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Particle Targeting in Complex Biological Media

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Abstract: Over the past few decades, nanoengineered particles have gained increasing interest for applications in the biomedical realm, including diagnosis, imaging, and therapy. When functionalized with targeting ligands, these particles have the potential to interact with specific cells and tissues, and accumulate at desired target sites, reducing side effects and improve overall efficacy in applications such as vaccination and drug delivery. However, when targeted particles enter a complex biological environment, the adsorption of biomolecules and the formation of a surface coating (e.g., a protein corona) changes the properties of the carriers and render their behavior unpredictable. For this reason, it is of importance to consider the potential challenges imposed by the biological environment at the early stages of particle design. This review describes parameters that affect the targeting ability of particulate drug carriers, with an emphasis on the effect of the protein corona. We highlight strategies for exploiting the protein corona to improve the targeting ability of particles. Finally, we provide suggestions for complementing current in vitro assays used for the evaluation of targeting and carrier efficacy with new and emerging techniques (e.g., 3D models and flow-based technologies) to advance fundamental bio-nano science understanding, and accelerate the development of targeted particles for biomedical applications.

1. Introduction

Early studies using particulates as drug carriers were conducted in the 1960s,[1] and since then, there has been a plethora of carriers developed for drug delivery, including liposomes, micelles, dendrimers, and polymeric and inorganic particles.[2] The field of nanotechnology provides an avenue to engineer carriers with tailored composition, morphology, dimensions and surface properties, all of which can influence the biodistribution and pharmacokinetic profile of an encapsulated drug.[2a-c,3] In addition, the potential to control critical parameters such as dosage, bioavailability and spatiotemporal release of the drug has fueled research interest in the application of particles, such as for the codelivery of drugs, for cancer therapy as well as for a wide range of other biomedical areas.[4] For example, Doxil, a liposomal formulation of doxorubicin (Dox), was one of the first nanotherapeutics approved by the FDA in 1995 to treat patients with Kaposi's sarcoma. Approval in Europe followed in 1997 for the treatment of ovarian cancer, breast cancer and multiple myeloma.[5] Doxil offers prolonged drug circulation time and enables the loading of a high amount of drug within an enclosed carrier.

The need to spatially control the localization of administered particles (e.g., for imaging of tumors or for delivering vaccine antigens to lymph nodes) has led to research into strategies for targeting particles to specific locations in vivo. The targeting of particles has been referred to occur either "passively" or "actively", based on the mechanisms by which the drug carriers accumulate at their targeted sites.[6] Passive targeting is based on the accumulation of drug carriers in specific tissues (e.g., tumor tissues) via biological phenomena that include "vascular bursts" and the "enhanced permeability and retention" (EPR) effect. Both mechanisms allow macromolecules and particles (approximately 10 to 500 nm in diameter) to escape the vascular bed and accumulate inside the interstitial space.[7] To this end, prolonged carrier circulation time is critical, which is usually promoted by small

carrier size and low-fouling properties of the carrier, which reduce phagocytic and renal clearance. Active targeting, or ligand-mediated targeting, can build on the effects of passive targeting, and often involves the recognition and binding to receptors on specific cells and tissues of drug carriers surface modified with targeting ligands.[2b,8] Since targeting ligands on a particle surface have the potential to induce receptor-mediated cell internalization and intracellular trafficking, active-targeting can therefore facilitate intracellular accumulation of drug and carrier.[9]

Despite recent advances, the clinical translation of nanotherapeutics remains a significant challenge and their prospective benefits is a topic of much debate.[10] For example, in a recent article analyzing the delivery efficiency of nanoparticles to tumor tissues, Chan and coworkers found that in the 117 studies investigated, the median delivery efficiency was 0.7%, with actively targeted systems achieving a median efficiency of 0.9% compared with 0.6% for passively targeted ones.[11] Additionally, the median delivery efficiency remained largely static (at less than 1%) from 2005 to 2015. While this analysis has sparked considerable debate—for example, about whether delivery efficiency is a good metric and, more generally, what the goal of nanomedicine should be[12]—it highlights the potential limitations in the field, despite the great developments achieved in the understanding of bio-nano interactions[6,13] and our increasing ability to tailor particle design and synthesis.[14] Researchers have become increasingly aware of the complexities involved in developing carriers suitable for the clinic, and in particular, the challenges associated with developing actively-targeted carriers. In many cases, promising in vitro targeting results have not translated well in vivo. Despite being chemically well-designed, many multifunctional systems fail in vivo, often due to rapid clearance by the mononuclear phagocytic system (e.g., liver, kidneys, and lymph nodes). Similarly, targeted particles that perform well in simpler environments (e.g. in vitro in saline) can be compromised when exposed to more complex

biological environments (e.g., blood) due to the rapid (< 30 s) absorption of biomolecules on the particle surface and the formation of a protein corona that ultimately influences particle behavior.[15]

Recent advances in proteomic, biophysical and computational methods[16] have increased our understanding on the formation and influence of protein coronas on particle composition and interactions.[17] Reviews in this field cover topics such as: the bio-nano interface;[18] the protein corona;[19] how the protein corona affects colloidal stability, cellular interactions and toxicity of particles;[20] the effect of protein coronas on particle "stealth" properties;[21] and how directed synthetic evolution can be used to tailor particle compositions to achieve functional protein coronas.[22]

In this review, we focus on the effect of complex biological media on particle targeting. We begin with particle design, which includes a discussion of the commonly used targeting ligands for functionalization. We then highlight how particle properties and different biological environments affect the performance of targeted drug carriers, and hence, targeting outcomes. We discuss the role of protein corona formation and efforts towards reducing, tuning, and exploiting the protein corona to optimize particle targeting. We revisit important factors when evaluating particle targeting in biological environments in vitro (e.g., fluidic flow, heterogeneous cell models, and the role of the extracellular matrix (ECM)) and suggest improved and complementary in vitro methods and models to help better understand and predict the interaction of particles with biological environments. The overall aim of this review is to provide insight into the design-performance correlation (in regards to targeting) of particles in complex biological media (**Figure 1**), and to guide future efforts towards combining this knowledge with adequate models to increase fundamental bio-nano understanding while advancing the translation of targeted particles into the clinic.

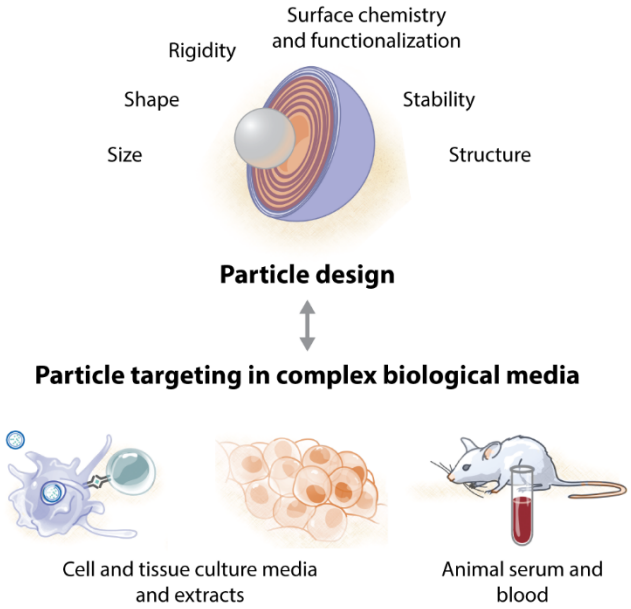

Figure 1. Particle targeting in complex biological media is determined by both the physicochemical properties of the particles and by the biological environment. Parts of this figure are adapted with permission.[13b,23] Copyright 2016 American Chemical Society.

2. Targeting Strategies for Drug Carriers

The targeting effect of drug carriers can be divided into passive and active targeting (**Figure 2**). Passive targeting was first observed in 1986 by two independent studies investigating increased blood vessel permeability and macromolecule retention in tumors.[7b,24] Due to the hypervascularization that can occur in tumor tissues, blood vessels supplying the tumor grow abnormally and can become leaky, allowing macromolecules to permeate into the tumor tissue. At the same time, an impaired lymphatic system around the tumor tissue can lead to enhanced retention and hence accumulation of macromolecules in the tumor.[25] However, EPR in tumors is highly heterogeneous and depends on a number of factors, e.g., vascular dynamics, systolic blood pressure, size and type of tumor (primary lesion vs. metastatic

cancer), which has led to a controversial debate about the clinical value of the EPR effect.[7d,26]

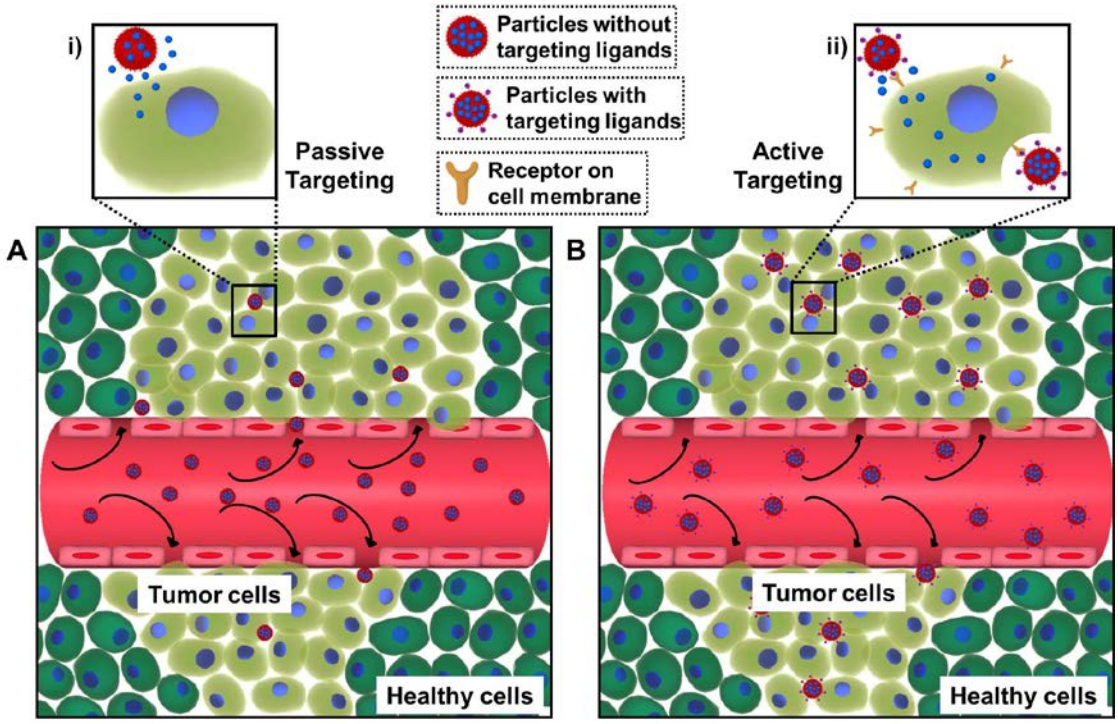

Figure 2. Schematic illustrations of passive targeting (A) and active targeting (B). Particles without a targeting ligand can accumulate in tumor tissue (light green) via the EPR effect (A) and release therapeutic agents, which can be taken up by tumor cells (i). Once particles have accumulated in the target tissue, particles with targeting ligands can achieve active targeting via specific ligand-receptor interactions (B). Active targeting can result in the enhanced accumulation of particles in the target tissue and enhanced cellular uptake of particles via receptor-mediated endocytosis (ii).

Due to this heterogeneity, there is still limited understanding of this effect in humans and also how realistically a preclinical tumor model represents the situation in cancer patients.[26a,b] Efforts towards a better understanding of the EPR effect in humans were made by Wong and coworkers,[27] who quantitatively analyzed pharmacokinetic data from clinical trials with Doxil and doxorubicin[28] to provide a model on the impact of the EPR effect on drug uptake

and accumulation in solid tumors. Using their model, they found that tumor uptake shows a characteristic maximum, with loss of drug from the tumor at longer times, when taking into account intravasation back into circulation.[27]

In contrast to drug accumulation via the EPR effect, actively-targeted drug carriers are designed to bind to specific receptors on the cell membrane via ligands tethered to the carrier surface.[2a,b] This interaction is intended to increase the drug concentration at a desired location, induce receptor-mediated cell internalization and minimize harmful side effects by reducing off-target accumulation. Active targeting often builds on passive targeting, which can enhance both extracellular and intracellular accumulation of the drug. In some cases, passive targeting can be ineffective, as observed for vascular endothelial targeting, and active targeting approaches are then necessary.[26c,29] Over recent years, there has been an increasing awareness that the terms “passive” and “active” targeting may be misleading, in particular when considering that more than 95% of administered particles accumulate in organs other than the tumor (e.g., liver, spleen, and kidney).[30] Bae and Park suggested the use of “blood circulation and extravasation” instead of “passive targeting”, and similarly, to replace “active targeting” with “ligand-receptor-mediated targeting”.[30a] “Active” targeting can also imply the existence of an active mechanism transporting a particle towards its intended target, which is not the case for most particle-based systems where ligand-receptor interactions require close proximity of the two binding partners.[30] It may therefore be more suitable to consider targeting ligands as “anchors” that attach a particle to a receptor that is already in close proximity, i.e., it is not actively “seeking out” its target.[30a] In summary, passive targeting attempts to describe the relatively higher accumulation of particles or macromolecules observed in tumor tissues compared to healthy tissues,[7d,25] while active targeting refers to a specific ligand-mediated interaction or binding between the particle and a cell receptor.[2a,b,31] To date, both terms (active and passive targeting) still find widespread

use in the literature[2a,31-32] including many research papers discussed herein, and are therefore used in this review.

2.1. Targeting Ligands

As mentioned above, actively-targeted drug carriers are designed to bind to specific receptors via targeting ligands. To date a number of overexpressed, disease-associated receptors and antigens have been reported that present attractive targets for ligand-mediated binding and accumulation of targeted drug carriers.[2a,b] The choice of ligand may depend on the degree of targeted receptor expression, the occurrence of endogenous competing ligands, binding affinity, and whether receptor-mediated binding results in particle internalization (**Figure 3**).[33] Monoclonal antibodies (mAbs) or their fragments are widely used as targeting ligands, either as antibody (Ab)-drug conjugates,[34] as pretargeting agents,[35] or tethered to the nanoparticle surface. Liposomes functionalized with mAb, or mAb-liposomes, were early examples of targeted nanoparticles (NPs), with studies published in 1980—six years before the clinical approval of the first murine-derived mAb (Muromomab-CD3 for an autoimmune disorder).[36] Since then, advances in Ab engineering (e.g., complementary determining region (CDR) grafting, phage display technologies, and expression in transgenic mice) have enabled the production of humanized and whole human Abs, as well as the engineering of Ab fragments (e.g., antigen binding fragment (Fab) and single chain antibody (scFv)) to reduce the immunogenicity associated with chimeric and murine-derived Abs.[37] Abs are key players in modern therapeutics with more than 50 different types approved by the FDA for clinical use.[38] Despite this success, there are a number of challenges associated with their use as ligands on drug carriers. Abs can be relatively large in size (150 kDa and around 5-6 nm in hydrodynamic radius[39]) for small nanoparticles and are sensitive to environmental conditions (e.g., ionic strength, temperature, and organic solvents), in which NPs are

commonly prepared.[2a,40] Furthermore, the Fc fragment of a whole Ab can interact with the Fc receptor expressed at the surface of several cell types and activate the mononuclear phagocyte system (MPS), which can lead to off-target effects and rapid clearance from blood circulation, respectively. This can be partly overcome by attaching the Fab fragment only.[2a,37,40]

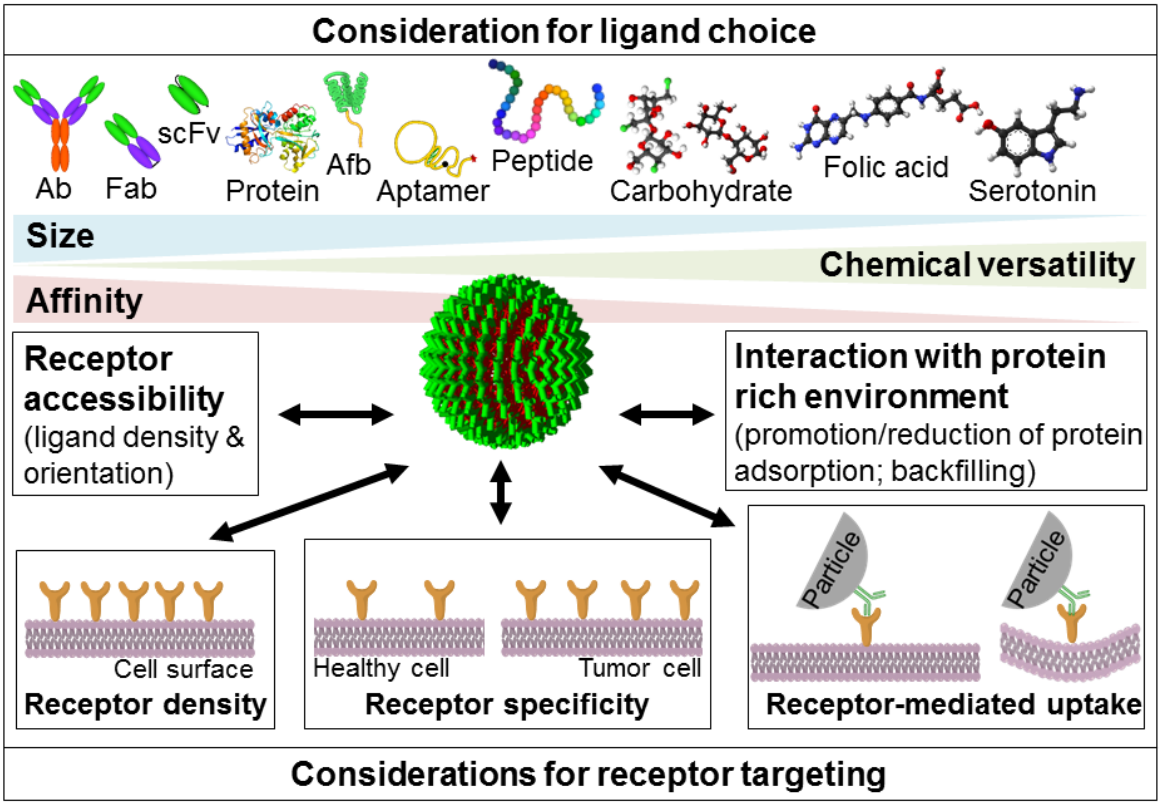

Figure 3. Schematic illustration of key factors that need to be considered in the design of targeted drug carriers.

