Strategies in the design of nanoparticles for therapeutic applications

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Abstract | Engineered nanoparticles have the potential to revolutionize the diagnosis and treatment of many diseases; for example, by allowing the targeted delivery of a drug to particular subsets of cells. However, so far, such nanoparticles have not proved capable of surmounting all of the biological barriers required to achieve this goal. Nevertheless, advances in nanoparticle engineering, as well as advances in understanding the importance of nanoparticle characteristics such as size, shape and surface properties for biological interactions, are creating new opportunities for the development of nanoparticles for therapeutic applications. This Review focuses on recent progress important for the rational design of such nanoparticles and discusses the challenges to realizing the potential of nanoparticles.

Therapeutic index
In the case of the anticancer drug doxorubicin, which displays dose-limiting cardiotoxicity, the therapeutic index is the amount of drug in the tumour compared with the amount of drug in the heart.

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In the past two decades, several therapeutics based on nanoparticles — particles in the size range 1–1,000 nm — have been successfully introduced for the treatment of cancer, pain and infectious diseases[1,2] (TIMELINE). These therapeutics harness the opportunities provided by nanomaterials to target the delivery of drugs more precisely, in order to improve their solubility, to extend their half-life, to improve their therapeutic index and to reduce their immunogenicity[1–4].

The first generation of nanoparticles used for such applications are primarily based on liposomes and polymer–drug conjugates (see BOX 1 for an early history of the field). Liposomes, which are spherical vesicles with a lipid bilayer membrane structure, can encapsulate both hydrophilic and hydrophobic agents, protecting the cargo (for example small molecule drugs, nucleotides, proteins, imaging agents or radionucleotides) during circulation in the body[2,5]. They can also be functionalized, for example, with ligands to cell surface receptors, to promote targeting to specific cells and tissues. In addition, they can be coated with polymers to prolong circulation half-life. The first liposome-based therapeutic, liposome-encapsulated doxorubicin (Doxil; OrthoBiotech), was approved by the US Food and Drug Administration (FDA) in 1995 for the treatment of HIV-related Kaposi's sarcoma, and was subsequently approved for the treatment of ovarian cancer and multiple myeloma. Encapsulating the cytotoxic anticancer drug doxorubicin into a liposome carrier increases its half-life and enhances its deposition in tumours. Furthermore, Doxil has shown significantly reduced cardiotoxicity compared with free doxorubicin[1,5–8]. Several other liposome-based therapeutics have been approved by the FDA for indications including fungal infections, for example, liposomal amphotericin B (AmBisome; Gilead) and postsurgical analgesia, for example, liposomal morphine (DepoDur; Pacira Pharmaceuticals).

Polymer–drug conjugates have also been extensively investigated, and several have received regulatory approval[1,2,9–11]. Polyethylene glycol (PEG), which can enhance the solubility and plasma stability of proteins, and reduce immunogenicity, has been the most widely studied polymer so far for this application. In 1994, PEG–L-asparaginase (Oncospar; Enzon) became the first such nanoparticle therapeutic to receive FDA approval, for the treatment of acute lymphocytic leukaemia[1,9]. Other examples of marketed PEGylated therapeutics include PEG–interferon-α2a (Pegasys; Roche) and PEG–interferon-α2b (Pegintron; Schering–Plough) for the treatment of hepatitis C, and PEG–granulocyte colony-stimulating factor (Neulasta; Amgen) for the treatment of neutropaenia.

Liposomes and polymer–drug conjugates have provided the foundations for the field of advanced drug delivery based on nanotechnology, but several key barriers remain. These barriers include elucidating the

underlying mechanisms for the molecular interaction of nanoparticles with cells and tissues based on surface chemistry and shape; and developing more efficient methods for the encapsulation of cargo coupled with activated release. Also, improvements in the cellular and the subcellular targeting of the nanoparticle are needed; and for oncology applications, addressing nanoparticle extravasation through extremely complex, heterogeneous tumour microenvironments are neeeded too[12–14]. Now, the development of the next generation of nanoparticle therapeutics — based on polymeric nanoparticles that combine the pre-eminent features of traditional delivery vectors, but yet offer new flexibility to overcome some of the key barriers in the field — is gaining momentum (FIG. 1). This Review focuses on aspects central to the rational design of polymer-based nanoparticles that are capable of having multiple functions, and discusses the impact of size, shape and composition on nanoparticle biodistribution and intended function.

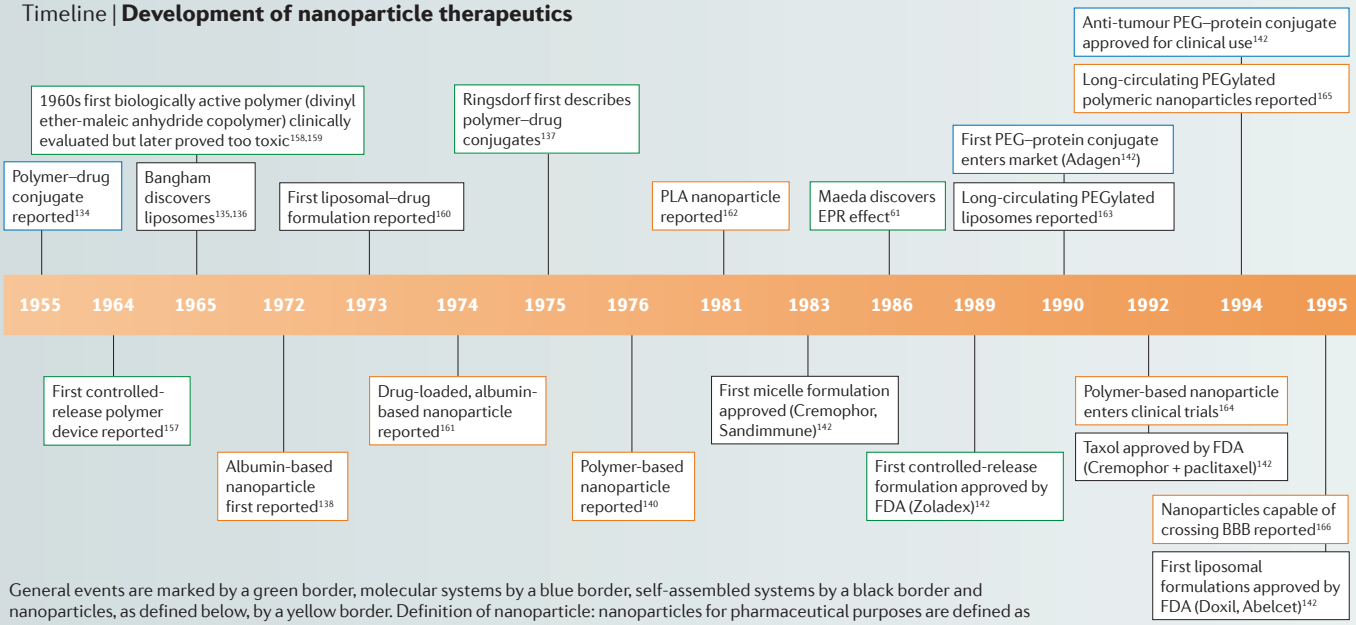
General nanoparticle characteristics

The size, surface characteristics and shape of a nanoparticle has a key role in its biodistribution *in vivo*. The effects of size have been studied extensively with spherically shaped particles and some general trends have been noted[15–19]. Particles less than 5 nm are rapidly cleared from the circulation through extravasation or renal clearance[17,20], and as particle size increases from the nanometre range to ~15 μm, accumulation occurs primarily in the liver, the spleen and the bone marrow[21–23]. Nanoparticle behaviour in the size range ~10 nm to ~15 μm varies widely in terms of biodistribution, and cellular uptake of nanoparticles in this range is heavily dependent on cell type[24,25]. Under normal circumstances, mechanical filtration by sinusoids in the spleen traps the nanoparticles, followed by their removal from the circulation by cells of the reticuloendothelial system (RES)[26]. In addition, Kupffer cells in the liver, which are also part of the RES, have a key role in nanoparticle removal[26].

