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Engineering Particles for Therapeutic Delivery: Prospects and Challenges

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ABSTRACT Nanoengineered particles that can facilitate drug formulation and passively target tumors have reached the clinic in recent years. These early successes have driven a new wave of significant innovation in the generation of advanced particles. Recent developments in enabling technologies and chemistries have led to control over key particle properties, including surface functionality, size, shape, and rigidity. Combining these advances with the rapid developments in the discovery of many disease-related characteristics now offers new opportunities for improving particle specificity for targeted therapy. In this Perspective, we summarize recent progress in particle-based therapeutic delivery and discuss important concepts in particle design and biological barriers for developing the next generation of particles.

Building on many years of basic and translational research, advances in the fields of nanotechnology and biomedical science are now converging to revolutionize the treatment of a range of diseases.1,2 To date, several types of particlebased therapeutics have been approved by the FDA for clinical use, including liposomes, albumin nanoparticles, and polymeric nanoparticles. 3 For example, doxorubicin-loaded PEGylated liposomes (i.e., DOXIL) have demonstrated reduced cardiotoxicity compared to doxorubicin,4 and paclitaxel-bound albumin nanoparticles (i.e., Abraxane) have shown enhanced drug efficacy for metastatic breast cancer.4 Along with this generation of particle-based therapeutics, the selective delivery of established chemotherapeutic compounds to solid tumors via the enhanced permeability and retention (EPR) effect has been a key research endeavor. However, there are still a number of challenges that must be overcome in order to achieve efficient and specific therapies with particle-based delivery systems. Thus, it is imperative that materials scientists be guided by a better understanding of relevant biological mechanisms. In the past decade, significant innovations in biomedical science have led to the development of a range of specific targeting molecules (e.g., monoclonal antibodies) and new classes of therapeutics (e.g., RNA-based therapeutics). The concept of using antibodies to improve target selectivity in treating diseases has been increasingly recognized. Humanized monoclonal antibodies that are specific for tumor-associated antigens have been engineered, some of which have been established as “standard of care” agents for the treatment of several types of cancer.5 The increased understanding of the molecular and cellular mechanisms in human diseases, ranging from viral infection to cancer, has also broadened the scope of therapeutic targets. Many classes of emerging therapeutics, including peptides and small interfering RNAs (siRNAs), have demonstrated unprecedented potential.6 There are dozens of RNA-based therapeutics currently under clinical investigation.6 However, the poor stability and cellular uptake of these novel therapeutics has been a major impediment to their effectiveness. Consequently, in recent years, there has been growing interest in combining these novel molecules with nanoengineered particles to further increase the specificity of particle delivery and overcome the obstacles associated with application of these emerging therapeutics. Identification of the principles that govern particle motility at the tissue, cell, and organelle levels has started to inspire the design of next-generation targeted particles, which will ultimately overcome an array of physiological barriers to enhance the bioavailability of a range of therapeutics. Moving forward, research at the interface of nanotechnology and biomedicine will underpin advances in particle-based therapeutic delivery.

In this Perspective, we highlight recent developments in particlebased drug delivery, focusing on three keys aspects: (i) functionalization of particles with targeting molecules to promote specific interactions both in vitro and in vivo; (ii) mechanisms involved in particle internalization and intracellular trafficking; and (iii) emerging concepts and strategies in particle design for controlling cellular uptake and intracellular targeting.

The ability to target particle systems to specific tissues has long been a significant goal in the field of drug delivery, as it offers a viable approach to reduce side effects and improve efficacy. Early work in this area involved the use of the EPR effect, or “passive targeting”, to allow particles to accumulate preferentially in tumor sites. The EPR effect arises due to the high fluid flow and large leaky vasculature within many solid tumors. Treatments exploiting the EPR effect have shown some therapeutic benefit.4 However, this passive targeting strategy still faces some challenges. The longer circulation times of drug-loaded particles can lead to adverse effects, as has been observed with DOXIL, which can cause severe hand-foot syndrome.4 In addition, the size of the tumor vasculature is highly dependent on the tumor type and age, and consequently, the EPR effect is not applicable for all tumor stages.7 Therefore, the heterogeneous nature of tumors underscores the need to identify alternative targeting strategies to enhance the specificity of particlebased therapies.