Single domain Abs (sdAbs), bacteria-derived affibody molecules (Afbs), and similar affinity proteins have also been developed to address common challenges related to the use of whole mAbs.[41] These types of peptides are smaller in size (typically around 5–15 kDa) compared with full length Abs (~150 kDa), but maintain a similarly high binding affinity and can be engineered with multiple functionalities.[41a,b] For example, bispecific affinity proteins were recently engineered using combinatorial protein engineering to bind the cancer-associated

human epidermal growth factor receptor 2 (HER2 or ERBB2) with subnanomolar affinity, while having tunable affinity to albumin.[42] A similar affinity protein modified with a chelator has also shown promise for in vivo imaging.[43]

Endogenous proteins have also been used for the functionalization of particles in active targeting strategies. Transferrin (Tf), an iron-transporting 80 kDa glycoprotein, is one of the most prominent examples and targets membrane-bound Tf receptors (TfRs), which are commonly overexpressed in tumor cells.[44] Recently, Singh et al. highlighted that active targeting of drug loaded Tf-functionalized poly(glycidal methacrylate) (PGMA) particles is effective at low docetaxel dosages (2 mg kg^{-1}) in an orthotopic PC3 prostate cancer model.[45] At higher dosages (12 mg kg^{-1}) negligible difference between the targeted and nontargeted particles was observed, suggesting a dominant role of the EPR effect for NP accumulation in the tumor.[45] Endogenous proteins as targeting ligands can have similar limitations as some Abs, including low targeting specificity if receptors are also expressed on healthy cells, immunogenicity, size, and difficulties in finding compatible grafting strategies without compromising the targeting ability. In this context, peptides of usually less than 50 amino acids present a promising class of targeting ligands.[2a] Peptides are smaller in size, are typically more stable compared to their protein counterparts, and can be easier to modify and attach to particles using common functionalization procedures.[2a] A number of peptide moieties have gained particular attention for their potential cell-penetrating ability. Among these are arginine-glycine-aspartate (RGD) and internalizing RGD (iRGD) motifs, which bind to the $\alpha_v\beta_3$ integrin receptor expressed on tumor cells and angiogenic endothelial cells.[2b] Liposomes with dual peptide targeting moieties (RGD and a galectin-specific peptide), showed synergistic effects in vitro and showed promise as agents for imaging and inhibition of angiogenesis.[46] Targeting to the brain and overcoming the blood brain barrier (BBB) is another important application for peptide ligands.[47] Prades et al. designed a retro-

enantio 12 amino acid peptide sequence to target the TfR at a binding site different to Tf.[48] TfRs are heavily expressed in the brain microvasculature. Retro-enantio peptides share similar structure to the parent peptide but show higher stability from proteolytic cleavage. The retro-enantio peptide outperformed Angiopep-2, which has excellent BBB permeability, and delivered cargo, including carboxyfluorescein, iron oxide NPs and quantum dots (QDs), across the BBB.[48] In a different approach, Oller-Salvia et al. synthesized a peptidomimetic motif inspired from bee venom, that was resistant to serum proteases and increased the translocation of 12 nm gold (Au) NPs by 20 times in a human cell-based BBB model.[49]

Aptamers, also referred to as 'chemical Abs',[50] are short single-stranded (ss) nucleic acid sequences (ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) oligonucleotides) that can be engineered to have high (e.g., nano- to picomolar) affinity to their receptors. Compared to Abs and similar molecules, aptamers can often be synthesized at a lower cost and are easier to scale up for production, as well as being typically more stable at high temperature.[50a] A limitation with RNA-based aptamers is that the presence of the 2'-OH group on ribonucleotides makes them susceptible to cleavage by degradative RNases, which are ubiquitous in vivo. However, chemical modification of the 2'-OH group or the use of ssDNA aptamers can reduce degradation and increase their half-lives in biological environments.[50a] Conjugation of the A10 RNA aptamer, which specifically binds to the prostate-specific membrane antigen (PSMA), to poly(lactic-*co*-glycolic acid)-*block*-poly(ethylene glycol) (PLGA-b-PEG) copolymer particles (150 nm in diameter), yielded specific drug carriers that efficiently delivered docetaxel in a tumor xenograft nude mouse model.[51] A DNA aptamer AS1411 has been used to target nucleolin, a protein that is highly expressed in the plasma membrane of tumor cells and on the surface of endothelial cells in angiogenetic blood vessels.[50a] Guo et al. showed a substantial increase in tumor growth inhibition of glioma xenografts in mice when treated with AS1411-decorated and paclitaxel

loaded PLGA-PEG particles compared to the free drug or to particles without the aptamer AS1411.[52]

Small molecules are an alternative class of targeting ligands. The advantages of small ligands over the targeting moieties discussed above typically include simplicity, cost, availability and low immunogenicity. Many of them are amenable to common synthetic functionalization procedures, and can provide tunable, high ligand densities on particle surfaces due to their small size. Folate, a vitamin B derivative, has been extensively used to target over-expressed folate receptors on tumor cells.[53] Carbohydrates are an emerging class of targeting ligands due to their natural abundance and ease of chemical functionalization. A number of different cell- and disease-associated receptors that can be targeted by carbohydrates have been identified.[54] In addition, carbohydrates are attractive because of their 'low-fouling' nature, which is particularly important for applications in protein rich environments (e.g., in blood).[55] The typically low binding affinity of carbohydrates to their receptors can be improved by multivalent presentation on a particle surface, thereby resulting in high binding avidity.[56] Galactosyl moieties have been applied to direct particles to asialoglycoprotein receptors, which are dominant on the surface of hepatocellular carcinoma HepG2 cancer cells.[57] Lactosylated particles have also been designed for the targeting and detection of galectin-1, a carbohydrate binding protein that plays a central role in cell proliferation, migration and tumor angiogenesis.[58] In addition, the functionalization of PEGylated hydroxyethyl starch nanocapsules with mannose molecules led to an increase in binding and uptake by mature dendritic cells.[59]

The functionalization of nanoparticles with targeting ligands adds a level of complexity to particle design. Along with the challenges associated with the ligands themselves (e.g., immunogenicity, selectivity, accessibility, stability), the functionalization procedure can cause instability or particle aggregation, and can increase manufacturing costs.

It may be promising to look for alternative avenues for the incorporation of a targeting moiety, such as the specific recruitment of functional proteins that can provide the targeting ability from the biological environment itself (as discussed below in Section 4.3).

3. Designing Drug Carriers for Targeting

In the past few decades, a wide range of actively-targeted drug carrier systems has been designed with considerable variation in targeting outcomes.[2a,b,8,13b] Great effort has been placed into the study of factors that influence targeting ability to aid in the design of improved carriers for delivery. In general, the targeting ability of drug carriers is affected by both the intrinsic particle properties and the biological identity that is imposed on the drug carrier when it enters a biological environment.[60] In this section, we discuss how particle properties and the formation of a protein corona influence the targeting ability of drug carriers. We highlight studies on actively-targeted drug carriers performed in complex biological milieus, and compare carriers that maintain targeting ability with those that were impeded by the biological environment. This section specifically focuses on the influence of protein coronas on the targeting ability of actively-targeted drug carriers. For more in-depth discussion on the nature of protein corona formation, readers are referred to other reviews.[19a,b,21b,60-61]

3.1. Effects of Particle Properties on Bio-Nano Interactions

The physicochemical properties of particles, such as size, shape, rigidity, roughness, surface charge, and surface chemistry, can result in different targeting outcomes (**Figure 4**).[2b,62] Many studies have been carried out to optimize these properties to achieve better targeting results. Among these properties, particle size is an important factor, since it influences in vivo circulation time, the biodistribution of the particles, and the internalization mechanism of

particles by cells.[2b,63] While smaller particles are typically easier to internalize, microparticles with dimensions larger than 1 μm can also be internalized by standard cell lines such as HeLa cells (i.e., cells that are not professional phagocytes).[62a] As particle size increases, the surface area per particle also increases, which can lead to greater interactions with opsonins in the blood, and result in faster clearance of the particles by macrophages in organs such as the spleen and the liver.[64] On the other hand, smaller nanoparticles (less than ~5 nm) can be rapidly eliminated from circulation by the kidney.[65] Clearance from circulation is therefore a major barrier for either smaller or larger particles in their goal to reach specific or targeted sites.

Particle size can influence the endocytic pathway used by cells to internalize particles. Micrometer-sized particles enter the cell via mechanisms such as pinocytosis and micropinocytosis, while smaller particles (<500 nm) can be internalized through clathrin- and cavaolae-mediated endocytosis.[66] Clathrin- and cavaolae-mediated cell entry can be facilitated by surface functionalization with ligands that target these endocytotic pathways. In a study with gold nanoparticles, greatest cellular internalization and cellular response under in vitro conditions was demonstrated with Ab-functionalized Au NPs of 40–50 nm diameter.[67] Particle size also influences ligand multivalency on particle surfaces. Jiang et al. suggested that larger particles with greater surface area and lower surface curvature allowed a higher protein-to-particle ratio and increased the Ab density on the particle surface.[67]

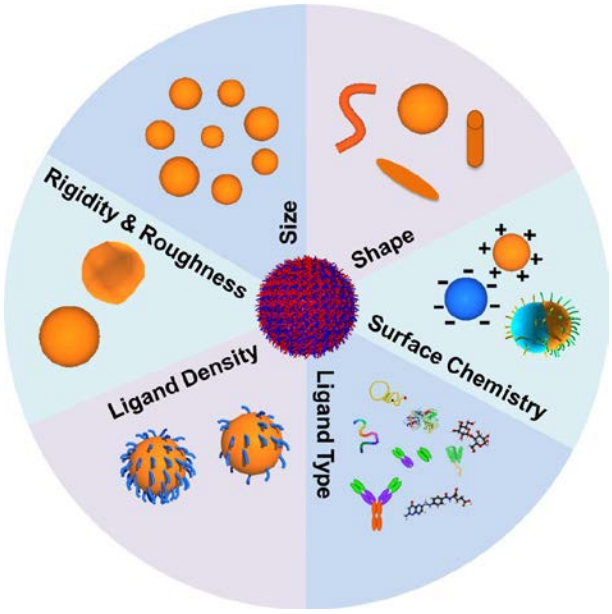

Figure 4. Key physicochemical properties that influence the targeting outcome of particles.

The geometry or shape of engineered particles is also an important factor that can influence the outcome for targeted particles. Particle shape has been reported to play an important role in cellular internalization, biodistribution, and circulation of particles, either independently or coupled with particle size, under defined conditions.[68] Champion et al. showed that the shape of particles, with their size within the range of the cell volume, played a dominant role in the phagocytosis of polystyrene (PS) particles by macrophages.[69] They found that the shape of particles at the particle-cell contact point influences the structural arrangement of actin, which is essential for initiating phagocytosis.[69] Similarly, Sharma et al. showed a profound impact of the particle shape on phagocytosis of PS particles by macrophages, where oblate ellipsoids were more easily internalized than prolate ellipsoids or spheres.[70] Cell attachment and internalization were influenced independently by particle shape. Prolate ellipsoids showed the highest cell attachment, but the lowest internalization rate after binding to cells, while oblate ellipsoids had the highest internalization rate after attaching but a lower overall cell attachment.[70] Shimoni et al. reported that the human cervical cancer cell line HeLa displayed a preference for spherical polymeric capsules

compared with rod-like capsules.[71] The polymeric rod-shaped capsules exhibited lower and slower cellular internalization with increasing aspect ratio (AR).[71] In a study by Decuzzi et al., the shape of particles was found to influence the biodistribution of intravenously administered, silicon-based particles (**Figure 5**).[72] The discoidal particles were observed to accumulate less in the liver than particles with other shapes, which highlights the possibility of using shape to help optimize the accumulation in the targeted organ whilst minimizing the elimination of particles by the liver.[72] Similar to particle size, the shape of particles can result in different ligand densities on particle surfaces due to diverse geometries governed by different shape.[68a] This could lead to varying levels of interactions between particles and their targets. Additionally, the adhesion of particles to the vascular wall depends on many parameters that can be influenced by the shape of particles, including margination tendency, hydrodynamic drag force and contact area.[73] Therefore, particles with different shapes can exhibit differing adhesion strengths to the vascular wall, which can influence both passive and active targeting outcomes.[29,73]

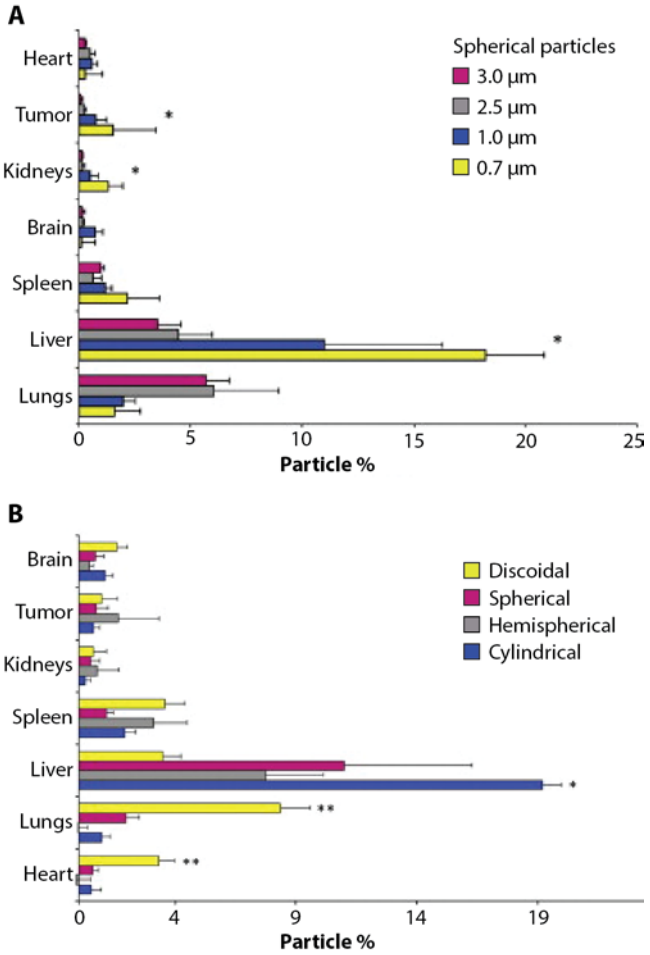

Figure 5. The effect of particle size and shape on the biodistribution of silicon particles in mice. (A) The biodistribution of spherical silicon particles of various sizes. (B) The biodistribution of silicon particles of various shapes and at constant volume. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used to quantify the amount of accumulated Si in the corresponding organs. Reproduced with permission.[72] Copyright 2010 Elsevier B.V.