The propensity for accumulation of nanoparticles in cells of the RES is dictated by specific proteins that are adsorbed *in vivo* to the particle surface[23,27–30], which can be influenced through modifications of surface characteristics[31,32]. This process of protein adsorption, known as opsonization, begins immediately after the nanoparticles come in contact with plasma. The exact nature of the types and quantities of proteins, and their conformations, dictate the body's reaction. The mechanisms involved are not well understood; however, the main opsonins are known. Immunoglobulin and complement proteins are the predominant contributors to the recognition of foreign particles by the cells of the RES (that is, macrophages). Complement activation can complicate targeted drug delivery by inducing hypersensitivity reactions[33,34]. Finally, particulate matter larger than ~15 μm is removed from the circulation by mechanical filtration in capillaries[21] and can be lethal depending on the dose.

Current methods for addressing the negative attributes associated with opsonization have focused almost exclusively on slowing the process by rendering the particle surface more hydrophilic or by neutralizing

Timeline | **Development of nanoparticle therapeutics**



General events are marked by a green border, molecular systems by a blue border, self-assembled systems by a black border and nanoparticles, as defined below, by a yellow border. Definition of nanoparticle: nanoparticles for pharmaceutical purposes are defined as solid colloidal particles ranging in size from 1 nm to 1,000 nm. They consist of macromolecular materials and can be used therapeutically as drug carriers, in which the active principle (drug or biologically active material) is dissolved, entrapped or encapsulated, or to which the active principle is adsorbed or attached. Abraxane, paclitaxel protein-bound particles for injectable suspension (Abraxis/AstraZeneca); Adagen, PEG–adenosine deaminase (Enzon); BBB, blood–brain barrier; Copaxone, glatiramer acetate for injection (Teva Pharmaceuticals); Cremophor, polyoxyethylated castor oil (BASF); EPR, enhanced permeability and retention; FDA, US Food and Drug Administration; Gliadel, polifeprosan 20 with carmustine implant (Eisai); PEG, polyethylene glycol; PLA, polylactic acid; Sandimmune, cyclosporine injection (Novartis); Zoladex, goserelin acetate implant (AstraZeneca).

the particle's surface charge. The predominant strategy has been to adsorb or graft a hydrophilic polymeric coating, such as PEG, to the surface of the particle[15,35–37]. These polymer chains, depending on their density, act as a steric brush that imparts resistance to protein adsorption. However, the PEG effect is transient, so eventual opsonization and macrophage clearance still occurs. For a review on PEGylation see REF. 38.

Although studies have shown the positive effects that can be achieved by dictating which proteins adsorb to the surface of nanoparticles[39–41], methods that have been used in the design of potential nanoparticle therapeutics so far are limited in scope. In one study, liposomes that had albumin covalently bound to the surface *ex vivo*, displayed extended circulation times compared to their naked or PEGylated counterparts[40]. The authors attributed this behaviour to reduced opsonin binding on exposure to plasma[40]. In a separate but related study, the same authors observed improved pharmacokinetic and pharmacodynamic properties of albumin-coated liposomes containing doxorubicin compared to PEGylated liposomes[39]. Comparing albumin-coated liposomes to their PEGylated equivalents, they observed decreased accumulation in the liver, the spleen and the heart, increased accumulation in the tumour and an overall greater than twofold increase in the therapeutic index of the drug, which displays dose-limiting cardiotoxicity.

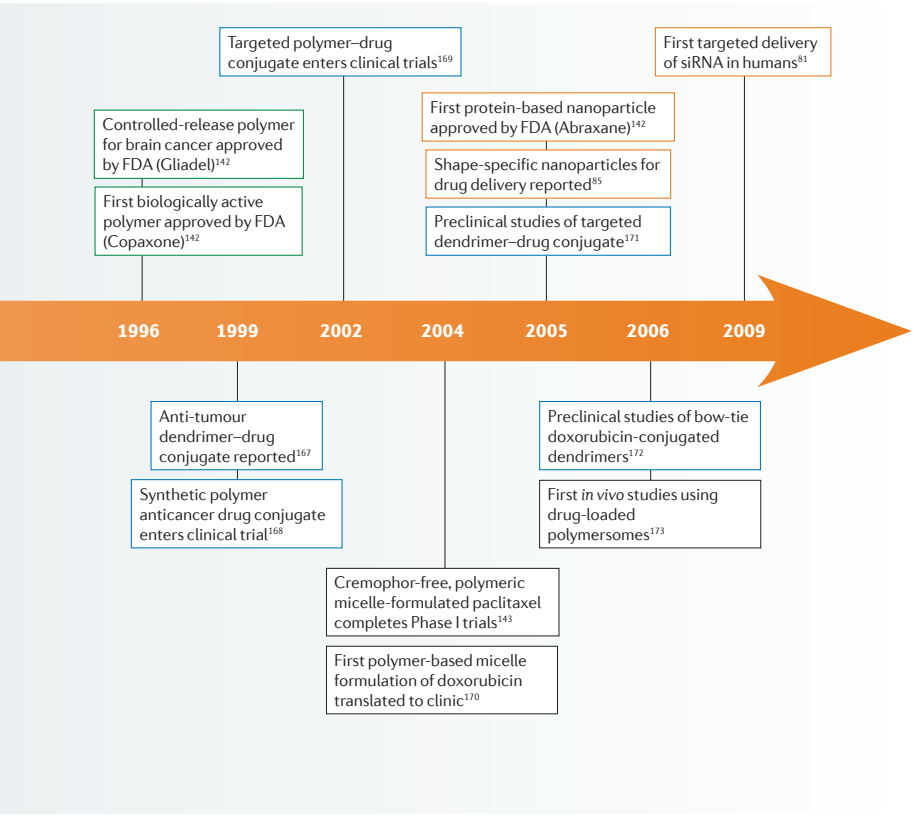
Particle size is also known to influence the mechanism of cellular internalization[42–44] — that is, phagocytosis, macropinocytosis, caveolar-mediated endocytosis or clathrin-mediated endocytosis (FIG. 2) — which in turn dictates the microenvironments that an engineered nanoparticle experiences on internalization. Detailed knowledge of the mode of entry into the cell is invaluable as this information could be used to design an engineered nanoparticle that is targeted to specific intracellular microenvironments, as discussed in more detail later.

