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Review

Designing the nanoparticle–biomolecule interface for "targeting and therapeutic delivery"

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ABSTRACT

The endogenous transport mechanisms which occur in living organisms have evolved to allow selective transport and processing operate on a scale of tens of nanometers. This presents the possibility of unprecedented access for engineered nanoscale materials to organs and sub-cellular locations, materials which may in principle be targeted to precise locations for diagnostic or therapeutic gain. For this reason, nano-architectures could represent a truly radical departure as delivery agents for drugs, genes and therapies to treat a host of diseases. Thus, for active targeting, unlike the case of small molecular drugs where molecular structure has evolved to promote higher physiochemical affinity to specific sites, one aims to exploit these energy dependant endogenous processes. Many active targeting strategies have been developed, but despite this truly remarkable potential, in applications they have met with mixed success to date. This situation may have more to do with our current understanding and integration of knowledge across disciplines, than any intrinsic limitation on the vision itself.

In this review article we suggest that much more fundamental and detailed control of the nanoparticle–biomolecule interface is required for sustained and general success in this field. In the simplest manifestation, pristine nanoparticles in biological fluids act as a scaffold for biomolecules, which adsorb rapidly to the nanoparticles' surface, conferring a new biological identity to the nanoparticles. It is this nanoparticle–biomolecule interface that is 'read' and acted upon by the cellular machinery. Moreover, where targeting moieties are grafted onto nanoparticles, they may not retain their function as a result of poor orientation, and structural or conformational disruption. Further surface adsorption of biomolecules from the surrounding environment i.e. the formation of a biomolecule corona may also obscure specific surface recognition. To transfer the remarkable possibilities of nanoscale interactions in biology into therapeutics one may need a more focused and dedicated approach to the understanding of the in situ (*in vivo*) interface between engineered nanomaedicines and their targets.

Contents

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1. Introduction

The emerging alliance between nanomaterial science and the biological and medical sciences is founded upon fundamental scientific principles, and may be expected to grow and deepen for many decades to come. It is perhaps worth mentioning that this alliance has been difficult to construct, involving mistaken directions and misunderstanding between different scientific disciplines and cultures that still hamper progress. The pillars upon which this alliance is now built are as follows.

Firstly, the endogenous transport processes at cellular level are actively driven at the nanometer length-scale [1–3]. Many small molecules can partition passively in living organisms (at cellular level and beyond), distributing according to physiochemical principles [4] independent of mechanistic biological transport processes. In the simplest manifestation of this, biological barrier crossing properties of similar small molecules are often determined by their physical properties such as hydrophobicity or polarity (there are also small molecules for which carrier mediated transport or carrier mediated/passive combinations are present). Proteins and biological assemblies of many kinds, on the other hand, are transported mainly once they are recognized (often by receptors) and are actively trafficked by processes that require expenditure of cellular energy. These endogenous processes promote entry into cells, and are present also across cell barriers and between organs. Indeed, the presence of physico-chemically driven distribution for many small molecules is in general unsuited to selective targeting outcomes, whereas larger objects energetically indisposed toward passive transport can thereby be selected and processed more effectively. They are processed more effectively via endogenous transport processes, active at the nanometer scale.

This brings us to the second major issue, that of the primary immune system. Specific clearance processes (e.g. the activation of macrophages, and other specialized cells) to eliminate foreign bodies operate extensively in all tissues, and have the important role of removing infective agents, and undesirable cellular debris. Precisely because many endogenous processes operate at the scale of some tens of nanometers (e.g. Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL) particles are around 22 nm and 10 nm, respectively [5]) these clearance mechanisms are considered muted for objects less than several hundreds of nanometers. There are of course other factors that affect these processes, for example the nature of the proteins (e.g. opsonins or dysopsonins) that are presented at the surface of the object alters its circulation time in blood [6], and we shall return to these in some detail later. There are also particular situations (discussed later), such as the action of the Enhanced Permeability and Retention (EPR) effect, where the general arguments above are not applicable, but these can be considered as being disease and tissue specific. The overarching principles stated above are durable.

Furthermore, there are practical reasons of a compelling nature to apply the principles of nanotechnology to deliver or target medicines. Thus, small molecules (upon which much of the classical pharmaceutical industry was built) and proteins (the basis of the biopharmaceutical industry) both suffer from an inherent lack of flexibility. In the former, efforts to improve drug targeting require changes to the physicochemical properties of the drug molecules, which need to be implemented on a case by case basis and may impinge on their functional impact. Proteins, though a considerable advance in this regard, have the limitation that they need to be re-engineered in order to achieve both targeting and therapeutic functions at the same time. The efforts in doing so are fraught with huge unresolved challenges, ranging from incomplete re-folding of the engineered proteins, to low yields and also complex regulatory challenges. Succinctly put, engineered nanoparticles have the benefit that their interface can in principle look like proteins to achieve targeting, but their interior, which can be designed to carry the therapeutic agents, is entirely within the control of the scientist or engineer.