Another set of factors that substantially impacts the targeting ability is the surface characteristics of the particles, including surface charge, hydrophobicity, roughness, and additional surface modifications.[2b,63a,74] These surface characteristics influence the coupling efficiency of targeting ligands onto the surface of particles, which directly determines the

particle multivalency.[2b,63a] For example, maleimide groups on particle surfaces are often exploited for the attachment of thiol-functionalized targeting ligands, such as Abs. If unreacted or not sufficiently quenched, maleimide groups can play a critical role in the adsorption of proteins and biomolecules and impede the targeting ability of particles.[75] Furthermore, these characteristics play significant roles in the non-specific interactions of particles with different tissues or cells, which indirectly influence the targeting outcome.[2b,63a] The surface charge of particles is a major parameter that contributes to nonspecific cellular binding and internalization. Positively charged particles promote cell membrane binding, followed by enhanced cellular uptake,[76] due to interactions between cationic particle surfaces and negatively charged phospholipid head groups, proteins and glycans on cell membranes.[2b] Moreover, charged particles bind serum proteins more easily and induce opsonization, such as complement activation, which leads to the quicker clearance of particles by the immune system.[2b] He et al. suggested to engineer particles with a ζ-potential below +15 mV to reduce phagocytic uptake, and to allow for longer circulation time and higher tumor retention.[77] Similarly, the hydrophobicity of the particle surface influences the targeting results by affecting the interactions of particles with both cells and proteins. Particles with more hydrophobic surfaces were reported to have higher cellular uptake and protein adsorption levels, which led to higher levels of opsonization and shorter half-lives.[2b]

Particle surface charge and hydrophobicity can be tuned by coupling ‘stealth’ polymers, such as PEG, onto their surfaces. The highly hydrophilic PEG has been extensively studied and widely used in various particle systems to prolong in vivo circulation time.[78] Additionally, PEG can be modified with different functional groups, including methoxy, carboxy and amine groups, which can modulate the particle surface charge.[79] Particles with carefully tuned surface charge and hydrophobicity often exhibit longer circulation times, and can have improved retention at the targeted site. In addition, surface roughness and rigidity

affects the behavior of particles, and can also influence targeting outcomes by affecting the cellular binding and in vivo biodistribution profile of particles, respectively.[2b,80] The in vivo degradability of particles, particularly particles with a polymer shell, is another factor that can affect the targeting outcome of surface-modified particles.[81] Radioactively labeled Au NPs (^{198}Au) with a polymer coating that was labeled with a different radioisotope (^{111}In) were found to partially disintegrate upon injection into rats, resulting in a different biodistribution of both radioisotopes.[82] Considering that polymer coatings commonly serve as matrices for the attachment of targeting ligands, degradation or desorption of the polymer shell could lead to complete loss of targeting functionality.

Besides the physicochemical properties of the bare particle, the properties of the targeting moiety on the particle surface, including affinity, density, and orientation or availability, can significantly influence the targeting ability of the drug carrier. Specific interaction between a targeting ligand and its complementary cell membrane receptor can result in receptor-mediated endocytosis of the particles.[18] When coupled with many targeting ligands on its surface, a particle can act as a multivalent ligand, with the ability to strengthen the binding and attachment to the cell membrane.[83] Therefore, the strength of interactions between a particle and the cell is not only determined by the affinity of the ligand to its target, but also by the ligand multivalency of the particle. As the multivalency of a particle is governed by the density of ligands on the particle surface, ligand density is important in targeting. A higher ligand density can result in higher particle multivalency and stronger particle-cell interactions. However, densely grafted targeting ligands may also affect particle surface properties, disrupt receptor clustering, and lead to an undesirable in vivo biodistribution profile and faster clearance of the particles from circulation.[84] Furthermore, in a recent study, nanoparticles with exactly one or two antibodies attached were compared. While the nanoparticles with two antibodies performed better in vitro, the nanoparticles with

just a single antibody attached performed better in vivo, possibly due to size-related effects.[85] Fakhari et al. also showed that higher grafting densities of a ligand targeting ICAM-1 did not translate to better targeting to cancer cells in vitro.[86] Therefore, it is important to control ligand density on the particle surface to balance targeting capacity and potential clearance from circulation. Besides ligand density, the availability of the ligand is another important factor to consider when designing targeted particles.[87] Hindrance, due to the ligand being buried by another ligand or by other molecules adsorbed onto the particle surface, or as a result of the incorrect orientation of the ligand on the surface, could affect the ability of the ligand to bind to its receptor. The attachment of targeting ligands onto particle surfaces does not guarantee successful targeting and that the influence from both the particle itself and the environment should be evaluated.

3.2. Formation of Protein Coronas

With the advances in nanotechnology and particle engineering, the physicochemical properties of many types of particles can be carefully designed and precisely tuned for improved targeting and drug delivery efficacy. However, when administered in vivo, these precisely engineered particles can behave unexpectedly, often affecting their targeting ability and eliciting undesired physiological responses.[19c,88] Upon contact with the biological milieu, nanoparticles, due to their surface properties and large surface-to-volume ratio, rapidly adsorb biomolecules resulting in a coating commonly referred to as a ‘protein corona’ or a ‘biomolecular corona’ (**Figure 6A**).[16a,b,89] The biomolecules adsorbed include a large number of proteins as well as lipids and oligosaccharides.[90] This biomolecular corona, which provides particles with a ‘biological identity’, not only alters the properties of particles but also determines subsequent bio-nano interactions and physiological responses.[19c]

A protein corona forms rapidly (< 30 s)[15b] upon the exposure of particles into a biological environment.[91] It covers the bare or functionalized particle surface, and subsequently alters particle properties, including size, surface charge, hydrophobicity, roughness and particle stability (Figure 6B). It has been widely reported that particles with proteins adsorbed on their surface have a greater hydrodynamic radius.[15b,92] The more proteins adsorbed on the particle surface, the greater the hydrodynamic radius of the particle typically is.[19a] Thus, in situ size measurement via different methods, including dynamic light scattering (DLS) and differential centrifugal sedimentation (DCS), have been widely used as an indirect quantification of protein adsorption.[19a] In terms of surface charge, the formation of protein coronas in a serum or plasma environment has been shown to result in a moderately negative ζ-potential of particles, regardless of their original surface charge.[15b,93] By using bioinformatics analysis, Tenzer et al. confirmed that the major corona components for different particles were proteins with an overall negative charge at physiological conditions.[15b] Moreover, the moderately negative surface indicates a different stabilization mechanism of corona-covered particles from that of bare particles.[89b] Usually, highly charged bare particles show strongly charge dependent, colloidal stability.[89b] However, the formation of a protein corona can greatly decrease the surface charge to a level that can cause

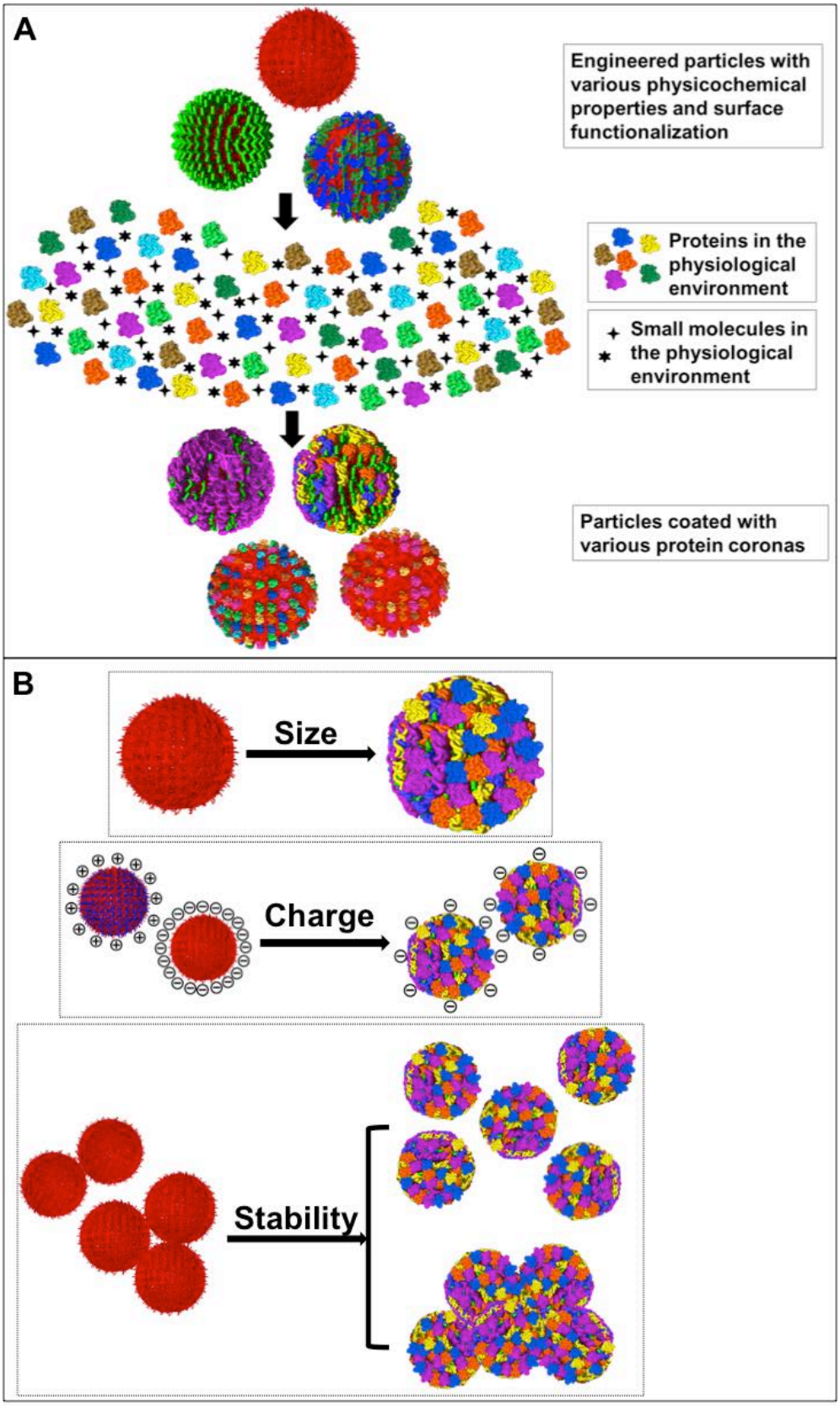

Figure 6. The formation of protein coronas depends on the nature of the particles (A) and influences the physicochemical properties of particles such as size, charge and colloidal stability (B).

colloidal instability.[89b] Walczyk et al. suggested that the corona itself could provide the stability for corona-covered particles.[89b] Similarly, Gebauer et al. reported that the protein corona formed around silver NPs could be utilized to stabilize the particles against agglomeration.[94] In contrast, Au NPs can aggregate when exposed to cell-conditioned media (media that has been previously exposed to cells, which alters its composition, for example through growth-related depletion and secretion of biomolecules).[95] The protein corona therefore affects the physicochemical properties of particles, and can influence their behavior in vivo.

As the outer layer on a particle surface, the protein corona can directly influence the targeting ability of particles. The adsorbed proteins may function as a shield and bury the pre-coupled targeting ligands underneath, which can block the recognition and interaction between the targeting ligands and their respective receptors (**Figure 7A**).[96] It was reported that using a non-cell-based platform, which eliminates the influence of cellular processes, the protein corona established a barrier and screened the interactions between the ligand and its target.[97] This simplified approach confirmed that protein coronas can significantly reduce particle targeting capability by screening the active sites of targeting ligands, thereby reducing the targeting efficiency of particles.[97] The smaller the ligand, the more likely it could be hindered. Many commonly used targeting ligands (e.g., Tf, sdAbs, and aptamers) have been reported to lose their targeting capabilities in the presence of a protein corona.[15a,98] Besides, the adsorbed proteins can infiltrate into the gaps between targeting ligands, especially for particles with a low functionalization density or particles coupled with ligands via a linker. These infiltrated proteins can change the arrangement and orientation of targeting ligands, and hence vary the targeting efficiency of particles. Combined, the adsorption and infiltration of proteins work together in altering the accessibility of targeting

ligands and lead to a different targeting outcome for engineered particles.[87] Dai et al. have reported that the adsorption of proteins from

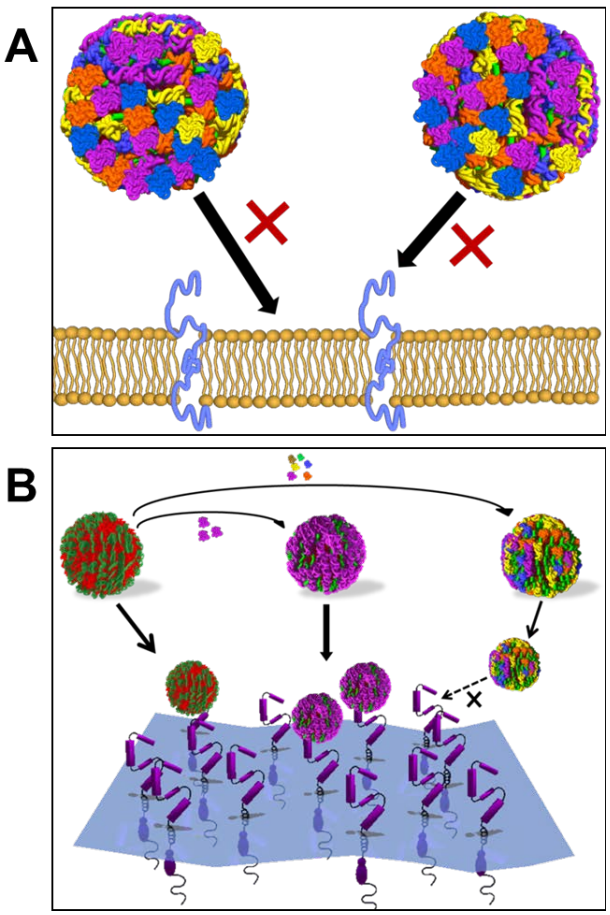

Figure 7. Influence of protein coronas on bio-nano interactions. (A) A protein corona on a particle surface can block both the nonspecific interaction between the particle and a cell membrane (left) and the specific recognition between targeting ligands on the particle and their complementary receptors on the cell membrane (right). (B) Protein coronas of different compositions can either facilitate or inhibit the specific recognition between targeting ligands on particle surfaces and the receptors on the cell membrane. Reproduced with permission.[87] Copyright 2015 American Chemical Society.

various biological sources on engineered layer-by-layer (LbL) particle surfaces had different effects on ligand accessibility, which resulted in either enhanced or inhibited targeting outcomes (**Figures 7B, 8**).[87] Adsorbed proteins may have direct interactions with targeting ligands, cause structural variations of the targeting ligands, and lead to a change in affinity of

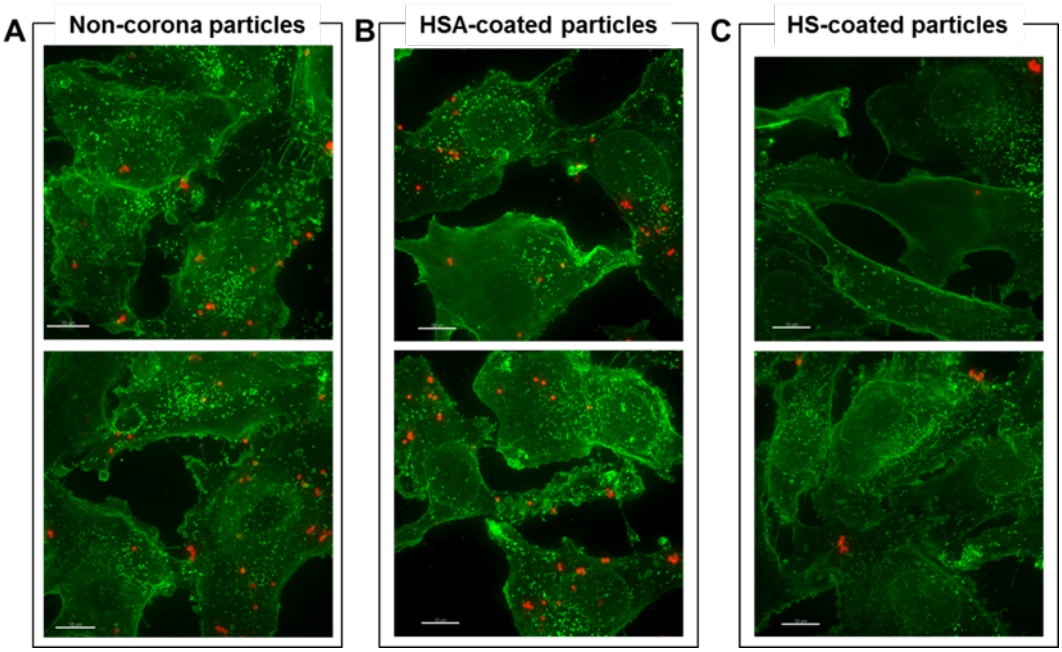

Figure 8. Fluorescence microscopy images of the cellular association of affibody-functionalized particles with targeted cells in the absence (A) or in the presence (B and C) of a protein corona. The protein corona was formed in either human serum albumin (HSA) solution (B) or human serum (HS) (C). Scale bars are 10 μm. Reproduced with permission.[87] Copyright 2015 American Chemical Society.

particles to their targets. It has been shown that proteins may undergo conformational changes during the formation of protein coronas, which can result in denatured proteins within the corona.[19c,99] Protein and peptide-based targeting ligands may also undergo structural rearrangements during formation of the protein corona.[19c] The structural rearrangement of ligands may lead to the denaturation or misfolding of the ligands, and result in the loss of their targeting ability.