As noted above, the impact of size on biodistribution and cellular internalization has largely been elucidated using spherically shaped particles. However, current findings[24,45–54] indicate that particle shape is just as important, if not more so, than size in controlling key aspects of both these phenomena. For example, a recent report investigated the effect of particle size and shape on the rate of particle internalization in HeLa cells using non-spherical particles[50]. A clear correlation between the shape and size of the particles on the rate of internalization was observed. Furthermore, it was shown that particles with similar volumes but different shapes were internalized at vastly different rates. It was also shown recently that the geometry of interaction between a cell and a particle can induce or inhibit internalization[51], and that shape has a significant impact on biodistribution[48]. Filamentous engineered nanoparticles that have single dimensions as long as 18 μm exhibit circulation half-lives of ~5 days, which is even longer than the half-life of 'stealth' liposomes[55].

Methods for incorporating cargo into engineered nanoparticles, with regard to the ultimate delivery of the cargo to the desired location *in vivo*, can be classified into two broad categories. In one category, the cargo is physically entrapped in or absorbed onto the nanoparticle through non-covalent interactions. The second category includes examples in which the cargo has been directly attached to the nanoparticle matrix by degradable or non-degradable covalent bonds. The use of stimuli-responsive materials, which will be discussed in more detail below, allows for the release of the cargo once the engineered nanoparticle reaches its intended location *in vivo*. The bulk composition of the engineered nanoparticle must be carefully chosen based on its biocompatibility[56,57], its immunotoxicity[31] and its ability to solubilize or sequester the cargo of interest.

Beyond these basic features of nanoparticle design, a multitude of approaches for targeting specific cellular populations or for altering the biodistribution of engineered nanoparticles *in vivo* are being developed[58,59]. Targeting has been achieved using three predominant strategies that rely on either passive or active modes of action, which can be further characterized as non-selective or selective.

The first form of targeting is only relevant to oncology applications, and relies on the accumulation of engineered nanoparticles in tumours by the enhanced permeability and retention effect[60,61](see also BOX 1). This accumulation is a passive, non-selective process that occurs due to leaky, underdeveloped tumour vasculature that allows macromolecules of a certain size range to accumulate in the tumour. These macromolecules exit the circulation owing to leaky vasculature, but remain at the tumour site as a result of inefficient draining into the lymphatic system.



Box 1 | **Early history of nanoparticle therapeutics**

The first synthesis of what would now be considered a nanoparticle therapeutic — a polymer–drug conjugate — can be traced back to the 1950s, when Jatzkewitz prepared a polyvinylpyrrolidone–mescaline conjugate that contained a short peptide spacer between the drug and the polymer[133,134]. When mescaline was conjugated directly to the polymer (without the dipeptide spacer), no release of mescaline was observed *in vivo*. However, when the peptide spacer was incorporated, release was observed and for a longer duration than free mescaline when administered in the same way (17 days compared with 20 hours). Another early seminal event occurred in the mid-1960s, when Bangham discovered liposomes[135,136]. These two events mark the birth of the field of nanocarriers, and together these two classes of nanocarrier represent most of the marketed nanoparticle therapeutics (see main text) and continue to be investigated extensively.

In the 1970s, Ringsdorf conceptualized targeted drug conjugates, expounding the key principles that underpin much of the current thinking and goals in the field of nanoparticle therapeutics[137]. Important examples of two other classes of nanoparticle therapeutics also emerged. The first of these, an albumin-based nanoparticle, was reported in 1972 (REF. 138). This was the precursor to the first protein-based nanoparticle to receive regulatory approval — albumin-bound paclitaxel (Abraxane; Abraxis/AstraZeneca) — which was approved by the US Food and Drug Administration (FDA) for the treatment of metastatic breast cancer in 2005 (REF. 139). The second class, polymer-based nanoparticles, was first reported in 1976 (REFS 140,141). Here, polymer-based nanoparticles are different from polymer–drug conjugates in that the former consist of a particle formed by a network of polymer chains, whereas the latter consist of a single polymer chain. Despite a tremendous amount of interest in this class, so far, no therapeutics based on such nanoparticles have been approved by the FDA.

In the 1980s, a groundbreaking discovery was made by Maeda and co-workers while investigating the polymer–drug conjugate poly(styrene-co-maleic acid) conjugated to the cytotoxic drug neocarzinostatin (SMANCS)[60,61]. Compared with free neocarzinostatin, a significantly enhanced accumulation of the SMANCS conjugate was found at the tumour site, which the authors ascribed to the unique structural features of tumour vasculature. This phenomenon became known as the enhanced permeability and retention (EPR) effect. The enhanced permeability allows macromolecules to escape the circulation owing to the inherent leakiness of the underdeveloped tumour vasculature. In addition, the lack of an efficient lymphatic system leads to retention of those macromolecules in the tumour bed. Typically, to capitalize on the EPR effect a drug carrier must be in a narrow size range from approximately 10 nm to 100 nm. Entities smaller than 10 nm are rapidly cleared by the kidneys or through extravasation[17] and larger entities (~100–200 nm) are cleared by the reticuloendothelial system[21]. Polymer–drug conjugates are particularly well suited to take advantage of the EPR effect because the molecular mass of the polymer can be altered easily, allowing the effective size of the construct to be tuned systematically.

The first nanoparticle therapeutics were approved by the FDA in the 1980s. A mixture of cyclosporine and Cremophor EL (polyoxyethylated castor oil, which is capable of solubilizing extremely lipophilic drugs through the formation of micelles), marketed as Sandimmune by Novartis, was approved in 1983. Cremophor is also used in the preparation of the cytotoxic anticancer drug paclitaxel (Taxol; Bristol-Myers Squibb)[142]. There has been some debate over whether the source of dose-dependent toxicities experienced during the administration of Taxol should be attributed to paclitaxel or to Cremophor[143]. This aspect, combined with other technical difficulties associated with the use of Cremophor as a solubilizer, has led to the development of Cremophor-free, paclitaxel dosage forms such as Abraxane and polymeric micelle-formulated paclitaxel[143–145].

The first controlled-release polymer composition, an implantable form of goserelin acetate (a synthetic analogue of luteinising hormone releasing hormone), which is marketed as Zoladex by AstraZeneca, was approved by the FDA in 1989 for the treatment of certain types of prostate and breast cancers[142]. These controlled-release polymer–drug compositions, also known as 'drug depots', allow a drug or other molecule of interest to be trapped inside a polymer matrix and released over an extended period of time as it slowly diffuses out of the polymer matrix. Langer's laboratory pioneered the use of these types of materials[146,147], beginning with non-degradable polymer matrices in early work and leading to therapeutics such as a biodegradable depot form of the cytotoxic drug carmustine (Gliadel; Eisai), which was approved by the FDA for the treatment of brain cancer in 1996.

The second, more advanced method of targeting that has been investigated for oncology applications is the attachment of ligands to the surface of the engineered nanoparticle. The ligands are known to bind to receptors that are overexpressed on the surface of rapidly dividing cancer cells. For example, because of the high metabolic demands engendered by rapid proliferation, many types of cancer cells overexpress transferrin and folate receptors, which makes conjugation of transferrin, folic acid or antibodies to these receptors a successful targeting approach for engineered nanoparticles[4,62–67]. However, because these receptors are expressed to some degree on many types of non-target cells, toxic off-target effects are not totally eliminated.