Over the past decade, targeted drug delivery has been inspired by many important discoveries relating to pathological characteristics. Overexpression of the receptors that are involved in increased nutritional uptake, such as folate and transferrin receptors, has been associated with the development of malignant tumors. For example, folate receptors that deliver folic acid into cells have shown 100- to 300-fold overexpression in a wide spectrum of cancer cells.8 For this reason, folate has become a popular targeting molecule to functionalize a range of delivery systems. In a recent study, polymer cross-linked liposomes loaded with doxorubicin were modified with folate.9 It was shown that folate-functionalized liposomes bound tumor cells differentially as a function of the folate receptor expression levels on the cell membrane.9 These carriers were shown to be 50-fold more potent than the untargeted agent toward a panel of cancer cells overexpressing the folate receptors in vitro. Recently, the first clinical investigation using transferrin-functionalized nanoparticles for siRNA delivery (CALAA-01) was reported.10 These particles were generated via a unique twovial formulation approach, which enabled the rapid self-assembly of siRNA and a cyclodextrin-containing polycation complex, sterically stabilized with adamantine-PEG and functionalized with transferrin for targeting. Tumor biopsies from melanoma patients obtained after treatment with CALAA-01 showed a favorable safety profile and effective siRNA knockdown by the particles.10

Another class of frequently overexpressed tumor-associated molecules is growth factor receptors. The epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (HER2) that are involved in tumorgenesis are the most extensively studied, given the development of specific monoclonal antibodies against these tumor-associated receptors. There has been increasing interest in functionalizing particles with these antibodies for targeted delivery. The use of antibodies not only provides high affinity toward their targeted cells but also potentially inhibits tumor growth by blocking ligand□ receptor binding and downstream signaling. In a recent study, drugloaded liposomes were modified with anti-HER2 or anti-EGFR antibodies.11 It was shown that the targeting molecules significantly enhanced liposome uptake by multiple breast cancer cell lines that overexpress the antigens, resulting in increased cytotoxicity in vitro and improved antitumor activity in vivo. Another recent study has provided further evidence that antibody functionalization is an important strategy in improving the specific delivery of particles in vitro and in vivo (Figure 1A,B).12 In this study, long-circulating filomicelles were conjugated with a panel of antibodies that specifically bind to endothelial cells. A 10-fold increase in binding to endothelium in vivo was observed for the filomicelles functionalized with targeting antibodies compared with those bearing a nonspecific antibody (immunoglobulin G, IgG). It is worth noting that currently used targeting molecules, such as folate and HER2 antibodies, are not uniquely specific for cancer cells but also recognize receptors expressed on healthy tissue. This could lead to nonspecific targeting and subsequently increase toxicity. Recently, by screening thousands of EGFR monoclonal antibodies for tumor specificity, an antibody that only binds overexpressed, mutant, or ligand-activated forms of EGFR in cancer cells was identified. In subsequent phase I clinical studies, this antibody showed excellent tumor targeting without observable normal tissue uptake.13 It is anticipated that conjugation of particles with such highly lesion-specific antibodies will further enhance the specific targeting of particles.

Suitable targets for drug delivery are molecules that are exclusively present in the targeted tissue. On the basis of this rationale, a paradigm shift for identification of potential targets has recently been suggested. In this approach, antibodies that recognize tissue-specific proteins can promote preferential tissue distribution. Given the fact that, in certain cases, tissue-specific proteins can display different turnover rates between the normal and malignant cells within the tissue; this may help differential cell targeting using tissue-specific antibodies. This has been exemplified by a series of studies on the highly tissuespecific A33 antigen, which is primarily expressed in intestinal epithelia cells and on