Additionally, the formation of protein coronas plays important roles in particle biodistribution, biocompatibility and cytotoxicity, and even drug release (**Figure 9**),[100] all of which influence the outcome of particle-based approaches in biomedical applications. The conformational changes of adsorbed proteins may result in the particles being recognized as 'foreign components' by the body and lead to rapid clearance of the particles by the immune system.[19c,99,101] For instance, Yan et al. has reported that unfolded albumin adsorbed on particles triggers class A scavenger receptor-mediated phagocytosis in differentiated macrophage-like (dTHP-1) cells.[99] Mortimer et al. also reported the role of unfolded albumin in cellular uptake of the protein-NP complex by dTHP-1 cells, and highlighted that denatured albumin and other serum proteins can promote the clearance of particles from circulation via the MPS.[101] In addition, opsonins, which are present in plasma, can promote the recognition of particles by the immune system and the phagocytosis of particles by macrophages.[19c] Opsonins reported to activate phagocytosis and inflammatory responses include immunoglobulin G (IgG), complement components, and fibrinogen.[102] The fast clearance of particles by the body's immunological response results in a reduced number of particles reaching their desired targets and leads to poor targeted delivery efficacy. However, interestingly, there are instances where interactions with phagocytes may be beneficial, for example in the case of priming or loading tumor-associated phagocytic cells.[103] Nevertheless, as mentioned above, upon adsorption of proteins, the physical characteristics of particles (e.g., size, surface charge, hydrophobicity) can be altered, which leads to changes in their circulation and biodistribution patterns, and consequently their targeting outcomes. However, there are also some components in plasma that may be recruited and used for improving the targeting of particles. These include apolipoproteins, which can cross the blood brain barrier, and when recruited by particles, may be useful for targeting particles to the brain.[104] In

Section 4.3, we will discuss in more detail how the recruitment of specific proteins on the particle surface can affect targeting.

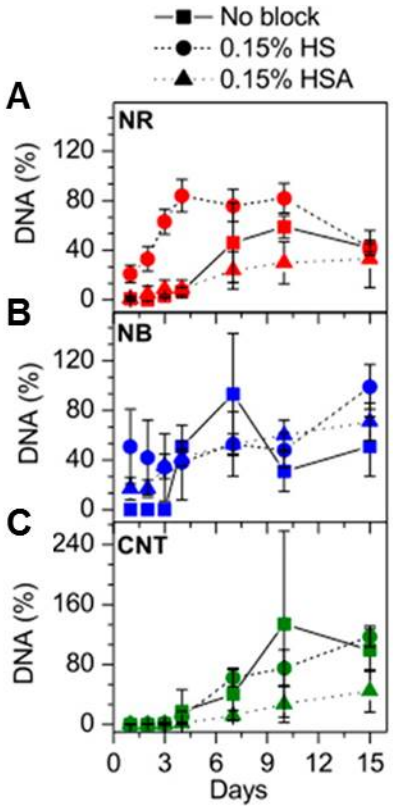

Figure 9. The influence of the protein corona and protein exchange conditions on DNA release from CTAB-coated gold nanorods (A), gold nanobones (B) and carbon nanotubes (C). Coronas were formed using 5% human serum, then loaded with DNA (particle-HS-DNA). DNA release when particles (particle-HS-DNA) are exposed to HS, HSA or no protein/block is shown. Reproduced with permission.[99b] Copyright 2013 American Chemical Society.

3.3 Performance of Targeted Drug Carriers in Biological Environments

Targeted drug carriers, particularly ligand-targeted liposomes, have been investigated for their targeting ability in vivo for more than two decades, which was before protein corona formation became a central theme in the development of targeted drug systems.[89c,105] For example, Kirpotin et al. compared anti-human epidermal growth factor receptor 2 (HER2) immunoliposomes with non-targeted liposomes and found that the overall tumor

accumulation after intravenous injection was high for all systems whether functionalized or non-functionalized.[106] The liposome accumulation in tumor tissue appeared to be dominated by extravasation of long-circulating, PEGylated liposomes from the tumor vasculature via passive targeting. However, ex vivo analysis of cells within the tumor tissue showed a 4-fold increase in uptake of targeted liposomes by epithelial cell adhesion molecule (EpCAM)-positive tumor cells compared to EpCAM-negative stroma cells. In addition, cell association was much higher for immunoliposomes compared to non-targeted liposomes, which supported the observed higher anti-tumor activity of immunoliposomes in the in vivo experiments.[106] Similar results were obtained in a study with poly(lactic acid) (PLA) particles injected in ovarian cancer xenograft mice.[107] Comparing anti-HER2 Ab-targeted and non-targeted paclitaxel-loaded particles, no significant difference in overall tumor accumulation was observed; however, the therapeutic effect was highest for the anti-HER2-PLA particles.[107] These two studies demonstrated successful targeting in an animal model, as well as the benefit of combining passive and active targeting strategies for effective anticancer treatment.[106-107] In these cases however, it was not determined whether targeting ability in vivo was influenced by the formation of a protein corona.

The loss of targeting ability of Tf-functionalized silica particles (**Figure 10**) has reiterated that successful receptor-mediated binding of a targeted NP in in vitro cell experiments may not necessarily translate to effective in vivo performance.[15a] Salvati et al. found that receptor-mediated uptake of the targeted particles depended on a number of factors,

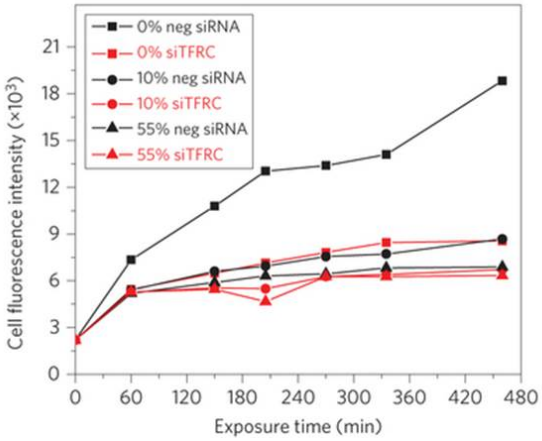

Figure 10. The effect of the biological environment on the binding of PEGylated human Tf particles (SiO_2–PEG_8–Tf) to A549 cells. As a measure of specificity, the cells were silenced for 72 h with a negative siRNA control (neg siRNA) and siRNA against the transferrin receptor (siTFRC), before exposure to nanoparticles. Median cell fluorescence intensity obtained by flow cytometry from A549 cells exposed to SiO_2–PEG_8–Tf in serum-free MEM (0%), complete medium (10%) and MEM supplemented with 55% serum (55%). The uptake is reduced in cells silenced for TfR showing that the binding is specific. At increasing serum content the uptake decreases and specific binding is lost. Reproduced with permission.[15a] Copyright 2013 Nature Publishing Group.

including PEG-linker length, the method of coupling Tf to the particle surface, and the complexity of the medium used to assess receptor-mediated NP uptake by A549 cells.[15a] In buffer, a significant difference in uptake of Tf-functionalized particles by TfR-expressing cells and TfR gene silenced cells was observed. In contrast, no difference in uptake was observed when particles were incubated in medium with up to 55% serum.[15a] To investigate the potential shielding effect of ligands by adsorbed biomolecules, Mirshafiee et al. performed a model targeting study and mimicked ligand receptor binding in the presence of a protein corona.[97] A silica surface modified with azide-terminating alkyl chains was reacted in a copper-free click reaction with strained cyclooctynes that were attached to fluorescent

silica particles (alkyne-modified). Incubation of alkyne-modified particles with either 10% or 100% fetal bovine serum (FBS) significantly lowered the reaction yield and only a few particles were covalently attached to the surface.[97] Even though this finding might not fully translate to biological receptor-mediated interactions (e.g., due to size of receptor or ligand, reversible and dynamic binding, binding avidity), it highlights the importance of validating receptor-mediated binding of targeted particles in more realistic biological environments. Since then, a number of actively-targeted drug carriers have been challenged in biologically relevant environments with different targeting outcomes (**Table 1**). Abs are among the most investigated ligands for targeting. For example, receptor-specific targeting of LbL-assembled poly(methacrylic acid) (PMA) capsules or core/shell particles functionalized with humanized A33 monoclonal antibody (huA33 mAb) was demonstrated in the presence and absence of a protein corona.[108] Targeting experiments towards colorectal cancer cells were performed with a mixed cell population of A33 antigen positive cells (LIM2405+) and A33 antigen negative cells (LM2405–).[108] Specific binding to LIM2405+ cells was observed for bare Ab-decorated particles and for Ab-functionalized particles with a protein corona derived from 100% human serum (HS).[108] The formation of a protein corona showed negligible influence on specific Ab-mediated cell association.[108] This has also been observed with Au NPs decorated with trastuzumab/Herceptin (mAb binding to the HER2 receptor) via a thiol-bearing 5 kDa PEG-linker. The particles maintained their targeting ability to HER2+ cells even after incubation of the particles with HS.[109] Protein adsorption was reduced by saturation of the NP surface with low-fouling PEG polymers. Interestingly, the targeting capability strongly depended on the size of PEG, and only polymers smaller than the Herceptin linker resulted in successful receptor-targeted binding (**Figure 11**).[109] Similarly, Xing et al. found that fine-tuning of ligand presentation and stealth polymer coating was crucial in maintaining the targeting ability of DNA aptamer-functionalized liposomes[110] (see

also Section 4.2). Zarschler et al. also observed receptor-mediated uptake of silica particles functionalized with an epidermal growth factor receptor (EGFR)-specific sdAb in a physiologically relevant environment.[98a] In the presence of protein coronas, overall particle uptake by EGFR+ and EGFR gene-silenced cells decreased with increasing concentration of HS. Despite this, receptor-dependent particle uptake could still be achieved even in HS, which indicates that sdAbs can be effective targeting ligands in high protein content environments.[98a]

Table 1. Examples of targeting ligands studied in the presence of protein coronas.[a]

Ligand	Carrier System	Target	Environment	Targeting Largely Maintained?
Cyclooctyne	Silica NP (75 nm)	azide	FBS (Non-cell-based model)	No[97]
Tf	PEGylated silica NP (50-100 nm)	Tf-receptor	FBS and HS	No[15a]
Trastuzumab mAb	PEGylated Au NP (50 nm)	HER2 oncoprotein	HS	Yes[109]
	PLA NP (240 nm)	HER2 oncoprotein	In vivo (SKOV-3 mouse model)	Yes[107]
Anti-CD11c mAb	MS NP (35 nm)	CD11c antigen	FCS	No[111]
Anti-huA33 mAb	PMA capsules/particles (2 µm)	A33 antigen	HS	Yes[108]
hCTMO1, anti-MUC-1 mAb	PEGylated liposomes (120 nm)	MUC-1 receptor	CD-1 mouse plasma (in vitro) CD-1 mouse model (in vivo)	No, but liposomes with in vivo formed PC maintained targeting ability[112]
Anti-CD44 mAb	MNP (30 nm), GNT (13 x 92 nm)	CD44 antigen	Mouse blood (in vitro) MDA-MB-231 mouse model (in vivo)	Yes (in vitro and in vivo)[113]
Anti-HER2 Fab or scFv	PEGylated liposomes (110 nm)	HER2 oncoprotein	In vivo (BT-474 mouse model)	Yes[106]

Anti-HER2 Afb	PMA particles (1 µm)	HER2 oncoprotein	HSA and HS	Yes for HSA; No for HS[87]
sdAb	Silica NP (50 nm)	EGFR	HS and FCS	Yes[98a]
MUC-1 aptamer	Chitosan NP (130 nm)	MUC-1 receptor	FBS	No[98b]
GlcNAc; Lac	Au NP (15 nm), Au NR (65 nm x 16 nm)	WGA; Gal-3	FBS	Yes[114]
Man	HES-NC (170 nm)	C-type lectin	HP	Yes[59]
Folate	Fe_3O_4-SiO_2 (18 nm)	Folate receptor	FBS	Yes[115]
GM3	Au NP (35 nm)	Siglec1 (CD169)	FBS (in vitro) Mouse model (in vivo)	Yes (in vitro and in vivo)[116]
HA	MPN capsules (1.4 µm)	CD44 receptor	HS	Yes[117]
Nanobody (2Rb17c)	Au nanostars (75 nm)	HER2 oncoprotein	FBS (in vitro) Mouse model (in vivo)	Yes (in vitro and in vivo)[75]
Biotin	Zwitterionic SiNPs (120 nm)	Streptavidin (non-cell based surface); Biotin-receptor (tumor cells)	HP	Yes[118]

[a]Abbreviations: NP: nanoparticle; FBS: fetal bovine serum; Tf: transferrin; PEG: poly(ethylene glycol); HS: human serum; mAb: monoclonal antibody; Au: gold; HER2: human epidermal growth factor receptor 2; PLA: poly(lactic acid); MS: mesoporous silica; FCS: fetal calf serum; PMA: poly(methacrylic acid); MUC-1: mucin-1; PC: protein corona; MNP: magnetic nanoparticle; GNT: golden carbon nanotube; scFv: single chain antibody; Fab: fragment, antigen binding; Afb: affibody; HSA: human serum albumin; sdAb: single domain antibody; EGFR: epidermal growth factor receptor; GlcNAc: *N*-acetylglucosamine; Lac: lactose; NR: nanorod; WGA: wheat germ agglutinin; Gal-3: galectin-3; Man: mannose; HES-NC: hydroxyethyl starch nanocapsules; HP: human plasma; GM3:

monosialoganglioside 3; HA: hyaluronic acid; MPN: metal-phenolic network; SiNPs: silica NPs.

In contrast, a loss of targeting ability was found for anti-CD11c-coated mesoporous silica (MS) particles that were loaded with Dox.[111] While the Ab-targeted particles showed increased uptake by human osteosarcoma (MG-63) cells compared with non-targeted MS particles in serum-free medium, uptake was at the levels of non-specific binding in the presence of 10% FBS.[111] These examples highlight the importance of evaluating particle types individually and to consider a realistic model to study ligand-receptor interactions in complex biological milieus.

The biological source of the corona can influence the targeting outcome, as it determines the biological identity of the particle system. A recent study on the targeting ability of anti-HER2-Afb functionalized PMA particles showed different results for protein coronas derived from HS and human serum albumin (HSA) solutions.[87] Adsorption of the single model protein HSA enhanced Afb-mediated interaction of the particles with SK-OV-3 cells overexpressing the HER2 receptors.[87]

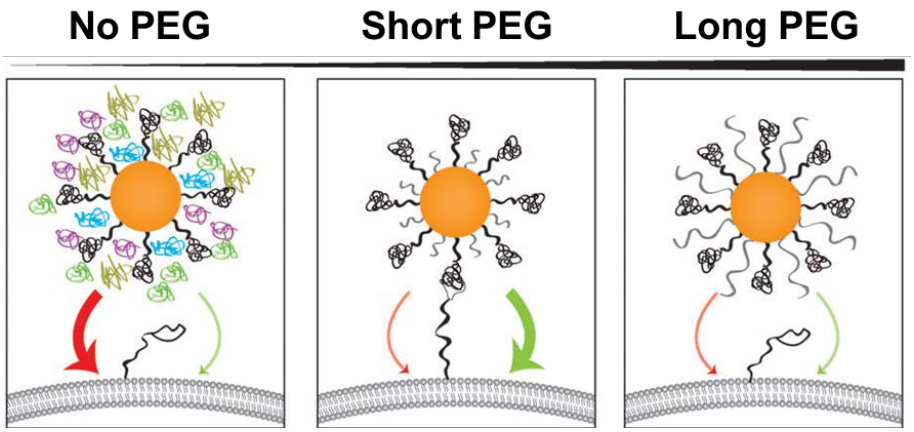

Figure 11. Schematic illustration of the influence of the size of PEG on the specificity of interactions between particles and cells. The size of the arrows indicates the likelihood of interactions, with red indicating the non-specific interactions and green indicating the ligand-

mediated interactions. Reproduced with permission.[109] Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

An opposite effect on targeting was observed for anti-HER2-Afb-PMA particles coated with a protein corona formed in HS, where the association with SK-OV-3 cells was drastically reduced.[87] Even though HSA is a popular model protein for studying the influence of a protein corona on interactions between (targeted) particles and cells, these studies show that opposite results may be obtained when using more complex biological solutions like serum.[87]

In addition to the biological source of the protein,[87,119] the protein corona might differ according to whether its formed in vitro or in vivo, even if formed from plasma of the same organism. To shed light on this aspect, Hadjidemetriou and colleagues compared the target-specific uptake of liposomes with a protein corona formed in vivo in mice, to liposomes with a protein corona from in vitro incubation with mouse plasma.[112] The active-targeting ability of the liposomes was provided by surface modification with the clinically-trialed hCTMO-1 Ab that binds transmembrane glycoprotein mucin-1 (MUC-1).[112] Cellular internalization of liposomes was reduced in the presence of a protein corona, and the reduction was more significant for liposomes with a corona formed under in vitro treatment.[112] This finding is promising as Ab-functionalized liposomes with a protein corona formed in vivo were still specifically internalized by MUC-1 overexpressing cells, and not by MUC-1 negative cells.[112] The results in this study indicate that the interaction of particles with the physiological environment is highly complex, and crucially depends on both the particle and the chosen biological model. However, as Hadjidemetriou et al. pointed out, even a protein corona from in vivo particle circulation may not provide the essential information required for successful clinical application.[112] In a different study targeting the MUC-1

receptor, drug-conjugated chitosan particles were decorated with a MUC-1 aptamer.[98b] While targeting to MUC-1 overexpressing cells caused increased cytotoxicity compared to MUC-1 negative cells in the absence of a protein corona, no difference in cytotoxicity was observed for both cell lines after particle incubation with bovine serum albumin (BSA). Considering the complexity of a protein corona, it is not surprising that aptamer-functionalized chitosan particles did not maintain their targeting ability in this study while Ab-modified liposomes preserved their targeting ability to the same receptor.[98b,112] The only common parameter in both studies was the targeted receptor MUC-1, while the systems differed in NP material, ligand properties, cell lines, as well as the methods of forming the coronas.[98b,112] In addition, the aptamer has a smaller size (8-25 kDa), compared to a full IgG Ab, which is approximately 150 kDa.[50a,98b,112] A smaller ligand might be more easily hindered by the formation of a protein corona.