Recently, the third form of targeting has made substantial progress in the ability of nanoparticles to target certain cell populations with high selectivity for both oncology applications and other therapeutic areas. These active, selective targeting strategies rely on highly specific interactions of antibodies, aptamers, peptides and oligonucleotides with cell surface receptors known to be expressed only on target cells. Both active targeting strategies are discussed in more detail below.

General biological barriers

To achieve intracellular drug delivery, strategies for overcoming various biological barriers — from the system level, to the organ level, to the cellular level — are

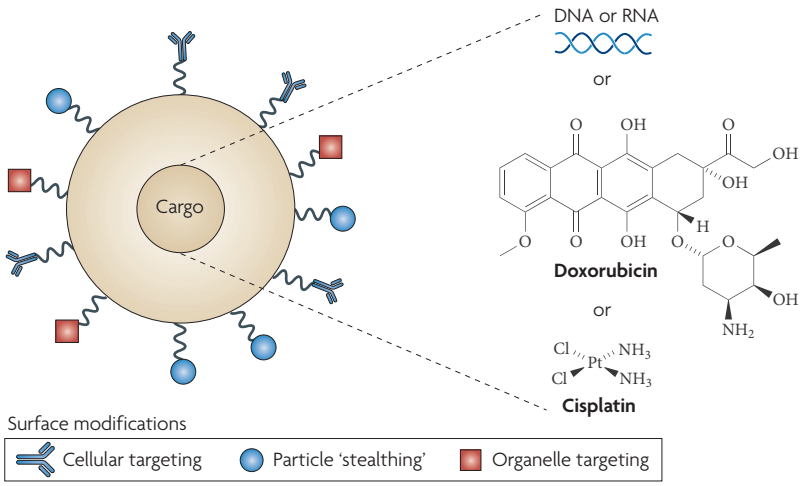


Figure 1 | **Schematic representation of an engineered nanoparticle.** The various surface modifications that are commonly pre-engineered, such as cellular targeting, particle 'stealthing' and organelle targeting, are highlighted. Ligands to extend circulation half-life and to reduce immunogenicity (usually polyethylene glycol (PEG) chains) are linked to the surface of the nanoparticle together with ligands to promote targeting. These ligands can be antibodies, aptamers or small molecules known to bind to surface proteins expressed on target cells or that are capable of guiding particle localization once inside the cell. Chemotherapeutics or other biologically relevant cargo are encapsulated inside the nanoparticle. Release of the cargo at the intended site of action is typically achieved through the incorporation of a stimuli-responsive material that changes state on exposure to the targeted environment (FIG. 4).

needed. The initial barriers encountered depend on the desired mode of administration (that is, inhalation, oral, intravenous or intraperitoneal injection). The degree of success in utilizing each of these modes of entry can be strongly influenced by the attributes of the engineered nanoparticle. For example, size can be a principal determinant for effective pulmonary delivery[68], whereas successful strategies for oral administration must address carrier stability during the harsh conditions in the gastrointestinal tract and simultaneously target a specific site for entry[69]. Intravenous injections must overcome the RES if prolonged circulation is to be achieved. Also a method for escaping the endothelium is required in order to exit the circulation into the desired tissue. Intraperitoneal injection allows tissue-specific delivery; however, nanoparticles can be rapidly cleared by the lymphatic system unless special steps are taken to avoid this.

Organ level. For intravenously injected engineered nanoparticles, avoidance of multiple organ-level clearance mechanisms, such as those operating in the spleen and the liver, must be compensated for if the carrier is to reach its intended destination. Fenestrations in the spleen typically do not exceed 200–500 nm in width[26], so particles larger than ~200 nm must be engineered to have some degree of deformability in order to remain in the circulation. A method for attenuating the activity of cells of the RES is also usually necessary to prolong circulation times.

Several strategies can be used to circumvent carrier removal by macrophages. First, decoy carriers can be pre-injected to saturate the phagocytic capacity of the RES, followed by injection of carriers containing the active ingredient[70–73]. Second, altering the hydrophilicity of the carrier surface has been shown to reduce the rate of protein opsonization, which ultimately marks carriers for sequestration and removal[15,35–38]. Third, specific proteins can be adsorbed or covalently linked onto the surface of the carrier that can help minimize or avoid complement activation[74,75]. Finally, markers of self, such as cell surface proteins, can be attached to the surface of the carrier[26].

In view of these desired characteristics of engineered nanoparticles, red blood cells could be considered as prototypical. First, they are capable of traversing biological barriers that are impenetrable to objects less than one-tenth their size and manage to avoid clearance by macrophages for up to 3 months. A number of factors are thought to contribute to their extended circulation, including their shape, deformability (which allows them to navigate through much smaller sinusoids in the spleen) and the presence of ligands, such as CD47 and CD200 that bind to inhibitory receptors expressed by macrophages[76,77]; absence of these markers leads to immediate removal of red blood cells by macrophages[73].

Cellular level. There are several biological barriers at the cellular level that an engineered nanoparticle must overcome, starting with the cell membrane, which blocks diffusion of complexes larger than ~1 kDa. Several endocytotic mechanisms can be engaged to facilitate the internalization of a carrier (FIG. 2). The details of the exact mode of endocytosis are important because they determine the path of trafficking through various possible subcellular compartments. For example, engineered nanoparticles internalized through clathrin-mediated endocytosis are destined for a lysosomal compartment, whereas those internalized through a caveolin-mediated process are not[78]. In clathrin-mediated endocytosis internalization, endosomal escape must occur before fusion with a lysosome to prevent degradation of the cargo under harsh lysosomal conditions. In either case, endosomal escape is usually necessary to allow access of the carrier to the desired subcellular compartment, whether it is the cytosol, the mitochondria or the nucleus.

Ligands conjugated to the surface of engineered nanoparticles can influence the mode of cellular internalization. Ligands such as folic acid, albumin and cholesterol have been shown to facilitate uptake through caveolin-mediated endocytosis, whereas ligands for glycoreceptors promote clathrin-mediated endocytosis[78]. Alternatively, macropinocytosis, a non-caveolin-mediated, non-clathrin-mediated process, can be engaged by incorporating cell-penetrating peptides, such as a *trans*-activating transcriptional activator (TaT) peptide into the design of engineered nanoparticles[79]. What is not well understood yet is the interdependent role(s) of particle size, shape and flexibility with ligand type, density, multiplexing and region-specific labelling on the nanoparticles.

The nuclear membrane is the final barrier for many types of engineered nanoparticles. Recent advances have been made in the ability to target specific organelles, which will be discussed later.