It is worth noting, as discussed in more detail later, that in practical situations in which nanoparticles interact with living organisms, the nanoparticle surfaces are initially exposed to a biological fluid, such as blood, lung fluids or the bile fluids in the digestive tract, depending on the route of exposure. For example, nanoparticles injected intravenously would be exposed to blood plasma, containing in excess of 3700 proteins, and many other complex biomolecules, which bind competitively with the surface of the nanoparticles. Whatever the detailed nature of the exposed 'bare' nanoparticle surface (in terms of its chemistry, charge and hydrophobicity), it is likely to be of quite high free energy relative to its environment [7]. Indeed, typical (unmodified) nanomaterial surfaces currently studied have surface free energies many times the thermal energy (k_bT). The nanoparticle surface is therefore immediately modified by the adsorption of biomolecules, such as proteins, lipids, etc., that lower the bare surface energy by a combination of particle surface charge compensation, water displacement, screening of hydrophobic patches and other mechanisms, so that its residual interactions are closer to the thermal energy. This leads to a tightly bound immobile layer formed by the proteins with higher affinities for the particle surface (the hard corona) and a weakly associated mobile layer (the soft corona) [7–9]. In practice, it is surprising to note that, even though biological fluids contain a large variety of proteins (in the example, more than 3700 proteins are present in blood plasma), typical final coronas contain much limited numbers and types of biomolecules (typically only few hundred) [10–13]. Early binders (which are typically the more abundant proteins) are quickly displaced by the proteins with higher affinities for the particle surface, leading to a layer of strongly bound (mostly identifiable) proteins which constitutes the final hard corona [10,11,14]. These tightly associated proteins can play a leading role in the biological interactions of the nanoparticle, and can constitute a nanoparticle–biomolecule interface "readable" by the living organism.

It has been shown that the size (and to a less well understood degree, the shape) of nanoscale objects is highly influential in their biointeractions, i.e., in terms of the selectivity and specificity of their interactions with biomolecules and cells [10,15]. In addition, the surface characteristics of the nanoparticle (e.g. charge, hydrophobicity etc.), and its state within the organism (i.e. the absorbed biomolecules associated with it, termed the bio–nano interface), play a special role in nanoparticle–biological interactions for two main reasons. Firstly, a particle lying within the correct size-range only confers the right to engage with cellular machinery; it is primarily the bio–nano interface that is 'read', interpreted and acted upon by the processing machinery of organisms, at all levels. Even though there are strong physical interactions (van der Waals and others) between the core of the particle and its surrounding environment, the interface between the synthetic architecture and the biological environment constitutes a means of communicating specific relevant signals to the biological processing machinery [16,17]. Secondly, there is a very large total available interfacial area, comparing to, for instance, a same mass of bulk material (e.g. several milliliters of 25 nm nanoparticles dispersed at 1 mg/mL, and with a density typical of organic materials exhibits about 1100 cm^2 of surface area). Clearly, any function displayed at the surface will show enormous effect amplification beyond typical expectations based on the mass of the material alone. These two principles will thereby ensure that the complexities of the bio–nano interface, and its implications in targeted delivery, will be a topic of detailed scientific study for many years to come.

The purpose of this review article is to highlight some key aspects essential for the realization of successful targeting of nanoparticles to specific organs, tissues or cells, outlining some of the obstacles that have prevented achievement of this goal to date. Even though several considerations are made in the context of particle uptake, which constitutes only the first of several recognition events which occur at the bio–nano interface, similar concepts and challenges apply also to the

following steps such as trafficking inside cells, as well as transport across organs and tissues. We describe the role that advances in the synthetic engineering of the bio–nano interface can contribute and suggest some future approaches.

2. Targeting strategies for drug delivery

Targeting is an approach that is intended to increase the specificity of delivery of therapeutic effect, such that it maximally reaches an intended location, thereby reducing the side effects related to unspecific accumulation in other organs or cellular compartments. A highly developed targeting strategy would exploit all of the nano-scale advantages discussed above. Thus one would seek to evade the immune system in order to increase circulation time of the targeted particle and to simultaneously access endogenous transport and trafficking processes. This can allow target site association for a sufficient length of time to confer the therapeutic advantage. Avoiding particle-clearance processes for as long as possible allows more opportunity for circulation, thereby increasing the chance of targeting events occurring, and possibly also lowering the rate at which the reticuloendothelial system must deal with the particles.