Another class of molecules that has been investigated for their targeting potential in complex biological environments is carbohydrates. Carbohydrate-lectin interactions are responsible for a number of physiological processes, such as cell signaling, inflammation, virus infection and cancer progression.[120] Abnormal glycosylation patterns on cell surfaces and overexpressed receptors have promoted glycan structures as alternative targets for delivery.[54b] The inherent low affinity (around micromolar to millimolar) of many carbohydrate-lectin interactions can be overcome by presenting multivalent binding motifs on particle surfaces to yield high-avidity binding.[56] Besides their role as targeting moieties, carbohydrates have gained considerable interest as particle coatings due to their hydrophilic, low-fouling character, which can decrease protein adsorption and nonspecific binding.[55b,121] Recent studies highlighted that nanocarriers decorated with carbohydrates as small as mono- and disaccharides (M_W = 180–360 g mol^{-1}) maintain their targeting abilities in complex biological environments.[59,114] García et al. showed that Au NPs decorated with *N*-

acetylglucosamine (GlcNAc) or lactose (Lac) exhibited comparable low-fouling character as PEG.[114] Remarkably, the targeting ability of glycan-decorated Au NPs was preserved in physiological medium supplemented with 10% FBS, while the non-specific cellular uptake of particles by phagocytic cells was negligible.[114] These findings, taken together, are promising, as they suggest a long circulation lifetime and targeting specificity of glycan-decorated Au in complex biological milieus.[114] Kang et al. reported similar observations using particles composed of hydroxyethyl starch (HES), an inherently low-fouling material, and functionalized with mannose (Man) groups via a PEG linker for targeting.[59] Minimal protein adsorption on the Man-functionalized HES particles was observed upon incubation in human plasma (HP) and the specific targeting capacity of these particles to C-type lectin was partially maintained. In cell studies, Man-decorated HES nanocarriers were specifically internalized by dendritic cells (DCs) via a receptor-mediated mechanism and the presence of protein coronas derived from HP had a minimal effect on cell association or internalization.[59] The impact of a low-fouling carrier surface was recently supported by targeting studies employing zwitterionic silica particles.[118,122] Particles functionalized with zwitterionic cysteine and biotin showed reduced protein adsorption, and biotin-mediated cell uptake after incubation with HP was higher than for targeted particles without the zwitterionic coating.[118] Strategies and opportunities for tuning the protein corona on the carrier surface with regard to the performance of an active-targeting drug carrier will be discussed in detail in the next section.

4. Tuning the Protein Corona through Particle Design

Protein coronas, as discussed above, can influence targeting in diverse ways, such as directly shielding the ligand-receptor interactions, causing faster elimination of particles by increasing their size and inducing agglomeration, or promoting phagocytosis and inflammatory

responses, which indirectly affect the targeting outcome of particles.[15,94,99] Therefore, to retain the targeting ability of particles in a biological environment, the goal is to better understand the formation and effect of protein coronas in order to eliminate their negative impact on in vivo particle targeting and behavior.[21b,89c,123] Low-fouling materials (e.g., PEG, zwitterionic polymers and carbohydrates) have been extensively used to confer the carrier surface with stealth properties and can significantly reduce the amount of adsorbed proteins on the particle surface.[124] However, even a small amount of adsorbed proteins can significantly vary the properties and behavior of particles.[15,19a,87,91-92] Therefore, the properties of particles need to be finely tuned to reach a compromise between low-fouling and targeting.[109,125] It is noted that, in vivo, the adsorption of dysopsonins such as albumin and clusterins can prolong the circulation time of particles and reduce nonspecific uptake, while the adsorption of opsonins (e.g. IgG) typically shortens the circulation time of particles.[19a,c,88,91,123b] Thus, the specific recruitment of dysopsonins or clusterins may be more advantageous than preventing protein adsorption altogether.

The protein corona can also be exploited to improve the properties and behavior of particles in other ways. For example, the protein corona has been reported to reduce the cytotoxicity of particles[126] and to elicit specific targeting ability.[127] Protein coronas around Au NPs have been used to enhance their cargo loading capacity, and to modulate both passive and triggered cargo release by heat or laser excitation.[100b,128] Ju et al. recently reported that the presence of a protein corona can improve the targeting specificity of capsules prepared through metal-phenolic complexation (a recently introduced method for the assembly of dynamic thin films and gels).[117,129] Moreover, the presence of specific proteins within the corona may help particles cross biological barriers, including the BBB.[104] Therefore, the protein corona is not necessarily detrimental. On the contrary, the adsorbed proteins can improve the surface properties of some particle systems and, when rationally designed and

carefully controlled, may facilitate targeted drug delivery while minimizing harmful off-target effects. In the following section, we will highlight studies that have aimed at reducing protein adsorption, modulating protein corona composition, and recruiting specific proteins from the biological environment to achieve optimized targeting outcomes.

4.1 Reducing Protein Adsorption

Since the protein corona can act as a barrier and shield the ligands from interaction with their receptors, the amount of proteins in the corona is a key factor that governs the extent of shielding. Reducing the amount of adsorbed proteins has therefore become a strategy for alleviating some of the negative aspects of protein coronas,[78a,124a,c,130] and this can be achieved by rendering the particle surface low-fouling.[21b] **Table 2** summarizes pertinent aspects of example materials that have been shown capable of decreasing non-specific interactions between a particle and its environment.

The surface properties of particles, such as surface charge and hydrophobicity, have been reported to influence both the amount and the type of adsorbed proteins,[19c,88,92a,93] and can be modulated by carefully tuning the particle surface. Modification of the particles by the attachment of low-fouling polymers is a commonly used strategy.[124a,c,125] PEG is currently the most widely used polymer for this approach, and has been reported to prolong the circulation time of particles with increasing molecular weight (and thickness) of the PEG layer.[131] The near neutral and highly hydrophilic nature of PEG has been reported to provide particles with a hydration and steric barrier, which can reduce non-specific binding of serum proteins to the particle surface.[78a,124a,125] PEGylated particles are therefore able to adsorb less opsonins from the biological system, and have longer circulation times in vivo.[124a] Particles with long circulation times have been reported to exhibit higher passive targeting ability to the tumor microenvironment due to the EPR effect.[124a] As active targeting often builds on passive targeting, the long systemic circulation lifetime of PEGylated particles also facilitates

active targeting.[124a] PEGylation has been reported to directly mitigate the negative effects of protein coronas on the targeting ability of ligand-functionalized particles.[109-110] Dai et al. reported that backfilling the surface of ErbB2 receptor-targeted NPs with PEG molecules helped to reduce protein adsorption and to re-establish specific binding of particles to SKBR3 cells (Figure 11).[109]

Table 2. Examples of materials and strategies that have been explored to generate stealth particles.[a]

Material		**Strategy**	**Performance**
PEG		Surface conjugation	Less non-specific protein adsorption; prolonged blood circulation time; increased in vivo half-life; mitigated negative effects on active targeting[78a,109,124a,c,125,131a,132]
		Surface deposition	Increased surface PEG density; end group modification of PEG molecules enables further attachment of targeting ligands or tuning composition of protein coronas[124b,133]
		Multilayered PEG capsules assembled via the LbL technique	Specific deconstruction property; low-fouling property; negligible cytotoxicity; post-functionalized with targeting ligands[130,133-134]
		PEG hydrogel particles generated using the MS templating method	Controllable size; tunable elasticity; extended circulation time and increased blood retention; improved biodistribution profiles[124d,135]
PVPON		Single-component PVPON capsules assembled via the LbL technique	Tunable degradation characteristics; good cargo loading, retention, and release profiles; low-fouling property; high targeting specificity with targeting ligands post-attached on surface[136]
		Multi-component PVPON/PDPA capsules assembled via LbL technique	Low-fouling property; pH responsive; charge-shifting property; increased cargo encapsulation and initial retention[137]
Zwitterionic polymers	PMPC	Replica particles prepared using surface-initiated polymerization in MS templates	Redox-responsive disassembly; ultralow cell association properties[138]
	PCB	PCB-gold NPs prepared via SI-ATRP	Prolonged circulation time; negligible production of polymer-specific

			antibodies[139]
HPMA		Polymer-based particles generated via self-assembly process	Possible to conjugate with different therapeutic compounds; minimized interactions with plasma proteins; prolonged circulation time[140]
POEGMA		Replica particles prepared using surface-initiated polymerization in MS templates	Redox-responsive disassembly; ultralow cell association properties[138]
$PEtOxMA_{SH}$		Capsules generated via the LbL technique	Redox-responsive; negligible cytotoxicity; low-fouling property[141]
PVA		Surface coating	Long circulation half-life without accelerated blood clearance phenomenon upon repeated injections[142]
PAcM		Surface coating	Long circulation half-life without accelerated blood clearance phenomenon upon repeated injections[143]
Poly(oxazoline)		Surface conjugation	Long circulation and low hepatosplenic uptake of liposomes[144]
PDAAm		Surface coating	Long circulation half-life without accelerated blood clearance phenomenon upon repeated injections[143a]
PEEP		Covalent surface conjugation	Less non-specific protein adsorption and reduced non-specific cellular uptake[123b]
PAAs	PHEA	Surface coating	Outlasted particles in the circulation and reduced accelerated blood clearance phenomenon[145]
	PGA	Surface grafting	Effective inhibition of particle uptake[146]
Polysaccharides	Heparin and dextran	Covalent conjugation	Prolonged circulation half-life of particles[147]
	Chitosan	Surface functionalization	Reduced phagocytic uptake efficiency and retarded blood clearance of NPs[147a]
	PSA	Surface conjugation	Antigen masking and in vivo survival; prolonged pharmacokinetic profiles; prolonged particle residence within the brain[148]
Proteins		Surface pre-coating	Reduced non-specific cellular uptake[123b]

[a]Abbreviations: PEG: poly(ethylene glycol); LbL: layer-by-layer; MS: mesoporous silica; PVPON: poly(*N*-vinyl pyrrolidone); PDPA: poly(2-(diisopropylamino)ethyl methacrylate); PMPC: poly(methacryloyloxyethyl phosphorylcholine); PCB: poly(carboxybetaine); NP:

nanoparticle; SI-ATRP: surface initiated atom transfer radical polymerization; HPMA: poly[*N*-(2-hydroxypropyl)methacrylamide]; POEGMA: poly[oligo(ethylene glycol) methyl ether methacrylate]; $PEtOxMA_{SH}$: thiol-containing poly(2-ethyl-2-oxazoline); PVA: poly(vinyl alcohol); PAcM: poly(4-acryloylmorpholine); PDAAm: poly(*N*,*N*-dimethylacrylamide); PEEP: poly(ethyl ethylene phosphate); PAA: poly(amino acids); PHEA: poly(hydroxyethyl-$_L$-asparagine); PGA: poly($_L$-glutamic acid); PSA: poly(sialic acid).

To improve stealth properties, PEGylation has been further optimized by a series of studies that change the molecular weight, structure, end groups and grafting density of PEG molecules.[124a,132d,149] Generally, longer PEG chains and higher PEG densities are necessary to increase the half-lives of particles in vivo, as well as to reduce protein adsorption.[132b]

However, the overall effect of PEGylation is also dependent on the particles. For instance, in one study, it was observed that PEG with a molecular weight of 5 kDa provided surface coatings (for polymer-based particles) which had the highest reduction of protein adsorption.[124c] In contrast, for polymer-coated Fe/Pt NPs, the surface modification with 10 kDa PEG molecules gave better results than when using 5 kDa PEG.[125] In the case of PEGylated MS particles, Clemments et al. reported that particles had negligible protein adsorption regardless of the PEG chain length used.[132c] It has also been observed that particles with densely packed brush-like PEG molecules can show extended in vivo circulation times compared to those with sparser mushroom-like PEG molecules.[132d] The performance of PEGylated particles can thus vary with each particle system and it therefore needs to be optimized independently for each type of particle under investigation. For targeting purposes, as a common rule, the PEG molecules should not be longer than the linker of the targeting ligand to avoid interference with ligand-receptor interactions, as Dai et al. reported (Figure 11).[109]

Carefully choosing the type and amount of PEG molecules is essential to effectively reduce the nonspecific protein-particle interactions. Ochs et al. reported a strategy to achieve a high PEG density on the surface of polymer capsules by using heterobifunctional PEG molecules.[124b] A monolayer of PEG was deposited on poly($_L$-glutamic acid) (PGA)/poly($_L$-lysine) (PLL) multilayered capsules, providing the capsules with low-fouling properties.[124b] The terminating end of the PEG molecules was modified with variable end groups to provide additional functionality, e.g., attaching targeting ligands or tuning the composition of protein coronas.[124b,133] For an even higher PEG proportion, multilayered PEG capsules can be generated via LbL assembly and click chemistry, using alkyne- and azide-functionalized PEG molecules (PEG_{Alk} and PEG_{Az}) as building blocks (**Figure 12**).[130,134] When crosslinked with a disulfide cleavable linker, the PEG-based multilayered capsules exhibited specific deconstruction under reducing conditions and low-fouling properties with negligible cytotoxicity.[130] Further post-functionalization of the PEG surface with targeting ligands, e.g., RGD or scFv, was also achieved, providing multilayered capsules with targeting ability and enabling the capsules to be used as targeted drug carriers.[133-134] When preparing these types of multilayered capsules through LbL assembly, it has been shown that the assembly method used can substantially affect the resulting particles; therefore particles with tailored properties (but composed of the same materials) can be engineered through judicious choice of assembly technology.[150] Additionally, PEG hydrogel particles with controllable size and tunable elasticity can be engineered using a MS templating method.[124d,135] The PEG particles with a diameter of 110 nm showed extended circulation time, increased blood retention, and improved biodistribution profiles (**Figure 13**).[124d] These hydrogel particles are amenable to further functionalization with targeting moieties, which highlights their potential as targeted drug delivery carriers.

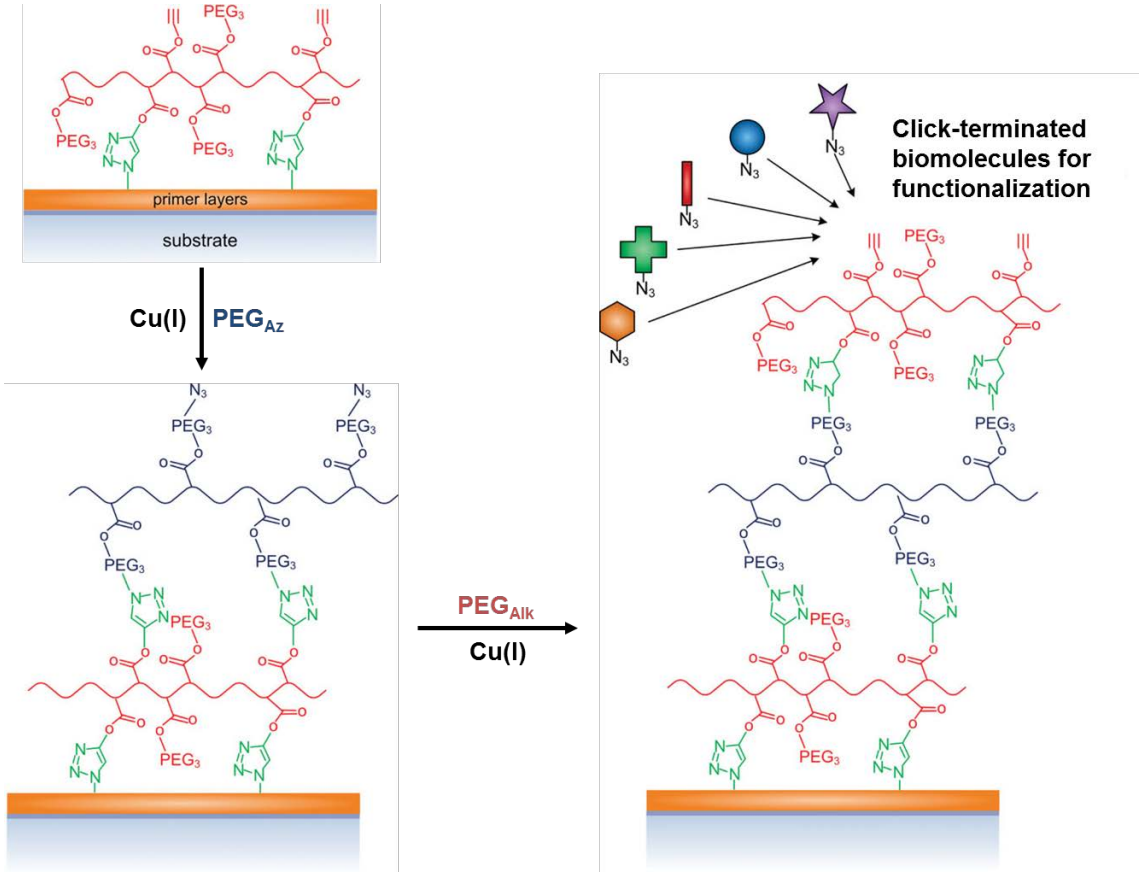

Figure 12. Schematic representation of the generation of multilayered films via the LbL technique and 'click' chemistry using the alkyne- and azide-functionalized PEG molecules (PEG_{Alk} and PEG_{Az}) as building blocks. Post-functionalization of the PEG surface is feasible by using various click-terminated biomolecules. Reproduced with permission.[134] Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Although PEG remains the most established low-fouling material used for modifying drug carriers, alternative materials and strategies have been explored for the development of particles with enhanced biocompatibility and improved in vivo performance (Table 2).[151] For example, low-fouling capsules can be prepared using poly(*N*-vinyl pyrrolidone) (PVPON) as the building block via LbL assembly.[14,136b,137] The neutral PVPON is both highly biocompatible and low-fouling.[136b] With post-modification and careful selection of cross-linkers, single-component PVPON