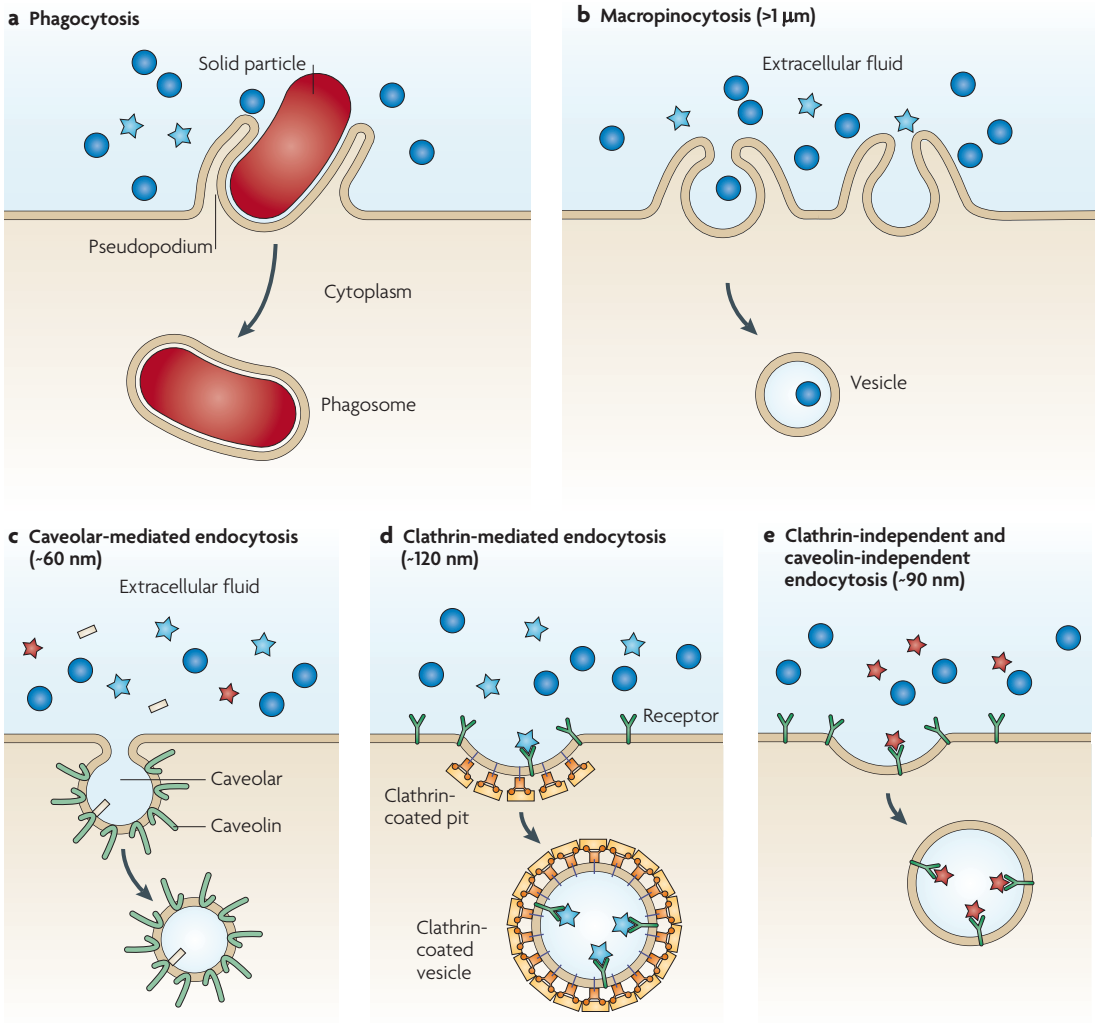


Figure 2 | **Modes of cellular internalization of nanoparticles and respective size limitations.** Internalization of large particles is facilitated by phagocytosis (**a**). Nonspecific internalization of smaller particles (>1 μm) can occur through macropinocytosis (**b**). Smaller nanoparticles can be internalized through several pathways, including caveolar-mediated endocytosis (**c**), clathrin-mediated endocytosis (**d**) and clathrin-independent and caveolin-independent endocytosis (**e**), with each being subject to slightly different size constraints. Nanoparticles are represented by blue circles (> 1 μm), blue stars (about 120 nm), red stars (about 90 nm) and yellow rods (about 60 nm).

Recent advances in nanoparticle design

The first attempted synthesis of an engineered nanoparticle can be traced back to the 1950s, providing more than half a century of experience to draw on in the rational design of modern engineered nanoparticles (BOX 1). The past decade has seen acceleration in the pace of new discoveries, some of which are highlighted in the following discussion.

Size and shape. Perhaps the most significant recent advances in engineering nanoparticles have come in the area of particle shape and its effect on cellular internalization and circulation times.

Recent publications illustrate the effect that particle shape can have during cellular internalization[16,51]. For example, the effect of shape and geometry of contact of spherical and non-spherical polystyrene microparticles during phagocytosis by alveolar macrophages was investigated[51]. With elliptical disk-shaped microparticles, it was discovered that when the macrophage first contacted particles along the major axis, the particles were rapidly internalized (< 6 minutes). However, when first contact was along the minor axis, the particles were not internalized, even after 12 hours. Spherical particles were rapidly and uniformly internalized because of their symmetry. This effect of shape was independent of particle size in the size ranges studied (0.1–100% of the volume of the macrophage). The only difference observed related to particle size was the extent of internalization, which was only observed with particles in which the volume of the particle was larger than the volume of the cell.

In an important study illustrating the dramatic role shape can have in the function of engineered nanoparticles, filamentous micelles (filomicelles) with single dimensions as long as 18 μm were reported to exhibit

circulation half-lives of ~5 days[48], which is significantly longer than even stealth liposomes[55]. This result is remarkable given that micrometre-sized rigid spheroids are cleared from the circulation almost immediately. This unique feature of filomicelles was attributed to two aspects of the carrier. First, having two dimensions on the length scale of nanometres (the diameter of filomicelles is ~20–60 nm) allows them to traverse extremely small openings, such as those found in the spleen. Second, their shape helps reduce the rate of phagocytosis by cells of the mononuclear phagocyte system. Shear forces of blood flow applied to portions of the filomicelle not in contact with the cell exert enough force to pull the carrier away from the cell before internalization. These filomicelles were synthesized from co-polymers of PEG and either non-degradable polyethylene or degradable polycaprolactone, which are materials commonly used to make engineered nanoparticles. Paclitaxel was loaded into the hydrophobic core of these filomicelles and they were shown to be more effective at reducing tumour size *in vivo* than free paclitaxel, indicating their promise in the development of advanced engineered nanoparticles[48]. Davis and colleagues have recently reported the first example of systemically administered small interfering RNA by targeted nanoparticles in humans[80]. The delivery vector is based on the self assembly of small interfering RNA, a linear cyclodextrin-based polymer, adamantane–PEG and adamantane–PEG-transferrin[81]. In their ongoing studies, the refinement of particle size is a crucial step in the design process and they report an optimal particle size of 50–70 nm for this particular delivery vector[81].