We can illustrate the current state of the art in terms of the translation of these ideas to the clinic by referring to the situation in cancer treatment, which as a result of its terminal nature has been used as a testing ground for potentially higher risk or less proven therapies, and as such is much further advanced than other drug-delivery areas. To date, the typical nanoparticles applied in cancer treatment are liposomal. Mixtures of albumin and Taxol have also been of interest, albumin being an endogenous protein that is believed to inhibit opsonization of the nanoparticles by macrophages [6,18]. The more developed concepts generally involve targeting of proteins or receptors that are over-expressed in the cells of a given cancer. Examples of cancer-related protein targets and their binders, or antibodies (often with smaller fragments such as FAB binding regions since the Fc region can activate the immune system) are listed in Table 1 below. Short peptide sequences have also been of some interest [19–21].

Indeed, there is little doubt that (for example with antibodies) one can considerably enhance the binding of nanoparticles to specific over-expressed targets in vitro, and to some degree in vivo, but the actual advantage gained thereby may be limited. Much confusion has arisen in the literature about this issue, which is only now clarifying. Thus, a recent review [36] explains in simple terms (Fig. 1) how the practical challenge goes considerably beyond simply targeting the receptor: even if one can avoid rapid clearance, probes that recognize solely tumor cells provide little improvement of tumor accumulation over a non-targeted probe unless one can penetrate the tumor tissue, and the means to achieve this are as yet limited. Other factors, as yet not clarified, suggest that overly strong receptor binding may block further endogenous trafficking processes (for example by failure to release the receptor inside the cell) therefore losing much of the potential efficacy.

Some examples in the literature (and reported informally elsewhere) appear to show an increased uptake in cells that over-express the corresponding receptor following in vivo delivery of targeted nanoparticles, and efforts to track the effect of uptake/drug release on tumor growth have also been made. However, it is not always clear if these effects are in actuality due to targeting, or to the fact that tumor vessels are composed of defective endothelial layers with wide fenestrations (the EPR effect) in which objects of certain sizes tend to preferentially accumulate [26] (for this – so called – passive targeting, no targeting moiety is needed). The whole field of nanoparticles applied to living organisms is in its infancy, challenged with poorly understood issues ranging from in vivo dispersability, to biological recognition and clearance, and is fraught with irreproducibility. As such, a more thorough and balanced comparative evaluation of targeting issues has not been widely attempted as yet, and it is difficult to be definitive.

2.1. Targeting at cell level

As discussed above, a common strategy to achieve targeting at cellular level is to functionalize the nanoparticle surface with biomolecules, whose receptors are over expressed in the cells to be targeted. Among these examples, human Transferrin (Tf) has been widely used as a cancer targeting agent [37], and thus can act as a useful model for our discussions here. Tf is an endogenous glycoprotein (79-kDa) that binds to the Transferrin receptor (TfR) at extracellular pH and an estimated $K_d = 1–100$ nM, subsequently triggering receptor mediated endocytosis [38]. Many cancer cell types undergo rapid cell division and have a need for additional iron (for heme synthesis), and therefore over-express the Tf receptor (TfR) [39].

The importance of understanding nanobioconjugates and the bio–nano interface at a detailed structural level can be demonstrated by looking at in vitro studies using nanoparticle-Tf conjugates. At cellular level, dextran stabilized iron oxide particles functionalized with Transferrin using different covalently bound linkers exhibited different particle internalization rates and trafficking behavior [22,40]. Linkage-dependant differences in uptake and localization have been commented upon, with various possible scenarios (different affinities, different stabilities of linker) recognized in the literature. For example, studies with the aim of improving MRI targeting report that different conjugates exhibited similar affinities for the TfR receptor, and that the differences in the observed uptake concentration dependence and the uptake time course for the different nanoparticles were linked to the chemical stabilities of the particle-protein linkers in situ. Nanoparticle uptake (by various cell lines including Wild-type rat 9L gliosarcoma cells, Breast carcinoma cell lines BT20, MCF7, BT549, and HBL100 and non-tumorigenic cell line MCF10A) was described as a transferrin receptor mediated process [22]. Transferrin-conjugated nanoparticles were however trafficked in a different manner to the unconjugated protein, going to lysosomes, without sign of export, whereas the protein was recycled as normal. This example shows another potential challenge of targeting, where recognition of specific receptors, even if achieved, does not necessarily imply that the targeted particle is trafficked in the same way as the targeting protein.