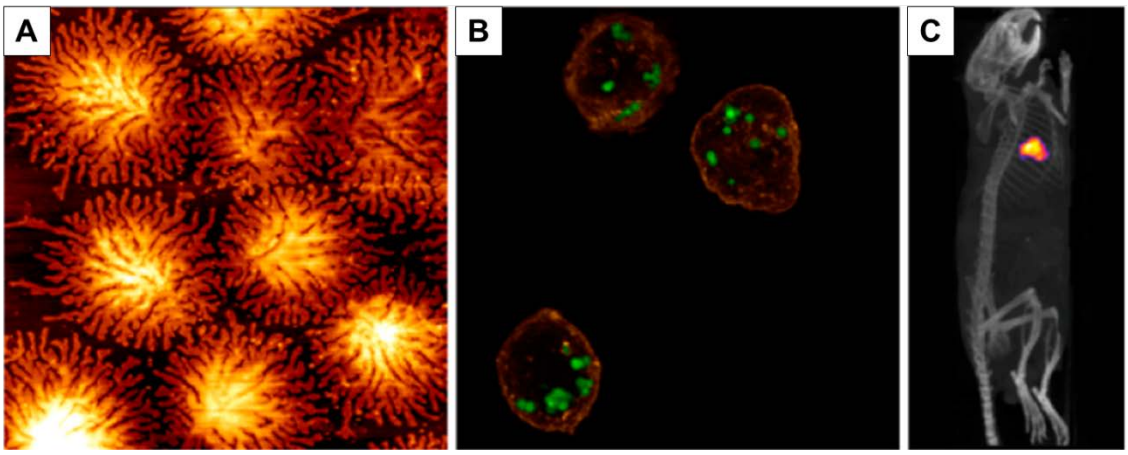

Figure 13. PEG particles generated using a MS templating method (A) and the in vitro cellular association (B) and in vivo biodistribution (C) assessment of the PEG particles. Reproduced with permission.[124d] Copyright 2015 American Chemical Society.

capsules that exhibited high targeting specificity, tunable degradation characteristics, as well as good cargo loading, retention and release profiles were prepared.[136a,c] Moreover, PVPON can be combined with other materials to generate multi-component capsules with specific properties. For instance, Ng et al. designed LbL assembled hybrid systems with poly(2-(diisopropylamino)ethyl methacrylate) (PDPA) inner layers and PVPON outer layers.[137] These hybrid capsules possess low-fouling character from the outer PVPON layers, as well as pH-responsive and charge-shifting properties from the inner PDPA layers, which improved the encapsulation and initial retention of the cargo.[137] Capsules can also be assembled from PDPA and PEG block copolymers which, after functionalization with peptides, can target atherosclerotic plaques.[152] Other neutral or zwitterionic polymers have also been used as building blocks to generate ultralow-fouling drug carrier systems.[153] Thiol-containing poly(2-ethyl-2-oxazoline) ($PEtOxMA_{SH}$) capsules assembled using LbL techniques displayed redox-responsive disassembly, negligible cytotoxicity and low-fouling properties.[141] Poly(methacryloyloxyethyl phosphorylcholine) (PMPC) or poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA) replica particles prepared using surface-initiated

polymerization in MS templates also exhibited redox-responsive disassembly and ultralow cell association properties.[138] In addition, Moyano et al. reported the generation of a series of zwitterionic particles as potential drug delivery vehicles and self-therapeutic systems that showed controllable hydrophobicity and highly reduced protein adsorption.[154] Zwitterionic poly(carboxybetaine) (PCB)-based nanomaterials, as demonstrated by Yang et al., showed prolonged circulation time after both the first and the second administration, indicating negligible production of polymer-specific Abs, which can be an issue for PEG.[139,151] This indicated that PCB could be an alternative to PEG to resist activation of immune responses as well as rapid clearance from blood circulation.[139] Many other synthetic or biological molecules, including poly[*N*-(2-hydroxypropyl)methacrylamide] (HPMA), poly(vinyl alcohol) (PVA), poly(4-acryloylmorpholine) (PAcM), poly(amino acids) (PAAs), and polysaccharides, have also been explored as alternative choices to PEG to develop stealth drug carriers (Table 2).[140a,142-143,145a,155] Recently, Rao et al. exploited red blood cell (RBC) membranes for coating of upconversion particles to avoid protein adsorption from the biological environment.[156] Cell membrane-capped particles with a folic acid (FA)-PEG-lipid conjugate inserted in the membrane targeted to FA receptors on MCF-7 cells after exposure to HP and showed enhanced in vivo tumor imaging.[156] Another example for bioinspired particle coating to overcome biological barriers and improve biodistribution are proteolipid vesicles (i.e., 'leukosomes') to target inflamed tissue.[157] Stealth particles with low protein binding promise not only improved blood circulation and biodistribution, but retained targeting ability in the presence of a complex biological environment.

4.2. Balancing Particle Stealth and Targeting

Improved circulation and biodistribution profiles in vivo is not just a result of having a low-fouling particle surface.[109] As mentioned above, the molecular size, conformation, grafting density and coupling methods used for low-fouling materials need to be carefully selected for

optimized particle surface functionalization. Indeed, even for just the 'stealth effect', the adsorption of distinct proteins can be exploited to prevent non-specific cellular uptake.[123b] For efficient targeting results, it is necessary that the particles can be recognized and internalized by their target cells.[125] Although it is widely accepted that particles with higher PEGylation density can exhibit enhanced low-fouling behavior in vivo,[124a,132b,d] the high PEG grafting density may cause poor cellular uptake by cancer cells, which is undesirable for the delivery of anti-cancer therapeutics.[125] Pelaz et al. suggested the use of zwitterionic surfaces as alternatives to PEG for both greater retention times and enhanced cellular uptake of particles.[125] As there is an interplay between stealth and targeting properties of particles,[125,158] it is important to tune both properties without compromising one or the other.

When coupled to the same surface, targeting ligands and stealth molecules may interact with each other, which could lead to changes in arrangement, conformation and orientation for both. Although a longer PEG chain was reported to increase the particle retention time in vivo,[132b] it needs to be shorter than the PEG-ligand-linker to avoid the interference with ligand-receptor interactions, as mentioned previously.[109] For example, targeting ligands can be attached to the carrier via bifunctionalized PEG linkers.[87,108-109] Thus, the linker chain maintains similar properties to the backfilled PEG molecules, including the high flexibility of the polymer. This suggests that ligand linkers can be "bent" as easily as the nonfunctionalized PEG molecules. Hindering of the specific recognition sites of targeting ligands by PEG molecules could lead to poor targeting results. This is more likely if the particle surface is saturated and the functional molecules are close to each other. Thus, optimizing the distance between different functional surface molecules is necessary for both the targeting ligands and the stealth molecules to maintain their functions.

A high PEG density on particle surfaces could, in turn, lower the surface density of the targeting ligands, and vice versa. As mentioned in Section 3.1, the surface grafting

density of targeting ligands is a key factor that governs the targeting outcome for particles.[83a] The ligands on particle surfaces need to reach a desired density for high targeting efficiency.[83a,86] Similarly, the grafting density of the stealth molecules should be high enough to render the surface low-fouling.[132d] However, in some cases, achieving an optimal balance of targeting ligand and stealth material density is challenging. Instead of post-PEGylation on ligand-functionalized particle surfaces, alternative approaches have also been pursued. Kang et al. reported the efficient combination of stealth and targeting behavior using particles with the targeting ligands tethered on the outer PEG layer.[59] The PEG layer could shield the non-specific interactions between the bare particle surface and biomolecules in the environment, while the terminating targeting molecules facilitated efficient targeting specificity.[59] This approach has been reported as an efficient method for attaching different targeting ligands, including Man, scFv, Tf, and polypeptides.[59,133-134,158] However, PEG itself could affect the efficiency of conjugating the ligand to the particle. Bargheer et al. reported that higher PEGylation of iron oxide NPs resulted in diminished binding of Tf onto PEGylated particle surfaces.[158] Therefore, PEGylation should also be optimized so it does not impede the ensuing attachment of targeting ligands. A post-PEGylation coupling approach results in the targeting ligand being the outermost layer of the particle. The targeting moieties not only interact with their specific targets, but may also have non-specific interactions with other components in its surroundings. Even though a high density of targeting ligands on particle surfaces can increase targeting efficiency, it can also inadvertently mask the PEG layer and compromise stealth properties of particles.[84] Thus, the functionalization degree of targeting ligands also needs to be at an optimum level to maintain a high targeting efficiency, while minimizing non-specific interactions.[84,86]

4.3. Recruiting Proteins to Promote Targeting

The biological system is extremely efficient, albeit complex, with each component playing a specific role. The adsorption of biomolecular components onto particle surfaces as they enter the biological system is often inevitable, and it is sometimes even desirable.[159] For example, it has recently been shown that protein adsorption is required for certain types of particles to exhibit stealth properties.[123b] If certain components with specific functions are adsorbed on particles, it may be feasible to exploit the properties of the adsorbed molecules to facilitate targeting (**Figure 14**). **Table 3** lists some proteins that have been shown useful for improving the targeted delivery of drug carriers. However, it has been suggested that the corona-promoted targeting strategies require the optimization of both the recruitment of specific plasma proteins, as well as the accessibility and orientation of these proteins within the corona.[160]

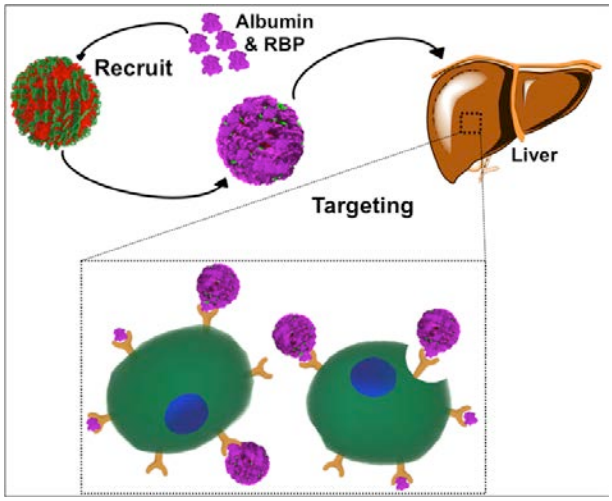

Figure 14. Schematic illustrating the recruitment of certain proteins to achieve targeting objectives (RBP: retinol binding protein).

Among the many thousands of proteins present in biological systems, one of the most abundant, albumin, has been widely studied as a model protein. Albumin has been used to

enhance the stability of different particle systems.[94,161] Particles with a robust albumin coating were reported to exhibit improved stability in biological media, and could be freeze-dried and redispersed without affecting their stability.[161a] Churchman et al. reported that a BSA coating on the surface of ZnO NPs improved their stability by forming BSA-ZnO NP complexes, and promoted the interaction of particles with model lipid membranes.[161b] It was suggested that the smaller size of the BSA-ZnO NP complexes compared to ZnO NP aggregates results in a larger total contact area between particles and the biomembrane, and leads to increased ordering of the lipids within the membrane.[161b] In addition, native albumin has been reported to prevent particles from being filtered by the reticuloendothelial system in vivo, due to its capability to pass through the filtration system without being phagocytized.[162] Dai et al. have reported that a layer

Table 3. Examples of proteins that have been explored to improve particle performance.[a]

Protein		**Recruiting Method**	**Performance**
Albumin	BSA	Non-specific adsorption	Improved stability of NPs[161]
		Specific adsorption	Prevented NPs from being filtered by the reticuloendothelial system in vivo[162]
	HSA	Non-specific adsorption	Enhanced the accessibility of targeting ligands and targeting results of functionalized polymeric particles; improved stability of NPs[87,94]
Apolipo-protein	ApoE	Specific adsorption	Helped particles to cross the BBB and to deliver therapeutics to the brain[104a,163]
	ApoJ (clusterin)	Specific adsorption	Created stealth surface of PEG- or PEEP-coated NPs and reduced non-specific cellular uptake of these NPs[59,123b]
	ApoA4 ApoC3	Specific adsorption	Decreased cellular uptake of NPs[93]
RBP		Specific adsorption	Directed NPs to hepatic stellate cells[162]
Vitronectin		Specific adsorption	Promoted efficient uptake of particles in cancer cells expressing high levels of vitronectin $\alpha_v\beta_3$ integrin receptor[164]
Aβ_{1-42} peptide		Specific adsorption	Captured toxic forms of Aβ_{1-42} peptides and improved Alzheimer's disease condition[165]

[a]Abbreviations: BSA: bovine serum albumin; HSA: human serum albumin; Apo: apolipoprotein; RBP: retinol binding protein; Aβ_{1-42}: amyloid-beta; NP: nanoparticle; BBB: blood-brain barrier; PEG: poly(ethylene glycol); PEEP: poly(ethyl ethylene phosphate).

of albumin coating enhanced the targeting of functionalized polymeric particles by improving the accessibility of the targeting ligand (**Figure 15**).[87] However, when forming the protein corona from an albumin-containing multicomponent biological environment like HS, the benefits of albumin can be impeded by other biomolecules adsorbed on the particle surface.[87] In this case, both the adsorbed albumin and the coupled targeting ligands can be buried by other adsorbed biomolecules.[87,160] Therefore, suitable spatial orientation of both the albumin and the targeting ligands is necessary to eliminate the shielding effects that can arise from the adsorption of other proteins and to ensure their accessibility to the targeted receptors.[87,160]

Another important family of serum proteins that can regulate the properties of particles and modulate bio-nano interactions are apolipoproteins (Apos). ApoE has been reported to help particles cross the BBB and deliver therapeutics to the brain.[104a,163] Kreuter suggested that particles (e.g., liposomes and silica NPs) with ApoE adsorbed on their surfaces could mimic natural low-density lipoprotein (LDL) particles and interact with LDL receptors on the BBB.[104a] Other Apos, such as ApoA-I and ApoB-100, were also reported to enable particles to cross the BBB and deliver drugs to the central nervous system.[104a] These proteins can be chosen to provide further surface functionalization on NP surfaces, and help them cross the BBB. Clusterin proteins (also known as ApoJ) were reported to influence the stealth properties of PEG- or poly(ethyl ethylene phosphate) (PEEP)-coated particles.[123b] In work by Schöttler et al., clusterin was identified as the major component on the surface of polymer-modified particles, and played an important role in reducing non-specific cellular

uptake of these particles (**Figure 16A**).[123b] Similarly, clusterin was found to dominate in the protein corona on the surface of PEGylated hydroxyethyl starch nanocarriers with targeting functionalities (**Figure 16B**).[59]

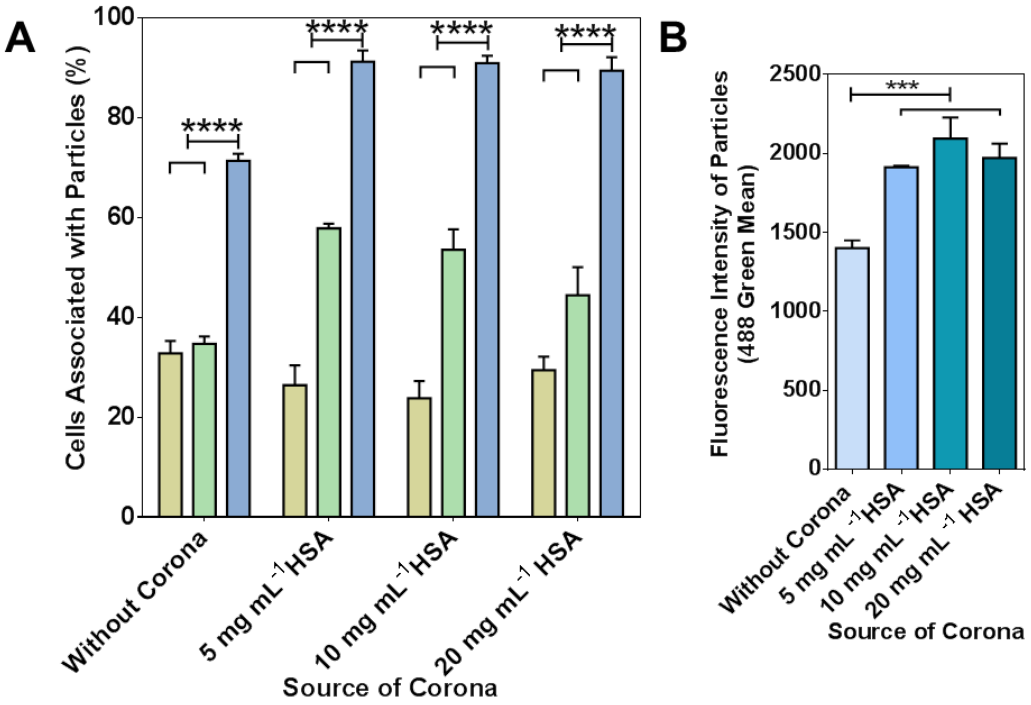

Figure 15. (A) Cellular association of affibody-functionalized particles with targeted cells after a 2 h incubation period in the presence of protein coronas derived from HSA solutions at different concentrations. (B) Accessibility of affibodies on particles coated with protein coronas derived from HSA solutions at varying concentrations. Reproduced with permission.[87] Copyright 2015 American Chemical Society.