New tools are emerging that allow a systematic study of the internalization kinetics and mechanism of a series of microparticles and nanoparticles in which a single parameter (shape or size) can be altered independently of all other particle attributes[50]. These particles were fabricated using particle replication in non-wetting templates (PRINT) technology[82–85], which is described in FIG. 3. In the above mentioned study, it was discovered that positively charged cubic particles with a cube side length as long as 3 μm were internalized *in vitro* by HeLa cells[50]. This clearly contradicts the current dogma that predicts that particles larger than ~150 nm are not internalized by non-phagocytic cells[42,86,87]. The rate of internalization of two particles with roughly the same volume but with extremely different three-dimensional shapes differed substantially. It was discovered that particles with an aspect ratio of 3 were internalized four times more rapidly than those with an aspect ratio of 1. Both were cylindrical, but particles with a high aspect ratio had a diameter of 150 nm and a height of 450 nm, whereas those with a low aspect ratio had a diameter and a height of 200 nm. Furthermore, the increased rate of internalization was shown not to be a result of a reduction in one of the dimensions of the particle, as particles with a diameter of 100 nm and an aspect ratio of 3 were internalized at a rate similar to those with a diameter of 200 nm and an aspect ratio of 1 (REF. 50). So far, the origins of the pronounced selectivity for one size and shape versus another are unclear. However, these data highlight the role of size and shape in the uptake of particles by targeted cells, such as the HeLa cell line studied, in addition to their impact on particle internalization by cells of the immune system, as already described.

Matrix chemistry. Another area in which significant advances have recently been made is in the design of stimuli-responsive carriers. Materials can be synthesized that respond either to an internal stimulus (such as the reducing nature of the cytosol compared with the extracellular space or the drop in pH known to occur in endosomes), or to an external stimulus (such as an applied magnetic field or exposure to a specific wavelength of light). These stimuli are used as triggers to break covalent bonds between the carrier and cargo, or to destabilize the carrier facilitating release of its contents once the carrier has reached a specific location (FIG. 4).

The reducing nature of the cytosol has been used extensively in protein–conjugate chemistry to trigger release of the payload on cellular internalization with cargos ranging from oligonucleotides to toxins and chemotherapeutics[88]. Gemtuzumab ozogamicin (calichaemicin linked to a CD33-specific human antibody) was the first FDA-approved therapeutic to contain a drug conjugated to its carrier through a reductively labile disulphide-based linker[89].

More recently, polymeric carriers containing disulphide crosslinks have been prepared that release chemotherapeutics physically entrapped in the polymer network on exposure to a reducing environment[90,91]. Reduction of the disulphide bonds leads to a decrease in the mesh density of the polymer, making it more porous, which allows the chemotherapeutic to diffuse out. Disulphide bonds have also been used to link targeting or 'stealthing' moieties to the surface of engineered nanoparticles[88,92]. In one example, when PEG chains were conjugated to the surface of liposomes through non-degradable bonds, the circulation time of the liposome was improved as expected; however, its activity decreased dramatically. When the PEG chains were conjugated to the liposome through a disulphide-bond cleavage site, circulation time was again improved but more importantly their therapeutic activity was maintained[92]. For further reviews detailing the use of other stimuli-responsive materials in engineered nanoparticles see REFS 93–95.

Cellular targeting. Tools for targeting specific cellular populations have been widely developed. This is true for both selective and non-selective active targeting methods and has been accomplished using various ligands, including antibodies, aptamers, peptides and small molecules. These methods almost exclusively target some type of membrane-bound protein (one exception is targeting carbohydrates on the surface of cancer cells with lectins; so-called reverse lectin targeting[96]).

Active, non-selective targeting methods for oncology applications that are directed at the most rapidly dividing cells focus primarily on transferrin and folate receptors. These receptors are expressed ubiquitously; however, their expression is often upregulated in cancer cells. The roles of transferrin[65,66] and folate[63,64] in targeted drug delivery have been reviewed recently. It should be noted,

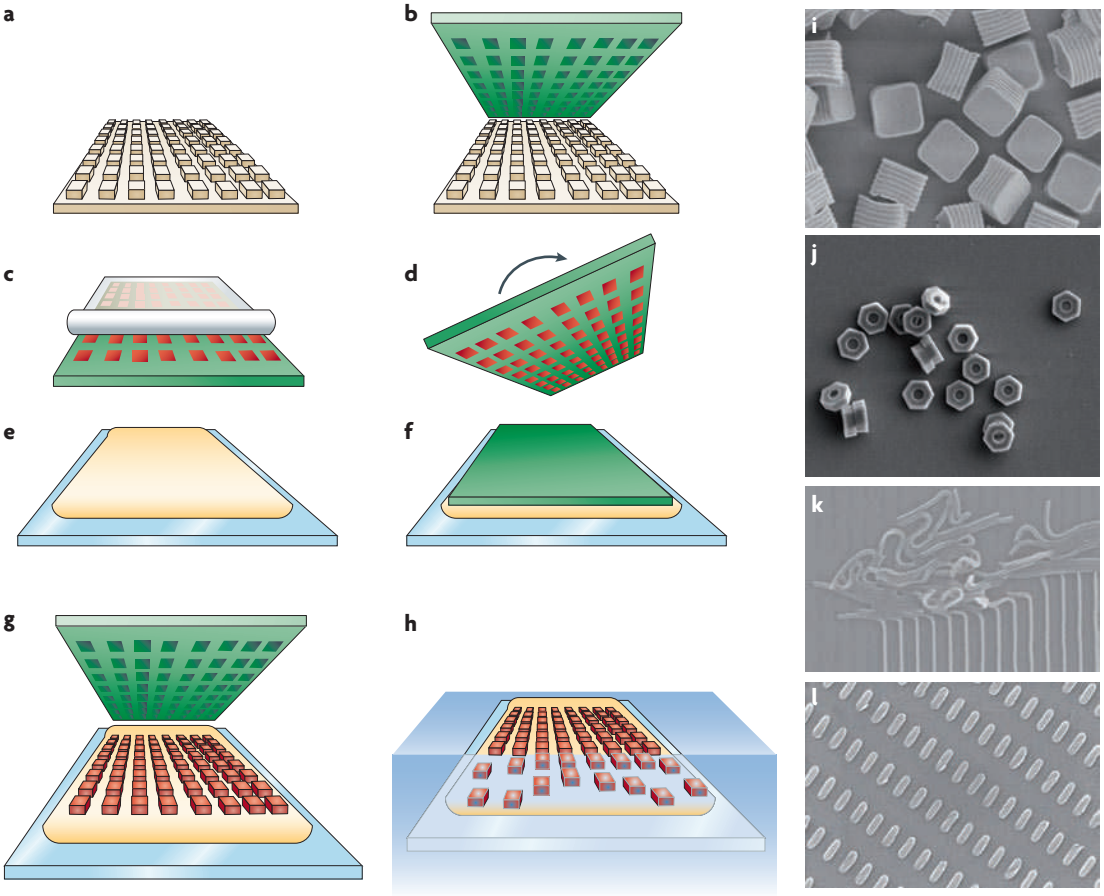


Figure 3 | **PRINT technology for generating microparticles and nanoparticles. a** | In the first step of particle replication in non-wetting templates (PRINT) technology, a silicon master template is prepared using techniques adapted from the microelectronics industry. **b** | Fluorocarbon-based moulds[151] of this master template are then generated. **c** | The moulds can then be filled with liquid precursors. These liquid precursors are then converted to solids through various methods[90,152,153]. The solidified particles can be harvested from the mould in a two-dimensional array on an adhesive film, such as medical grade poly(cyano acrylate) or on an excipient film such as povidone (polyvinyl pyrrolidinone). **d** | The mould containing the particles is turned over and placed onto a harvesting layer that has been rolled onto a glass slide using a Meyer rod. **e** | Next, the patterned perfluoropolyether mould containing the particles is run through a roller, pattern side down. **f** | The mould is then placed onto the liquid harvesting film. **g** | After the harvesting film is dried (if a povidone harvesting film is used) or polymerized (if a cyano acrylate harvesting film is used), the filled patterned mould is peeled away from the harvesting film to yield two-dimensional arrays of particles. **h** | Finally, individual particles are produced by dissolving the harvesting film. **i** | Scanning electron micrograph (SEM) images of PRINT micro/nanoparticles can be taken. From top to bottom the SEM images show cubic (cube-side length = 2 μm) microparticles prepared from a reductively-labile disulphide-based polyethylene glycol (PEG) diacrylate[90]; 3 μm 'hex nut' particles[83], 80 nm by 2,000 nm wormlike cross-linked PEG nanoparticles on a harvesting layer[154]; and 80 nm by 360 nm cross-linked PEG particles on a polymeric harvesting layer[154].