more than 95% of primary and metastatic colorectal cancers. Phase I clinical trials using a humanized A33 monoclonal antibody (huA33 mAb) have shown promising results in targeting colorectal tumors, with cancer cells showing slower A33 turnover rates compared with the normal intestinal epithelial cells.14 Recently, the potential for using particles functionalized with this antibody for colorectal cancer targeting was investigated in vitro using layerby-layer (LbL) capsules.15 Capsule binding was investigated in a mixed population of two human colorectal cancer cell lines, of which one is inherently A33 antigen negative (LIM2405□) and the other is stably transfected with A33 antigen (LIM2405þ). Flow cytometry showed that over 90% of the LIM2405þ cells were associated with huA33 mAbfunctionalized capsules, while less than 5% of the LIM2405□ cells showed association with the capsules (Figure 1C,D). Highly specific binding was observed to the targeted A33 positive cells, even when this population was only 0.1% of the total cells. In contrast, capsules modified with the nonspecific antibody IgG showed very low cell binding in both cell lines. These results suggest the potential for using such tissue-specific antibodies in order to target colorectal cancer. As additional disease biomarkers are emerging, such as overexpressed transmembrane protein CD47 in solid tumors,16 it is envisaged that materials scientists, chemists, biologists, and clinicians will continue to develop targeted delivery systems with enhanced efficacy and specificity.

Effective therapy not only requires transportation of therapeutics to specific tissues and cells but also requires delivery to specific molecular targets. As many therapeutic targets are localized at certain subcellular sites, there has been a surge in recent years into the investigation of the cellular uptake of particles. Studies have shown that particles with sizes between 10 nm and 5 μm are typically internalized into cells via endocytosis. Endocytosis is an energy-dependent process by which particles are engulfed and encapsulated within a lipid bilayer that isolates them from the cytoplasm of the cell. In many cases, the resulting endosomes then undergo a rapid maturation to late endosomes and lysosomes; however, this process is also highly dependent on cell type, internalization mechanism, and properties of the interacting materials. Typically, endocytosis can be classified into two categories: phagocytosis (cell eating), by which cells internalize only solid aterial, and pinocytosis (cell drinking), where cells take up a significant amount of liquid from the extracellular environment along with the internalized material.

While phagocytosis is generally limited to specialized cells such as macrophages and dendritic cells that interact directly with large material (>250 nm), pinocytosis occurs in almost all cells. Pinocytosis can be further subclassified into a number of distinct mechanisms, including macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin/caveolaeindependent pathways (Scheme 1).17 Like phagocytosis, macropinocytosis is associated with the uptake of large material and has been shown to form endocytic vesicles up to 5 μm in diameter. In contrast, clathrin and caveolae pathways are generally limited to smaller (<150 nm) materials. Understanding the molecular mechanisms of endocytosis is an active area of research, and the emerging discoveries have been recently reviewed.17

On the basis of the dynamic nature of endocytosis, it is not surprising that the cellular uptake of particles is dependent on many factors, including cell physiology and particle properties.18 Here, we focus on a few seminal studies on various particle systems to exemplify several important physicochemical parameters (Scheme 2). Particle size is a key property that affects the cellular uptake rate, as it influences the endocytic pathway. Rejman *et al*. studied the internalization of polystyrene (PS) particles by murine melanoma cells (B16□F10).19 They demonstrated that PS particles with diameters of 50 and 100 nm were rapidly internalized (less than 30 min) via a clathrin-mediated pathway; however, larger particles (200 and 500 nm in diameter) were only slowly internalized (2□3 h) and exhibited 8- to 10-fold decreases in internalization when compared to the smaller particles. In