Moreover, Schöttler et al. suggested that the efficiency of clusterin in hindering NP uptake could be further assisted by other Apos or other types of proteins.[123b] Ritz et al. reported that ApoA4 or ApoC3 precoated on particle surfaces significantly decreased the cellular uptake of particles, indicating the possibility of using these proteins as an endogenous alternative to PEG to mask non-specific bio-nano interactions.[93] By specifically recruiting or pre-adsorbing these endogenous proteins for surface functionalization, particles can display

improved biodistribution profiles, hence enhancing their possibility of reaching their designated targets.

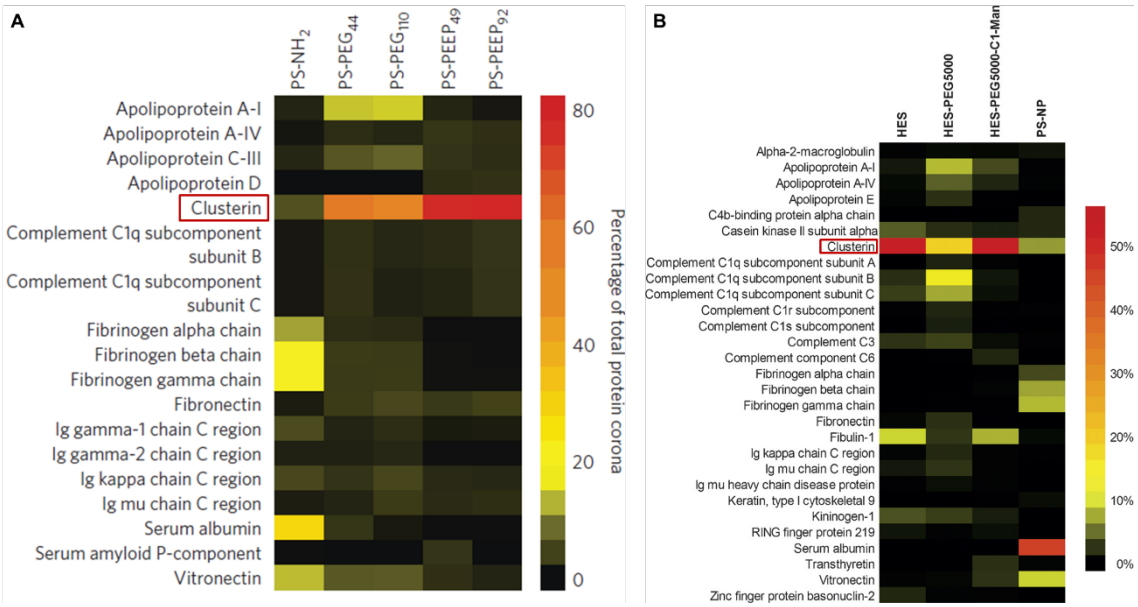

Figure 16. Heatmaps of the most abundant proteins in the protein corona of PEG- or PEEP-functionalized polystyrene (PS) particles (A) as well as the hydroxyethyl starch (HES) particles with different surface functionalization (B) determined by mass spectrometry. Clusterin is highlighted by the red rectangle in both heatmaps. Reproduced with permission.[59,123b] Copyright 2016 Macmillan Publishers Limited (A) and 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim (B).

As researchers have become increasingly aware of the influence of adsorbed proteins from biological milieus, the recruitment of proteins to improve specific targeting of particles has been investigated. Recently, Santi et al. showed that a rationally designed and in silico predicted peptide sequence tethered to Au NPs can specifically recruit and bind native Tf in a controlled orientation, while minimizing nonspecific protein adsorption from the biological environment.[166] Tf-Au NPs were internalized via transferrin-mediated endocytosis and the internalization rate was dependent on the amount of Tf-binding peptides on the Au NP

surface. Similarly, Zhang et al. reported a retinol-conjugated polyetherimine (RcP) NP system, which recruited native albumin and retinol binding protein 4 (RBP) in its protein corona and facilitated targeted delivery of therapeutics to treat hepatic fibrosis.[162] The recruitment of native albumin helped to evade phagocytosis by macrophages, while the RBP was found to direct the RcP NP to hepatic stellate cells (HSC).[162] The RcP particles loaded with an antisense oligonucleotide then effectively suppressed the expression of type I collagen, which is necessary for the amelioration of hepatic fibrosis.[162] In addition, lipid NPs from 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and DNA were reported to recruit vitronectin from human plasma components.[164] The vitronectin-enriched protein corona on the DOTAP/DNA particle surface promoted the efficient uptake of particles in cancer cells expressing high levels of the vitronectin $\alpha_v\beta_3$ integrin receptor.[164] Caracciolo et al. demonstrated that the protein corona of these lipid particles can potentially be used for the targeted delivery of therapeutics.[164] Other examples are PEGylated polymeric particles, which have been reported to interact with the amyloid-beta ($A\beta_{1–42}$) peptide in different environments.[165] The $A\beta_{1–42}$ peptide adsorption did not change the complement activation of particles nor the adsorption of other specific proteins, hence the safety and the clearance kinetics of the particles remained unchanged.[165] Brambilla et al. suggested that these long-circulating particles had the potential to capture toxic forms of $A\beta_{1–42}$ peptides from systemic circulation as a treatment for Alzheimer's disease.[165]

4.4. Tuning the Amount and Properties of Adsorbed Proteins

Although there are various proteins that have the capability to improve the outcome of targeted carriers, the biological environment is very complex and makes the specific recruitment of a desired protein difficult. Other proteins may adsorb on the particle surface as well, which can bury the desired proteins or change their conformation.[160] Therefore, as discussed above, to achieve protein corona-mediated particle targeting, both the recruitment

of certain proteins as well as their structure, orientation, and accessibility within the corona must be optimized.[160]

Since specifically recruited proteins are designed to improve the performance of particles, they must be relatively stable on the particle surface and must not be readily exchanged by other proteins in the biological environment. This can be accomplished by having the desired proteins present in the 'hard' corona, which is more stably bound to the particle surface than the 'soft' corona.[167] Moreover, the soft corona needs to be as sufficiently thin so as to not hinder the function of the recruited proteins.[160] Ashby et al. observed a relationship between particle properties and the dynamic nature of protein coronas by studying protein exchange rates on the surface of particles with different surface hydrophobicity and core diameter.[167] Higher surface hydrophobicity or larger core sizes resulted in a more dynamic protein corona with a larger portion of adsorbed proteins exhibiting fast exchange rates.[167] Therefore, both the size and surface hydrophobicity must be carefully controlled to ensure that recruited proteins remain stable and can perform their function in the corona. In addition, the surface modification of particles also plays an important role in determining the protein corona formation.[167-168] For particles with various surface functionalities, such as targeting ligands, stealth molecules, and other functional groups to recruit specific proteins, a rational design of all surface functional molecules is necessary for improved particle performance. This includes optimization of surface density, choice of end groups, molecular size and structure, as well as coupling methods of the functional molecules.[168a] It was reported that PEG conformation (e.g., due to different coupling methods) had a strong influence on the corona composition and properties.[168a]

Another important factor is the orientation and structure of proteins on the surface of particles. Generally, a protein contains one or more active sites/domains for their specific function.[169] The accessibility of these active domains is necessary for a protein to exhibit its

desired function.[160] Therefore, proper orientation of recruited proteins is required to make their active domains exposed and accessible.[160] In some cases, space between different protein molecules is necessary. Moreover, the interactions between proteins and particle surfaces can result in the denaturation of proteins, and thereby affect the properties and functions of the proteins.[99,102c] Thus, recruited proteins must maintain their proper structures to retain their desired functions. For this purpose, specific ligands or functional groups need to be introduced. Zhang et al. reported that the retinol on RcP particles recruited albumin via hydrophobic interactions, which maintained the native confirmation of albumin and hence its activity.[162] In contrast, unmodified hydrophilic particles bound albumin so that hydrophobic regions of albumin were exposed, which induced denaturation and rapid recognition and clearance of particles.[162] Moreover, retinol also recruited RBP onto the particle surface with the native function of the protein maintained, which directed the RcP particles to HSC cells.[162] Some selective polymers containing pyridine groups, such as poly(4-vinylpyridine) (P4VP) and pyridine grafted poly(hydroxyethyl methacrylate) (pHEMA), were reported to offer a microenvironment capable of maintaining protein structure and conformation.[170] These polymers can be candidates to maintain proper conformation and activity of recruited proteins.

Taken together, it is difficult to avoid the formation of a protein corona on a NP surface. Instead, an alternative approach is to exploit the protein corona to improve the properties and performance of targeted particles. As discussed above, certain types of proteins recruited from biological environments by particles can help improve the biodistribution profile of particles, and help direct them to targeted regions and tissues, or to facilitate crossing of challenging biological barriers. As these recruited proteins compete with the influence of other proteins, controlled orientation and molecular structure of the recruited proteins are essential for maintaining their native or desired functions. As these challenges

are being investigated and our understanding is increasing, it may soon be possible to rationally design drug delivery carriers with highly efficient targeting ability arising from both chemically grafted ligands and controlled recruitment of endogenous proteins.

5. Evaluating Targeted Drug Carriers in Vitro

In the previous sections, we discussed how the properties of particles and their interaction with biomolecules in a physiologically relevant environment can affect the targeting performance of the drug carrier. Many of these studies that determine the effect of the biological environment on targeted carriers and the targeting outcome take place under in vitro static conditions (e.g., conventional multiwell plates in a cell culture incubator). In some cases, the targeting assays are performed with the free receptor in buffered solution, or using cells in serum-free conditions that poorly reflect the complex biological environment targeted drug carriers are exposed to when administered in vivo (e.g., via systemic circulation). Before particles reach their target sites, they need to overcome several biological barriers, including the MPS, hemorheological limitations, intratumoral pressure, and cellular uptake/endosomal escape.[13a,171] In this section, we discuss more complex in vitro models and assays that can facilitate mechanistic studies of fundamental processes of targeted drug delivery to guide the design of drug carriers with improved efficacy (**Figure 17**). We highlight factors, including fluidic flow, heterogeneous cell models, and the role of the ECM, that are important for evaluating the targeting ability in vitro.[172]

5.1. Influence of Fluidic Flow

Once targeted drug carriers are exposed to the biological milieu, proteins will be adsorbed on their surface, forming a protein corona and affecting their targeting capabilities, as discussed above. Hadjidemetriou et al. showed that the protein corona composition on targeted liposomes differed when formed under static in vitro conditions or under in vivo blood

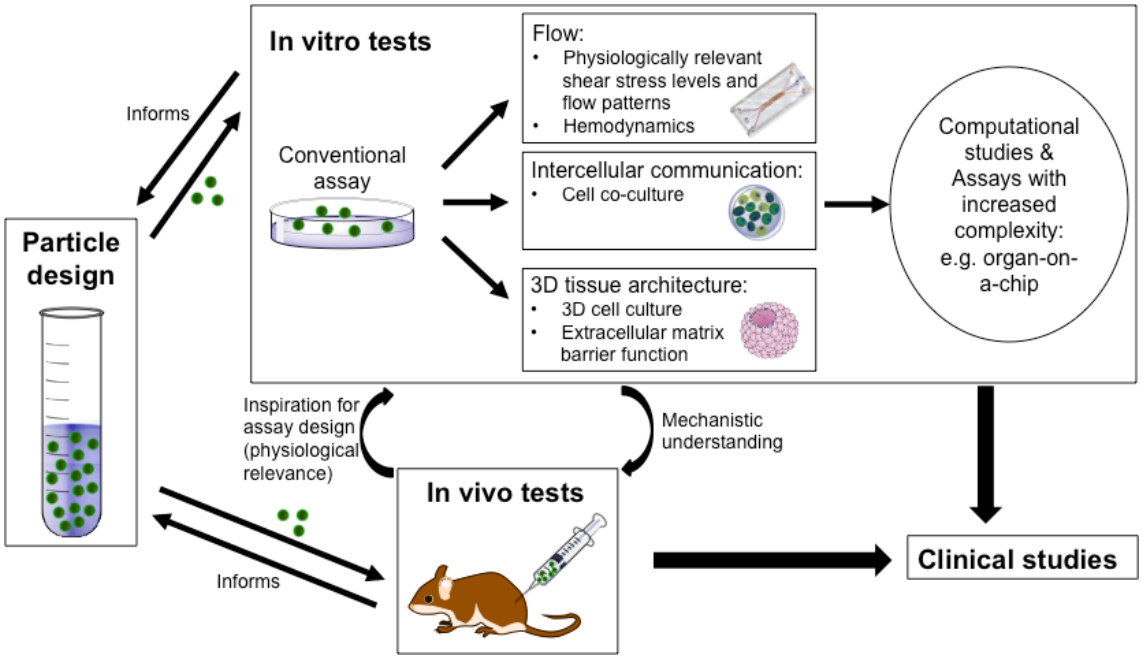

Figure 17. Schematic illustration of how particle design and particle evaluation can direct the development of targeted drug carriers for successful translation into the clinic.

circulation.[112] However, most of the studies so far have formed coronas under static conditions and neglected the influence of fluid flow in systemic circulation. It is important to include this type of effect as slight modifications of protein coronas can result in different physiological responses.[91] To recapitulate the situation in vivo more closely, Pozzi et al. compared the protein corona formed on liposomes exposed to FBS under static and dynamic incubation conditions.[173] Compared to static incubation conditions, there were more low-molecular-weight proteins adsorbed under dynamic incubation conditions.[173] Similarly, low-molecular-weight proteins were found to be dominant in protein coronas formed on liposomes in the blood circulation of rats and mice.[112,174] To further elucidate the impact of fluid flow on the formation of protein coronas, studies investigating how flow patterns and shear stresses influence protein adsorption are needed. Moreover, as the particles will be transported by blood when administered in vivo, the use of whole blood as the circulation

medium for in vitro assays can be beneficial for mimicking the in vivo environment more closely and to provide results more consistent with in vivo data.

Blood is a complex fluid consisting of RBCs, leukocytes, and platelets in protein-rich plasma.[175] In blood vessels, RBCs tend to align in the middle of the bloodstream, while leukocytes and platelets migrate to the vascular wall, generating an RBC-depleted plasma layer. This tendency of leukocytes and platelets is referred to as margination.[176] Similarly, injected particles also have a tendency for margination, which can enable enhanced cellular adhesion.[176] Margination of particles is affected by hemodynamic forces (e.g., flow rate, hematocrit content, vessel geometry), buoyancy, van der Waals and steric particle-endothelium interactions, as well as particle size, shape, and deformability.[177] A number of experimental and theoretical studies have investigated the margination behavior of particles in the presence of flow.[175b,177-178] Overall, microparticles show improved margination over NPs.[179] However, if the particles are below a critical size (< 100 nm), margination has been observed to increase again.[177,180] This behavior has been attributed to differences in the transportation mechanisms of particles with different size, which significantly affect the margination of particles.[178a,180] Moreover, blood composition is highly complex and blood cells can significantly affect the margination behavior of particles. In the presence of RBCs, Eniola-Adefeso and co-workers found that spherical particles with a large diameter (2 μm) showed significantly improved margination and binding affinity to activated endothelial cells compared to their nano-sized counterparts.[181] In addition, nonspherical particles (e.g., rod, oblate, disc) experienced torque resulting in rotation and tumbling under flow conditions, which increased the probability of particle-endothelial cell (EC) interaction and extravasation.[182] In contrast, in the absence of RBCs, rod-shaped particles of a smaller size favored wall deposition compared to their spherical counterparts in fibronectin coated microfluidic channels.[180] While nano-sized particles exhibited disadvantages in margination

compared to micron-sized particles, successful in vivo targeting studies using NPs suggest that these disadvantages can be counteracted by other effects. Such effects could include reduced recognition of NPs by the MPS, susceptibility to hydrodynamic drag forces, and collisions with blood cells after successful adhesion.[183]

Other important parameters in the blood stream are the varying flow rates and the different shear stresses. Upon intravenous administration, particles are exposed to very different flow rates during their journey, ranging from up to around 0.3 m s^{-1} in the aorta, to mm s^{-1} within capillary networks, and µm s^{-1} in the interstitium.[184] Increasing shear stress (as a result of high flow rates and/or small vessel diameters) has been found to decrease the cellular association of targeted particles in fluidic channels.[185] Therefore, particles aimed at vascular targeting need to tolerate high shear stress at their targeted sites. However, carriers for tumor cell targeting are exposed only to low shear stress in the interstitial tumor space. The effective strength of the particle-cell interactions must overcome hydrodynamic drag forces to allow for sustained cell binding.[68c,186] Cell-lined, fluidic systems have been used to optimize targeting ligand density and geometry of particles and to mitigate the impact of hydrodynamic drag forces.[187] In physiological cases, striking a balance is important: a bigger size can allow for the multivalency of particles to be increased, thus improving their adhesion to cells, but in turn larger particles are affected to a greater extent by hydrodynamic drag forces. The advantage of using particles with elongated shape to maximize multivalency, while minimizing the impact of drag forces has been recognized and validated in both experimental and theoretical studies.[73,187c,188] Kolhar et al. reported that rod-shaped nanocarriers showed higher specific binding compared to their spherical counterparts in both a Y-shaped microfluidic channel and in mice.[187c] This shape-enhanced targeting specificity was attributed to multivalent interactions favoring cell binding, while simultaneously compensating shear-induced detachment and entropic losses that reduce cell adhesion.