however, that Davis *et al.* discovered that ligands targeting the transferrin receptor exert their influence by increasing uptake of targeted nanoparticles by cancer cells and not by increasing particle accumulation in the tumour region[81]. Again, because these receptors are expressed to some degree on many types of non-target cells, toxic off-target effects can occur.

One representative example of an active, highly selective targeting strategy is the use of antibodies to target prostate-specific membrane antigen (PSMA)[97]. PSMA, a 100 kDa type II membrane glycoprotein, is highly expressed on virtually all prostate cancer cells, but is not expressed on healthy cells. In a recent study[97], an antibody to PSMA (J591) was conjugated to dendrimers and its binding to androgen-sensitive human prostate adenocarcinoma (LNCaP) cells was investigated. Antibody-targeted dendrimer staining of LNCaP cells was then compared with untargeted dendrimer staining or with antibody-targeted dendrimer staining in a cell line that does not express PSMA (PC-3 cells). The antibody-targeted dendrimer bound preferentially to LNCaP, whereas the untargeted dendrimer did not. Furthermore, the antibody-targeted dendrimer did not bind to PC-3 cells, which do not express PSMA.

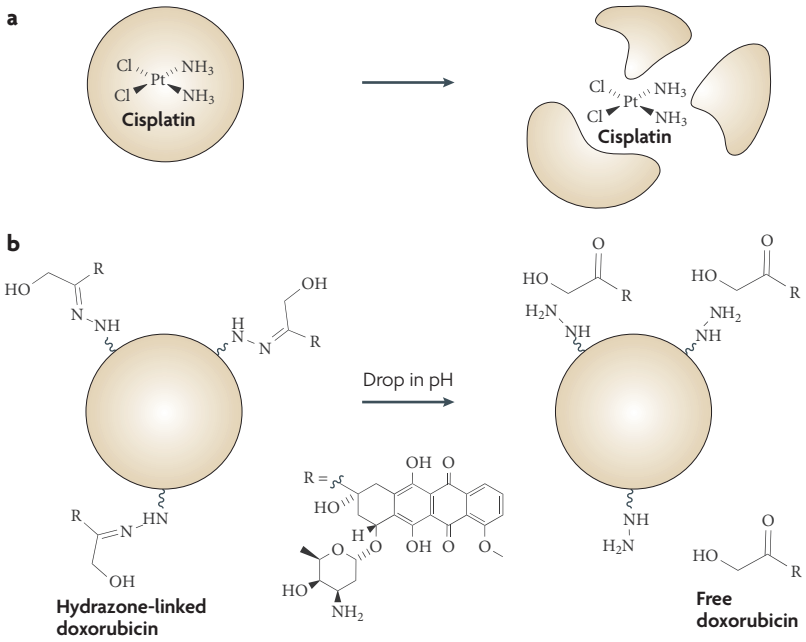


Figure 4 | **Stimuli-responsive engineered nanoparticles.** Nanoparticles that release their cargo (such as a drug) in response to environmental stimuli can be designed either by physically entrapping the cargo in the carrier (**a**) or covalently linking the cargo to the carrier (**b**). For cargos that are physically entrapped, the carrier degrades on entry into a predefined environment, thereby releasing its cargo. Release can be tailored to respond to a reducing environment, such as the cytosol[155] or a slightly acidic environment, such as a lysosome or even a tumour bed[94]. For covalently linked cargos, such as the hydrazone-linked doxorubicin shown, special linker groups must be designed that can be cleaved under the desired conditions, such as exposure to reducing or acidic[94] environments or exposure to a specific enzyme[156].

Perhaps even more remarkable was the finding that prostate cancer cells are not the only cells that express PSMA, but endothelial cells in the tumour neovasculature of multiple nonprostatic solid malignancies also express it[98] (BOX 2). This is in stark contrast to endothelial cells of benign tissues. As a result, efforts are currently underway to target the neovasculature of many types of solid tumours using [^{111}In]–J591 conjugates and the first successful human Phase I study was completed recently[99]. A total of 27 patients in the trial received the [^{111}In]–J591 antibody. Kidney, bladder, lung, breast, colorectal and pancreatic cancers, as well as melanoma, were all successfully imaged in the study, and in 20 out of the 27 patients (74%) at least one site of known metastatic disease was successfully imaged. In one of the patients, an undiagnosed brain metastasis was also identified. Indeed, in a magnetic resonance imaging (MRI) scan from 4 months earlier the brain metastasis was not evident and was not apparent during pretreatment imaging. Subsequent MRI scans confirmed the presence of the metastases in the observed region during imaging with the [^{111}In]–J591 antibody. No tumour regression was noted in any of the patients; however, the ability to image such a diverse set of tumours with high selectivity indicates that [^{111}In]–J591 could be a useful targeting ligand for engineered nanoparticles.

Cellular targeting with the aid of short peptide sequences capable of binding to cell surface receptors has also been shown to be selective[100]. The arginine-glycine-aspartic acid (RGD) peptide, discovered by Pierschbacher and Ruoslahti[101], was one of the first examples of a short peptide sequence capable of binding to proteins (integrins) on the cell surface. This motif is common in extracellular matrix proteins and promotes cellular adhesion to the extracellular matrix. Cyclic versions of the RGD motif were later discovered to bind more effectively to integrins, such as α5β1, αVβ1, αVβ3 and αVβ5, and these peptides are still under extensive investigation today as targeting ligands directed towards disrupting tumour angiogenesis[102–105]. Differences in protein expression in diseased versus healthy vasculature have only recently been fully appreciated for their potential as targets for engineered therapeutics. Progress is being made in the development of tools necessary to identify ligands that bind to such target receptors expressed by tumour endothelial cells[106–109], which facilitates the vascular mapping of tumours. Strategies that aim to target tumour vasculature have the added benefit of the target being readily accessible to circulating nanoparticles compared to strategies that target receptors on the tumour cells themselves[73,104,107,110,111].