another study, Jiang *et al*. synthesized a series of herceptin (i.e., a humanized anti-HER2 monclonal antibody)-functionalized gold nanoparticles within the size range of 2□100 nm. It was shown that although the different-sized nanoparticles bound effectively to human breast cancer SK-BR-3 cells, the internalization of 40 and 50 nm nanoparticles via the clathrin-mediated pathway was the most efficient.20 Shape has also recently been shown to play an important role in particle uptake. By using a series of particles fabricated via the particle replication in nonwetting template (PRINT) approach, it was found that higher aspect ratio (AR) particles were internalized in HeLa cells at a greater rate compared to submicrometer spherical particles of a similar internal volume.21 The different uptake efficiency was due to a greater utilization of multiple internalization mechanisms by the high AR cylinders through cellular interactions at multiple nonsymmetric axes.21 Similarly, Mitragotri and co-workers found that particle shape also influenced the rate of phagocytosis. By comparing 1 μm PS spherical and elliptical particles of equal internal volume, it was found that phagocytosis was sensitive toward the interaction axis for these particles, as the spheres were seen initially to be internalized more rapidly.22 This kinetic phenomenon was exploited for immune system evasion and improved particle biodistribution in vivo by Discher and co-workers.23 It was shown that flow effects and shear forces limited the ability of macrophages to internalize the flexible worm-like micelles, leading to long blood circulation times of 5 to 6 days. In addition, the effect of particle rigidity on cellular uptake was recently demonstrated by another study, where 150 nm hydrogel particles with intermediate Young's modulus (35 and 136 kPa) were found to be internalized by macrophages via multiple mechanisms. After 4 h incubation, nanoparticles with intermediate elasticity showed approximately 67% higher internalization compared to their softer counterparts (Young's modulus of 18 kPa) and 25% higher uptake compared to the more rigid nanoparticles (Young's Modulus of 211 kPa).24 Besides these emerging physical properties, surface charge has also been shown to affect particle internalization. Positively charged particles are typically internalized to a greater degree than are negatively charged particles, presumably due to the negatively charged cell membrane. Macropinocytosis and clathrin-mediated endocytosis have been shown to play a role in the internalization of positively charged 100 nm PS particles in HeLa cells. However, in the same cells, negatively charged 100 nm PS particles were internalized via an undetermined clathrin/caveolae-independent pathway.25 In addition, particle surface chemistry and functional group density play important roles in particle endocytosis. Harashima and co-workers performed a systematic study of the internalization of liposomes coated with octapeptides.26,27 Positively charged liposomes functionalized with either a high density of octaarginine (R8) or octalysine (K8) were internalized by NIH-3T3 cells via a macropinocytic pathway, whereas liposomes with a low density of R8 were internalized via a clathrin-mediated pathway. Interestingly, in polarized Madin-Darby canine kidney (MDCK) cells, the high-density R8-functionalized lipsosomes were internalized via both clathrin-mediated endocytosis and macropinocytosis to a similar extent.28 This study highlights that the intrinsic cell physiology is also deterministic in particle endocytosis, leading to the use of alternative endocytic pathways and variable cellular processing. Taken together, the complex effects arising from multiple parameters on cellular interactions require investigation on a case-by-case basis, allowing improved particle design informed by these characteristics.

Many drugs, such as peptides, proteins, DNA, and RNA, are cellmembrane-impermeable and degrade in the acidic environment of lysosomes. Therefore, for an effective therapeutic response, it is critical for cargo to escape from these endosomal compartments (Scheme 1). The mechanisms by which internalized particles can escape from endosomes are complex and not yet fully understood. Proposed mechanisms of endosomal escape include the proton sponge effect, membrane destabilization, and osmotic shock. The proton sponge effect is mediated by, for example, polymers with a high buffering capacity. During acidification of the endosomes, an increase of endosomal osmolarity occurs as a