Moreover, an adequate flow rate reduces the impact of carrier sedimentation and improves the predictive power of in vitro assays.[186,189] Brodha et al. reported a microfluidic setup and analysis routine to investigate the targeting ability of peptide functionalized particles to HuH7 cells under shear stresses mimicking elevated interstitial fluid pressure.[190] This strategy successfully reduced experimental artifacts arising from particle sedimentation.[190]

The studies mentioned above show that fluidic devices can be useful when optimizing the ligand density and carrier geometry. Careful design of in vitro assays, e.g., the application of different physiologically relevant shear stresses and vessel geometries (small vs. large, branched vs. linear), the use of complex liquids to recapitulate biological fluids, as well as validation through in vivo experiments, are necessary to increase the predictive power of such assays. In combination with computational modeling studies, such assays using fluidic devices hold potential to be cost-effective tools to accelerate drug carrier development while reducing the need of animal experiments.[191]

5.2. Heterogeneous Cell Culture Models

The cellular diversity found in vivo is poorly mimicked in most of the currently existing in vitro assays. A basic approach used to validate the specificity of targeted particles in a heterocellular environment is to culture the targeted cell line in mixture with cells lacking the targeted receptors.[108,192] However, this simplified approach does not fully mimic the in vivo heterocellular environment. In the body, heterocellular environments and concomitant intercellular communication has been shown to be critical for maintaining functional cell phenotypes.[193] For example, the M-cell is a potential target for oral vaccination because of its transcytotic capabilities.[194] Gullberg et al. established an in vitro co-culture model of human intestinal epithelial Caco-2 cells and B-cell lymphoma Raji cells for the expression, investigation, and development of the M-cell phenotype in a controlled in vitro manner.[195] This co-culture model showed that intercellular communication resulted in altered surface

receptor expression patterns and increased transcytotic rates compared to monoculture.[195] Evaluating in vitro targeting results using co-culture systems of this type may be easier to translate to in vivo.[196] Moreover, with regard to cancer metastasis, the influence of intercellular communication is important.[197] For instance, the tumor stroma, including cancer-associated fibroblasts and pericytes, contributes significantly to the tumorigenic process.[198] To this end, it is of interest to evaluate how targeted drug carriers perform in such a heterocellular environment.

Studies have shown that gene expression and signaling pathways vary substantially for three-dimensional (3D) tissue constructs compared to two-dimensional (2D) cell monolayers.[199] For example, Pickl et al. reported that the proliferation of SKBR-3 cells was inhibited to a greater extent by Trastuzumab when the cells were cultured in a 3D construct as compared to a traditional monolayer culture pattern.[179,199] It was shown that the organization of HER2 molecules, the receptor of Trastuzumab, was dependent on the cell culture conditions. In 2D culture, the formation of heterodimers of HER2 and HER3 was observed, whereas HER2 formed homodimers in 3D tumor spheroids. Moreover, ECM components were also found to contribute to the influence on the targeting efficacy of HER2-targeting agents (Trastuzumab, Pertuzumab, and Lapatinib).[199] It was observed that the HER2 downstream signaling was directly affected by the culture conditions. These studies highlight that it is critical to evaluate how different architectural phenotypes affect molecular signaling and consequently the efficacy of targeted drug carriers. Emerging concepts such as 3D bioprinting and the engineering of advanced materials with enhanced cell culture performance may aid in these investigations.[200]

5.3. Role of the Extracellular Matrix

Tumor cell-targeted particles have shown promising results in monolayer cell cultures, whereas these results have been challenging to translate to in vivo. The reduced efficacy of

particles is typically due to a combination of factors, including poor vascularization in solid tumors, varying composition and architecture of the ECM at different sites, as well as high interstitial flow pressure, which contributes to limited particle distribution and tumor penetration.[201] Simple but instructive models to investigate the influence of the ECM on transportation of drug carriers within tissues are acellular hydrogels mimicking the porous and tortuous structure of the ECM.[202] Both size-based and the interaction-based filtering mechanisms have been reported to play important roles in particle transportation.[203] Early studies investigated the influence of particle surface chemistry on particle movement within complex biomaterials such as F-actin and fibrin networks.[204] PEGylated particles were found to interact the least in comparison with bare carboxylated or BSA-coated microspheres.[204] Lieleg et al. confirmed the influence of electrostatics on the interaction of particles with ECM components.[203b] It was suggested that localized charge patches within the ECM can act as electrostatic bandpass filters that inhibited the transportation of both negatively and positively charged carriers.[203b] Mathematical modeling studies also showed that the movement of charged particles was slowed down by both attractive and repulsive interactions with ECM components.[205] In both studies the neutral particles had the highest mobility within the ECM.

More biologically complex models for investigating transportation limitations of particles are multicellular spheroids characterized by tight cell-cell interactions and ECM production, mimicking aspects of physiological tissue organization.[201d] In contrast to endothelial targeting where high particle avidity is desirable, high avidity may impede effective tumor penetration due to the ‘binding site barrier’ effect, an effect previously observed for monoclonal, high-affinity Abs.[206] Whereas high avidity of particles resulted in accumulation at the rim region, decreased avidity enhanced penetration of tumor spheroids.[207] Treatment with collagenase, changing the organization of ECM, led to a

significant increase in NP penetration, which highlights the importance of extracellular matrix organization.[208] In addition, the multicellular spheroid with ECM production is a fairly simple system that can provide new insights when studying and optimizing particles functionalized with tumor penetrating peptides.[209] However, it is important to note that the assay conditions used in 2D cell culture patterns need to be carefully redesigned and adapted to 3D cell culture models (e.g., longer incubation times and/or trypsinization is required to allow for spheroid penetration in 3D constructs).[210] In a recent study Priwitaningrum et al. found that the penetration of particles in tumor stroma is strongly impeded.[211] The penetration depths in a 3D spheroid model depended on several factors, including NP size, ζ-potential, and the spheroid model. Smaller silica NPs (30 nm) showed deeper penetration than larger particles (100 nm), NPs with a low ζ-potential (-40 mV) showed deeper penetration than NPs with a ζ-potential of -20 mV, and homospheroids were penetrated to a larger extent than heterospheroids.[211] This study supports the understanding that tumor stroma presents a biological barrier that drug carriers need to overcome to reach their targets.

5.4. Suggestions for Selecting Adequate Models

There is no easy solution or simple guide for the complex challenge of designing an efficient targeted drug carrier as often multiple conflicting requirements and properties needs to be balanced,[212] and different applications will require different particle designs. For example, for endothelial targeting using larger particles with efficient margination, microparticles can work well whereas for direct tumor cell targeting small NPs are typically needed for efficient extravasation and transport through the interstitium.[13a,b,29] The intended mode of delivery (e.g., inhalation, transdermal or intravenous injection) is also important, as this will determine the biological environment that the particles are first exposed to and can therefore affect targeting outcomes. Similarly, there is no ‘one size fits all’ for in vitro assays either. Here are some questions we suggest one should keep in mind when choosing and designing assays:

i) What are the fluidic flow rates and patterns the carrier will be exposed to?
ii) How does the fluidic flow affect the protein corona formation on the carrier surface? For example, does it induce agglomeration under certain conditions?
iii) How does fluidic flow affect the recognition between targeting ligands and receptors, as well as the subsequent cellular internalization of particles?
iv) How does hemodynamics influence margination and adhesion of carriers in various vessel geometries?
v) How does intercellular communication affect the expression of receptors on cell membranes; can it promote drug resistance?
vi) What is the influence of extracellular matrix and tissue-like architecture on the transportation of carriers in the interstitial space?

While fundamental in vivo studies are certainly important to understand the principles governing particle behavior within the human body, in vitro models that mimic a specific clinical context or disease are also necessary to increase our understanding and to facilitate the design of drug carriers. The studies highlighted in this section suggest that assays for the evaluation and optimization of targeted drug carriers can be improved by successively and systematically increasing the complexity of in vitro models. For example, the organ-on-a-chip technology has shown promise in this regard and is expected to contribute in bridging the gap between in vitro and in vivo.[213] In general, the difficulty that remains in the translation of nanomedicine delivery systems towards clinical applications emphasizes that it is time to challenge current thinking by adopting methods that complement the 'traditional' ones, and investigating new and creative strategies for improving our understanding of the behavior of targeted particles in complex biological environments.[10a,11,214]

6. Perspectives and Conclusion

There has been a trend towards using dual or multiple surface functionalities to improve performance and to design drug carriers with higher targeting efficiency (**Figure 18**).[215] While there are trade-offs involved when adding functionality to a particle system,[10a,216] multi-functional particles have the potential to perform several roles, including imaging, targeting, as well as drug encapsulation and delivery.[216-217] Moreover, the recruitment of certain proteins to improve the performance of particles also requires

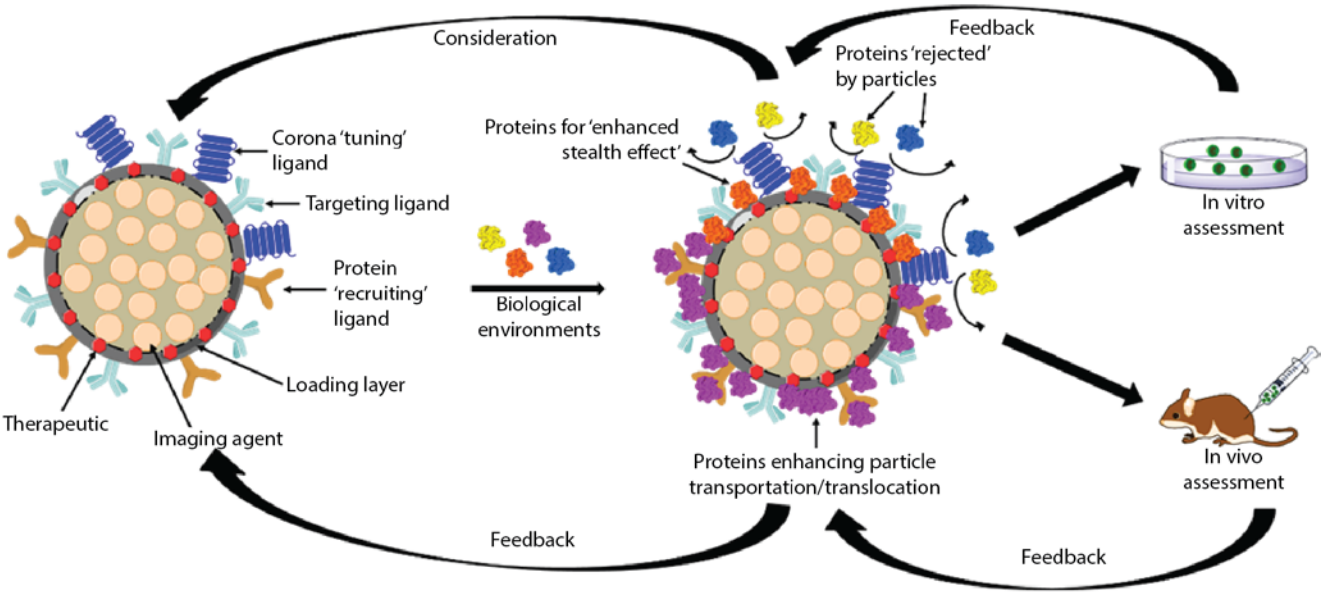

Figure 18. Schematic illustration of the design of the next generation nano-carrier systems with multiple functions, e.g., targeting, imaging, and delivery of therapeutics, by taking advantage of the protein corona.

a multi-functional particle surface, which may include targeting ligands, protein-recruiting materials or molecules, as well as other possible "corona-tuning" groups. Hence, it is important to balance the different surface functionalities to optimize (i) targeting ability, (ii) pharmacokinetics, and (iii) biodistribution, without compromising each other. There are also several other factors that are important for successful translation, including stability of drug

carriers as they need to be stable enough to survive transportation and storage.[218] Particles with well-controlled and tailored protein coatings can facilitate this. For example, it has been shown that some protein coatings not only stabilize Au NPs at high concentration in biological media but can also facilitate lyophilization and redispersion of particles (a common method for transportation and longer term storage).[161a] The benefit of a protein corona coating might also be applicable to other carrier systems to enhance their stability.

The 'personalized protein corona' (PPC) discussed by Hajipour et al. is another strategy that may enable the design of new and improved drug carriers.[219] Since many diseases affect both the concentration and the composition of plasma proteins, any drug carrier administered to patients will be exposed to biological environments that vary depending on the disease.[219-220] Furthermore, even patients with the same type of disease can vary in the concentration and composition of plasma proteins, due to differences in age, gender, medical history etc.[221] Therefore, for drug carriers administered to patients, different biomolecular coronas will form.[219] To achieve optimal performance, one may therefore need to consider the type of disease, as well as the age, gender, and medical history of patients in the early stages of drug carrier design.

With the ability of raising the drug concentration at a desired location while minimizing harmful side effects in healthy tissues, targeted drug delivery systems have been attracting increasing attention for the delivery of numerous therapeutics. In this review, we have discussed key factors to be considered during the design of targeted drug delivery carriers. We focused on both the challenges and potential benefits associated with achieving successful particle targeting in complex biological environments. In addition, we provided suggestions for the development of new, improved and complementary in vitro assays to facilitate the evaluation of drug carrier systems.

Influences and effects from the biological environment, especially the formation of protein coronas, affect every drug carrier system when it is administered in vivo. Therefore, even at the carrier design stage, the complexity and dynamic nature of the in vivo environment and associated bio-nano interactions need to be considered. The negative impact of a complex biological environment on particle targeting can be largely reduced—or even eliminated—through the rational design of particle properties and careful selection of particle surface functionalities (Figure 18). As it remains highly challenging to totally shield particles from biomolecular adsorption once they are in contact with the biological environment, utilizing the protein corona to improve particle performance is a promising avenue. One research direction related to this that is showing promise is the design of particles that constructively interact with and prime the immune system—instead of trying to hide from it—and represents the emerging interface of nanomedicine and immuno-oncology.[222] These research efforts can be accelerated by emerging broader themes towards increasing robustness and convergence in science,[10a] for example by facilitating quantitative comparisons through the use of best reporting practices and the development and adoption of nanomaterial standards.[223]

Finally, we highlighted a series of protein candidates that have already been recruited successfully to fulfill a targeting role or enhance circulation times. Both the composition and the amount of proteins on particle surfaces must be carefully tuned to maintain the active orientation and structure of the recruited proteins. Recent advances using “bio–nanointerface mapping” and “epitope mapping” approaches have provided valuable insight into these topics.[224] For example, it was recently shown (using epitope mapping) that only around 4% of targeting ligands grafted on silica nanoparticles have a favorable orientation for recognition by the targeted cellular receptor,[225] indicating that some commonly used methods for grafting targeting ligands can lead to poor or heterogeneous outcomes.

Since bio-nano interactions are complex and system-dependent, there is no unified standard among different carrier systems, and each system needs to be optimized individually to improve its performance. To this end, we have discussed trends and guidelines that can be adopted for optimizing drug carrier design, towards addressing the challenges imposed by the biological environment. Developing new and improved in vitro assays that can help elucidate bio-nano interactions and predict in vivo performance of targeted particles (and eventually perhaps even clinical performance) remains an important objective for the field. In vitro assays with greater biological complexity that capture key aspects of the in vivo environment may prove useful in these efforts, to bridge the gap between in vitro and in vivo, and to facilitate and accelerate the translation of well-designed carrier systems into improved patient outcomes.

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