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ENGINEERING PARTICLES FOR THERAPEUTIC DELIVERY: PROSPECTS AND CHALLENGES*

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Building on many years of basic and translational research, advances in the field of nanotechnology and biomedical science are now converging to revolutionize the treatment of a range of diseases.[1,2] To date, several types of particle-based 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™) has 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, have 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.

Here we highlight recent developments in particle-based 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 preferentially accumulate in tumor sites. The EPR effect arises due to the high fluid flow and large leaky vasculature within many solid tumors. Treatments exploiting the

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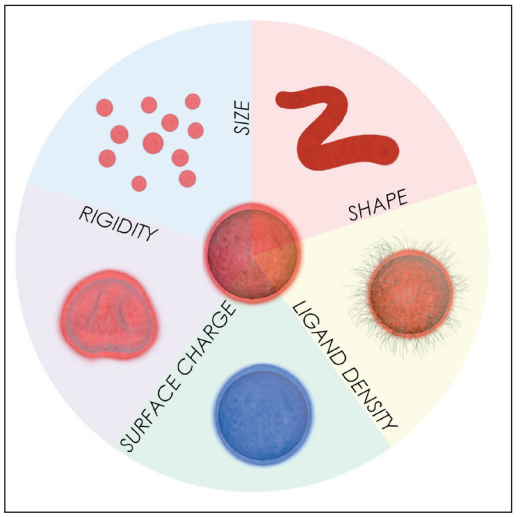
EPR effect have shown some therapeutic benefit.[4] However, this passive targeting strategy still faces some challenges. The longer circulation times of drug-loaded particles can lead to adverse effects, as has been observed with DOXIL™, which can cause severe hand-foot syndrome.[4] In addition, the 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 particle-based therapies.

Over the last 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. Recently, the first clinical investigation using transferrin-functionalized nanoparticles for siRNA delivery (CALAA-01) was reported.[8] These particles were generated *via* a unique two-vial formulation approach, which allowed for 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 the treatment of CALAA-01 showed a favorable safety profile and effective siRNA knockdown by the particles.[8]

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, drug-loaded liposomes were modified with anti-HER2 or anti-EGFR antibodies.[9] 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*. 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 increased toxicity. Recently, by screening thousands of EGFR monoclonal antibodies for tumor specificity, an antibody that 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.[10] 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. Based on 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 serve to help differential cell targeting using tissue-specific antibodies. This has been exemplified by a series of studies on the highly tissue-specific 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.[11] Recently, the potential for using particles functionalized with this antibody for colorectal cancer targeting was investigated *in vitro* using layer-by-layer (LbL) capsules.[12] Highly specific binding was observed to the targeted A33 positive cells, even when this population was only 0.1% of the total cells. 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,[13] it is envisaged that materials scientists, biologists and clinicians will continue to develop targeted delivery systems with enhanced efficacy and specificity. Effective therapy generally requires transportation of therapeutics to specific cells. Therefore, there has been a surge in recent years into the investigation of the cellular uptake of particles. Based on the dynamic nature of endocytosis, it is not surprising that the cellular uptake of particles is dependent on many factors,



Scheme 1. Key physicochemical properties of particles that influence particle cellular uptake.

including cell physiology and particle properties.[14] Here, we focus on a few seminal studies on various particle systems to exemplify several important physicochemical parameters (Scheme 1).

Particle size is a key property that affects the cellular uptake rate, as it influences the endocytic pathway. 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.[15] 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 sub-micron spherical particles of a similar internal volume.[16] The different uptake efficiency was due to a greater utilization of multiple internalization mechanisms by the high AR cylinders through cellular interactions at multiple non-symmetric axes.[16] Similarly, Mitragotri and coworkers 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 the phagocytosis was sensitive toward the interaction axis for these particles, as the spheres were seen initially to be internalized more rapidly.[17] This kinetic phenomenon was exploited for immune system evasion and improved particle biodistribution *in vivo* by Discher and coworkers.[18] 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 (35.84 and 136.28 kPa) were found to be internalized by macrophages *via* multiple mechanisms. After 4 h incubation, the both nanoparticles with intermediate elasticity showed approximately 67% higher internalization compared to their softer counterparts (Young's modulus of 18.04 kPa), and 25% higher uptake compared to the more rigid nanoparticles (Young's Modulus of 211.39 kPa).[19] 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 negatively charged particles, presumably due to the negatively charged cell membrane. In addition, particle surface chemistry and functional group density plays an important role in particle endocytosis. Taken together, the complex effects arising from multiple parameters on cellular interactions requires investigation on a case-by-case basis, allowing improved particle design informed by these characteristics.

As many therapeutic targets are localized at certain subcellular sites, effective delivery needs to be further optimized at subcellular levels. Many drugs, such as peptides, proteins, DNA and RNA are cell membrane-impermeable and degraded in the acidic environment of lysosomes. Therefore, for an effective therapeutic response, it is critical for cargo to escape from these endosomal compartments. 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.[20]

Recently, 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.[21] While the proton sponge effect has been observed for a number of polymers, it remains unclear why some particles comprised of cationic polymers can cause endosomal escape by this mechanism. For example, K8-functionalized liposomes showed accumulation in lysosomes following internalization *via* macropinocytosis.[22] In contrast, R8-functionalized liposomes were internalized *via* macropinocytosis and subsequently escaped from the endosome.[22] The difference in intracellular fate was attributed to the ability of R8 to facilitate the 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 to mediate 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 an increase 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 endo-lysosomal 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) (PMPC-PDPA), has been shown to destabilize in endosomal compartments and release cargo to the cytosol.[23] It is anticipated that a 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 particle-based 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 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 next-generation particle delivery systems and for continued progress in translational research.

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