result of polymer protonation. Ultimately, this process causes lysosomal rupture and particle release into the cytoplasm. A number of cationic polymers, such as polyethylenimine (PEI), have been shown to promote the proton sponge effect.29 Core□ shell nanoparticles comprising a poly(ethylene glycol) dimethacrylate (PDEAEMA) core and a poly-2-aminoethyl methacrylate (PAEMA) shell have been shown to escape the endosomes via the proton sponge effect and effectively deliver ovalbumin to the cytoplasm of dendritic cells.30 While the proton sponge effect has been observed for a number of polymers, it remains unclear why not all particles composed of cationic polymers can cause endosomal escape by this mechanism. For example, K8-functionalized liposomes showed accumulation in lysosomes following internalization via macropinocytosis.26 In contrast, R8-functionalized liposomes were internalized via macropinocytosis and subsequently escaped from the endosomes.26 The difference in intracellular fate was attributed to ability of R8 to facilitate liposome fusion with the endosomal membrane over a broad pH range, whereas K8 fusion is limited at low pH. This suggests that membrane destabilization is another important factor that mediates endosomal escape. With the rapid development of responsive polymer particle systems, an “osmolytic” approach has also been demonstrated to stimulate endosomal escape. In this approach, responsive particles can rapidly disassemble to smaller particles or individual polymers in endosomes, which leads to increases in endosomal osmotic pressure. Such osmotic pressure can further induce temporary osmolysis of the endosomal membrane to release the particles into the cytoplasm. Critically, the responsiveness of particles in the endolysosomal environment and the stability of particles in the extracellular conditions must be carefully balanced. A pH-responsive polymersome, poly-(2-(methacryloyloxy)ethyl phosphorylcholine)-co-poly(2-(diisopropylamino)ethyl methacrylate) (PMPCPDPA), has been shown to destabilize in endosomal compartments and release cargo to the cytosol.31 For effective gene delivery, nuclear translocation of the released DNA from the cytoplasm is another ratelimiting step (Scheme 1). Recent knowledge on nucleocytoplasmic delivery mechanisms has shown that DNA associated with specific nuclear localization signals (NLS), such as PKKKRKV, can be actively transported to the nucleus to improve transfection efficiency.32 It is anticipated that detailed knowledge of particle uptake mechanisms and intracellular trafficking will provide a roadmap of the cellular “highway” that regulates the motility of particles, open new possibilities to overcome cellular barriers, and direct improved particle design.

OUTLOOK AND FUTURE CHALLENGES

The past few decades have witnessed the evolution of particlebased therapeutics, from concept to clinical reality. Driven by innovations in enabling technologies and chemistries, many novel particle systems, such as filomicelles, PRINT particles, LbL capsules, and polymersomes, have been developed. The ability to control physiochemical properties of particles, such as surface functionality, size, shape, and release mechanisms, strongly supports the continuing promise of tailor-made particles for a range of biomedical applications. In combination with the development of biomarkers and novel therapeutics, the next generation of targeted particles is expected to yield effective new therapies. These advances will arise from the ability to formulate novel classes of particle-based of therapeutics, the ability to deliver drugs specifically at cellular and subcellular levels, and the ability to spontaneously deliver multiple drugs for combination therapy. However, a significant knowledge gap still exists, as understanding the dynamic and complex interactions between particles and biological systems is far from complete.

Studies have identified several important physiological concepts in particle delivery, including the mononuclear phagocytic system for particle clearance, enhanced retention and permeability effects for particle accumulation, and endolysomal compartments for particle entrapment. There are relatively few studies on how the physical and chemical properties (e.g., size, shape, deformability, and surface

functionality) of particles influence their biodistribution, cellular uptake, and intracellular trafficking. An improved understanding of the principles governing particle cell interactions will undoubtedly shed light on key issues, including triggered release, therapeutic efficacy, and particle toxicity. Given the complexity and heterogeneity of most human diseases, understanding the biological interactions dictated by the physicochemical properties of particles will be essential for the development of nextgeneration particle delivery systems and for continued progress in translational research.

Conflict of Interest: The authors declare no competing financial interest.