Short peptide sequences capable of binding to the antigen site of surface immunoglobulin receptors expressed by B cells were discovered by phage display peptide libraries[112]. These peptides were shown to bind selectively to SUP–B8 immunoglobulin, but not to immunoglobulin receptors of non-target B cells. The authors also reported interesting cytotoxic effects of the targeting peptide. The monomeric form had no effect on cellular proliferation; however, when the peptide was made multimeric through attachment to avidin, the peptide induced significant levels of apoptosis. The cytotoxic effect was attributed to cross-linking of the immunoglobulin receptors on the cell surface, which triggered specific phosphorylation of intracellular protein kinases. This effect is similar to the cross-linking effects seen when B cell lymphomas were treated with anti-immunoglobulin reagents[113,114]. These results provide a cautionary note by illustrating that nanoparticle binding can lead to unexpected biological consequences.

Organelle-specific targeting. Ultimately, the effectiveness of any engineered nanoparticle will depend on the efficiency of the carrier to deliver its cargo to the intracellular site of action. For example, carriers containing oligonucleotides as cargo, which need to cross the nuclear membrane to be effective, can be successfully targeted to specific cells and internalized. However, if they do not escape the endosome, the oligonucleotides will probably be degraded under the harsh lysosomal conditions. This highlights the need for strategies to direct engineered nanoparticles to specific subcellular compartments. Tools and principles for effective organelle targeting are emerging, such as those for targeted delivery to the nucleus[115–117], cytosol[118,119], mitochondria[120–123], peroxisomes[124] and endosomes/lysosomes[78].

Box 2 | **Targeting tumour angiogenesis**

Targeting the tumour vasculature with nanoparticle therapeutics represents an attractive strategy for treating many forms of cancer because of the inherent accessibility of vascular components to circulating nanoparticles[107]. Tumours must recruit vasculature to supply nutrients for cell growth and for the removal of waste by-products. This process, known as angiogenesis, is the result of a cascade of events beginning with upregulation and secretion of growth factors by tumour cells, which in turn stimulates infiltration of the tumour bed by vascular endothelial cells. Once activated, these migrating endothelial cells secrete matrix metalloproteinases (MMPs) that degrade the extracellular matrix (ECM) near the tumour allowing other endothelial cells to migrate towards the tumour. In addition to secreting MMPs, these cells, which normally only divide about once every 3 years, begin to divide at an accelerated pace[148]. This combination of ECM remodelling and cell proliferation provides the requisite space and cellular populations to facilitate vessel formation.

Numerous studies have been done to investigate the consequences of disrupting the process of angiogenesis in solid tumours, which illustrate the marked effects that can be achieved in slowing or ceasing tumour growth[149]. Angiogenesis is also known to have a pivotal role in tumour metastasis[148]. Great strides are being made in the ability to identify ligands to bind target receptors expressed by tumour endothelial cells[106–109] and such strategies have the advantage of having the targeted receptor readily accessible to circulating nanoparticles[73,104,107,110,111]. In a recent study, the rate of metastasis in an experimental model was significantly diminished by directed disruption of angiogenesis using targeted doxorubicin-containing liposomes[104]. Liposomes containing doxorubicin were effectively targeted to the tumour vasculature by the attachment of the arginine-glycine-aspartic acid (RGD) peptide to their surface that is known to bind αvβ3, an integrin crucial to cancer progression[150]. Targeted liposomes exhibited a 15-fold improvement in drug efficacy compared with the free drug, demonstrating the marked effects that can be achieved with successful targeting.

Delivery to mitochondria is largely based on electrostatic interactions between the engineered nanoparticle and the mitochondrial membrane, which has a membrane potential of 130–150 mV[125]. This potential is lower than other membranes in the cell and can be exploited by grafting cationic species, such as triphenylphosphonium cations, to the surface of the carrier[121]. This strategy was shown to be effective at delivering hydroxypropylmethacrylamide polymer conjugates to mitochondria *in vitro*[126]. Peptide ligands provide an alternative method for targeting mitochondria, which was shown recently by the successful targeting and localization of peptide-targeted quantum dots in mitochondria[127].

Finally, targeting engineered nanoparticles to the nucleus remains a significant challenge. The nucleus is separated from the cytosol by two membranes with pores of ~10 nm. These pores allow the free diffusion of macromolecules less than 30–40 kDa. They can dilate only slightly, so transport of larger macromolecules requires protein transport factors. The inclusion of a nuclear localization signal can activate the nuclear transporter importin, driving uptake into the nucleus[128]. In one study, gold nanoparticles as large as 39 nm coated with nucleoplasmin were efficiently targeted to the nucleus[129]. In another study, DNA–polylysine complexes as large as ~60 nm were shown to efficiently deliver DNA to the nucleus, but only when coupled to a nuclear localization signal peptide sequence[130,131]. The authors postulated that these larger structures were able to pass through the nuclear pores owing to the inherent flexibility of the conjugate. These two studies taken together clearly demarcate an upper size limit for transport into the nucleus.

Conclusions

Several particle characteristics have emerged as being central to the function of engineered nanoparticles and should therefore be used to guide future design efforts.

Particle size. For rigid, spherical particles, ones that are 100–200 nm in size have the highest potential for prolonged circulation because they are large enough to avoid uptake in the liver, but small enough to avoid filtration in the spleen. The design of non-spherical and/or flexible particles can, however, dramatically extend the particle's circulation time *in vivo*. The same general principles govern the biodistribution profile of these particles: for long-circulating particles, uptake by the liver and the spleen must be avoided. This can be accomplished practically by engineering deformability into particles >300 nm or by keeping at least one dimension of the particle on a length scale >100 nm to prevent accumulation in the liver and still maintaining at least two dimensions at <200 nm, thereby allowing the particle to navigate the sinusoids of the spleen.

Particle shape. In some instances, the effects of particle shape can be intimately coupled to particle size, as described for long-circulating non-spherical particles. Particle geometry also has a key role in particle internalization. Although preliminary data exist demonstrating the marked effects of particle shape, optimum parameters for engineered nanoparticles have yet to be determined.

Surface characteristics. This particle attribute has three vital roles in the function of engineered nanoparticles. First, surface chemistry is known to heavily influence the process of opsonization, which ultimately dictates the RES response. Several methods designed to circumvent the activation of the immune system are described above. Second, to achieve cellular targeting, ligands known to bind cell surface receptors of selected cells should be included in the design of engineered nanoparticles. Third, if organelle targeting is also required, those ligands must be incorporated into surface design.

Release of therapeutics. Achieving tailored activated release still represents a key barrier in the field of engineered nanoparticles. The predominant strategies so far incorporate materials that are enzymatically degradable, pH-sensitive or reductively labile, which facilitate bond breaking between drug and carrier, or destabilization of the carrier on reaching the intended site of action.

Summary. Great strides have been made in the design and application of engineered nanoparticles throughout the past 50 years; however, significant challenges remain. Our ability to shepherd cargo to sites in the body to achieve precisely defined therapeutic effects is still in its infancy[132]. Developing the requisite tools to dictate events occurring at the biotic/abiotic interface requires a highly interdisciplinary approach, which is benefiting tremendously from the increasing collaborations among scientists from the physical and the life sciences. As this trend continues, the potential of engineered nanoparticles with increasing complexity and efficacy will be achieved.

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Competing interests statement

The authors declare competing financial interests: see web version for details.

FURTHER INFORMATION

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