C., and Moghimi, S. M. (2002) Therapeutic synthetic polymers: a game of Russian roulette? *Drug Discov. Today* **7**, 998–1001
139. Shvedova, A. A., Castranova, V., Kisin, E. R., Schwegler-Berry, D., Murray, A. R., Gandelsman, V. Z., Maynard, A., and Baron, P. (2003) Exposure to carbon nanotube material: assessment of nanotube cytotoxicity using human keratinocyte cells. *J. Toxicol. Environ. Health* **66**, 1909–1926
140. Derfus, A. M., Chan, W. C. W., and Bhatia, S. N. (2004) Probing the cytotoxicity of semiconductor quantum dots. *Nano Lett.* **4**, 11–18
141. Savic, R., Luo, L. B., Eisenberg, A., and Maysinger, D. (2003) Nanocontainers distribute to defined cytoplasmic organelles. *Science* **300**, 615–618
142. Moghimi, S. M., Hunter, A. C., Murray, J. C., and Szwedczyk, A. (2004) Cellular distribution of nonionic micelles. *Science* **303**, 626–627
143. Nishiyama, N., Koizumi, F., Okazaki, S., Matsumura, Y., Nishio, K., and Kataoka, K. (2003) Differential gene expression profile between PC-14 cells treated with free cisplatin and cisplatin-incorporated polymeric micelles. *Bioconjugate Chem.* **14**, 449–457
144. Lam, K. H., Schakenraad, J. M., Esselbrugge, H., Feijen, J., and Nieuwenhuis, P. (1993) The effect of phagocytosis of poly(L-lactic acid) fragments on cellular morphology and viability. *J. Biomed. Mat. Res.* **27**, 1569–1577
145. Batrakova, E. V., Li, S., Alakhov, V. Y., Miller, D. W., and Kabanov, A. V. (2003) Optimal structure requirements for pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. *J. Pharmacol. Exp. Ther.* **304**, 845–854
146. King, M., Su, W., Chang, A., Zukerman, A., and Pasternack, G. W. (2001) Transport of opioids from the brain to the periphery by P-glycoprotein: peripheral actions of central drugs. *Nat. Neurosci.* **4**, 268–274
147. Kabanov, A. V., Batrakova, E. V., and Alakhov, V. Y. (2003) An essential relationship between ATP depletion and chemosensitizing activity of Pluronic block copolymers. *J. Control Release* **91**, 75–83
148. Langer, R., and Tirrell, D. A. (2004) Designing materials for biology and medicine. *Nature (London)* **428**, 487–492
149. Silva, G. A., Czeisler, C., Niece, K. L., Beniash, E., Harrington, D. A., Kessler, J. A., and Stupp, S. I. (2004) Selective differentiation of neural progenitor cells by high-epitope density nanofibers. *Science* **303**, 1352–1355

150. Anderson, D. G., Levenberg, S., and Langer, R. (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. *Nat. Biotechnol.* **22**, 863–866
151. Somia, N., and Verma, I. M. (2000) Gene-therapy: trials and tribulations. *Nat. Rev. Genet.* **1**, 91–99
152. Cavazzano-Calvo, M., Thrasher, A., and Mavilio, F. (2004) The future of gene therapy. *Nature (London)* **427**, 779–781
153. Jones, R. A., Poniris, M. H., and Wilson, M. R. (2004) PD-MAEMA is internalised by endocytosis but does not physically disrupt endosomes. *J. Control Release* **96**, 379–391
154. Murray, J. C., Moghimi, S. M., Hunter, A. C., Symonds, P., Debska, G., and Szweczyk, A. (2004) Lymphocyte death by cationic polymers: A role for mitochondrion and implications in gene therapy. *Br. J. Cancer* **91**, S75–S75
155. Omid, Y., Hollins, A. J., Benboubetra, M., Drayton, R., Benter, I. F., and Akhtar, S. (2003) Toxicogenomics of non-viral vectors for gene therapy: a microarray study of lipofectin- and oligofectamine-induced gene expression changes in human epithelial cells. *J. Drug Target.* **11**, 311–323
156. Lynn, D. M., Anderson, D. G., Putnam, D., and Langer, R. (2001) Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of degradable polymer library. *J. Am. Chem. Soc.* **123**, 8155–8156
157. Anderson, D., Lynn, D., and Langer, R. (2003) Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery. *Angew. Chem. Int. Ed. Engl.* **42**, 3153–3158
158. Szebeni, J. (2001) Complement activation-related pseudoallergy caused by liposomes, micellar carriers of intravenous drugs, and radiocontrast agents. *Crit. Rev. Ther. Drug Carr. Syst.* **18**, 567–606
159. Moghimi, S. M., Hunter, A. C., Dadswell, C. M., Savay, S., Alving, C. R., and Szebeni, J. (2004) Causative factors behind poloxamer 188 (Pluronic F68, Flocor)-induced complement activation in human sera. A protective role against poloxamer-mediated complement activation by elevated serum lipoprotein levels. *Biochim. Biophys. Acta* **1689**, 103–113
160. Chanan-Khan, A., Szebeni, J., Savay, S., Liebes, L., Rafique, N. M., Alving, C. R., and Muggia, F. M. (2003) Complement activation following first exposure to pegylated liposomal doxorubicin (Doxil): possible role in hypersensitivity reactions. *Ann. Oncol.* **14**, 1430–1437
161. La Van, D. A., Lynn, D. M., and Langer, R. (2002) Moving smaller in drug discovery and delivery. *Nat. Rev. Drug Discov.* **1**, 77–84

Received for publication September 17, 2004.

Accepted for publication November 19, 2004.