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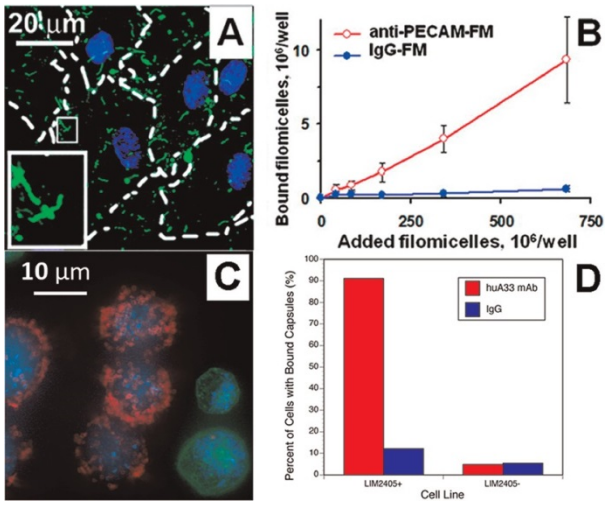
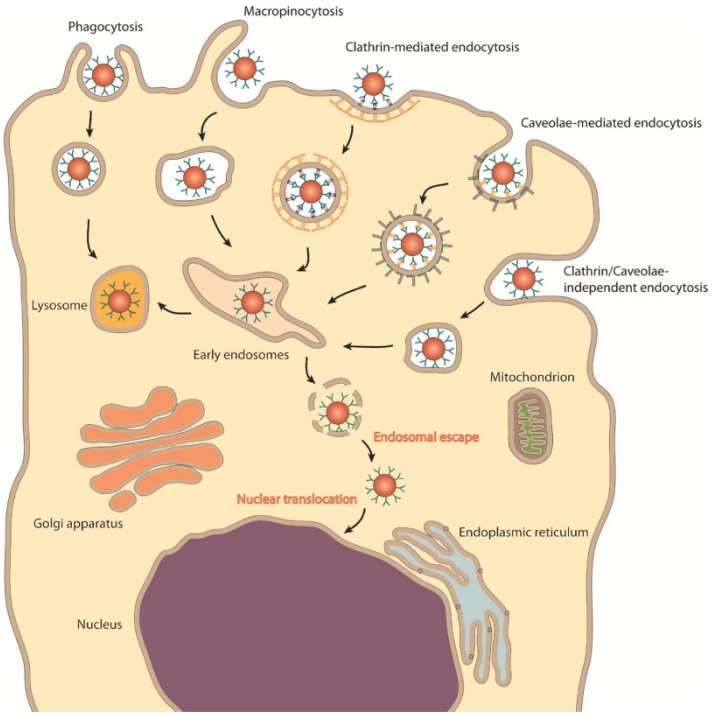
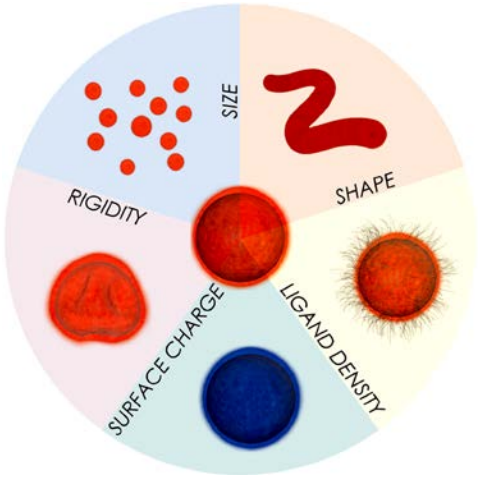


Figure 1. (A) Fluorescence micrographs of antiplatelet endothelial cell adhesion molecule-1 (PECAM)-functionalized filomicelles (green) bound to endothelial cells after incubation for 1 h at 37 °C. The cell nuclei were stained with DAPI (blue). Dashed lines mark the cell borders. The inset shows a magnified image of the filomicelles. (B) Quantification of binding of 125I-traced filomicelles coated with IgG or anti-PECAM to endothelial cells after incubation for 1 h at 37 °C. Reproduced from ref 12. Copyright 2011 American Chemical Society. (C) Fluorescence microscopy images of LIM2405þ cells (blue) and LIM2405□ cells (green) incubated with huA33 mAb-functionalized capsules (red). (D) Flow cytometry analysis of the binding of capsules to mixed cell populations. Comparison of huA33 mAb- (red) and IgG-functionalized (blue) capsules incubated at capsule/cell ratios of 100:1 with a 50:50 ratio of LIM2405þ/LIM2405□ cells. Reproduced from ref 15. Copyright 2010 American Chemical Society.



Scheme 1. Schematic of the cellular uptake and intracellular trafficking of targeted particles. Particles are internalized by cells via several endocytic pathways. Endosomal escape and nuclear translocation of particles are two major cellular barriers for effective delivery of membrane-impermeable therapeutics, such as peptides, RNA, and DNA.



Scheme 2. Key physicochemical properties of particles that influence particle cellular uptake.

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