However, NP exocytosis was claimed to have been observed in the case of transferrin physisorbed gold NPs. They were described as entering cells (ovarian carcinoma cell line HeLa, brain tumor cell line SNB19, and fibroblast cells STO) via a clathrin-mediated endocytosis pathway, with the endocytosed NPs being subsequently exocytosed at a rate that was in linear correlation with their size [41]. 50 nm was reported as the optimal cellular accumulation size for nanoparticles, due to the equilibration of the rates of uptake and exocytosis. Possibly these observations can be understood within the parameter framework of nanoparticle size and the bio–nano interface nature. Certainly if Tf functionalization does induce exocytosis of nanoparticles this is striking, as most nanoparticles do not show any evidence of exocytosis themselves and accumulate in lysosomes, likely a result of there being no signal present at the nanoparticle surface to instruct the cells to divert the particles from the endo-lysosomal pathway [42,43].

Clearly, there are many potentially confounding circumstances that suggest that one should interpret current data with caution. It

Table 1
Examples of ligand–receptor pairs that have been exploited in targeting cancer cells (at research stage — few have yet been transferred to clinical use).

Surface function	Target receptor	Citation
Transferrin	Transferrin receptor	[22–26]
Insulin	Insulin receptor	[27]
Folic acid	Folic acid receptor	[28,29]
EGFP-EGF1	Tissue factor (TF) related to thrombosis	[30]
GRP	Gastrin-releasing peptide (GRP) receptor	[31]
EGF	EGF receptor	[32,33]
Apo A1 and Apo E	Apolipoprotein receptors (HDL/LDL trafficking)	[34,35]

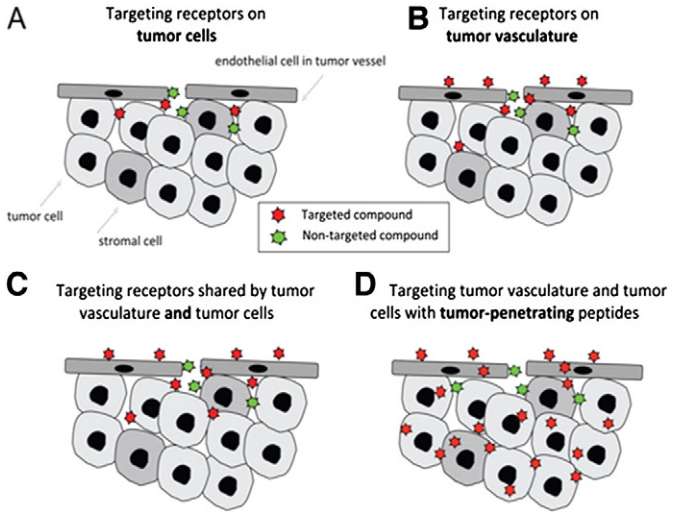

Fig. 1. Targeting approaches for cancer cells. (A) Probes that solely recognize tumor cells provide little improvement of tumor accumulation over a non-targeted probe. (B) Probes that recognize tumor vessels accumulate in the tumor, but entry into tumor tissue relies on passive mechanisms. (C) Probes that recognize both the vessels and tumor cells combine the (limited) efficiency of the two targeting mechanisms. (D) Tumor-penetrating targeting probes (so far only peptides with such characteristics are known) provide a particularly potent targeting system [36].

can be noted that there are a range of factors to consider from particle size, surface properties including charge density, which in turn influence the nanoparticle dispersion behavior in relevant media, even before considering optimal receptor targeting. As discussed above, most nanoparticles, whatever biomolecules they are coated with, adsorb to cells in culture, where they are taken up and trafficked to lysosomes without sign of export [42,44,45]. Moreover, it is important to distinguish between uptake and targeting, as simple uptake levels of targeted nanoparticles do not imply necessarily that uptake is occurring via the targeted receptor. It is possible that there are multiple uptake routes operating simultaneously even in the uptake of targeted nanoparticles. Thus, different ligands, though not acting in a receptor specific manner, can induce differential uptake rates. Also, it is difficult (though not impossible) to control the absolute amount and disposition of the ligand on the surface of the nanoparticles, and in the absence of this, it is difficult to separate out and quantify effects resulting from the amount of grafted ligand from effects due to physico-chemical non-specific adhesions of the particle conjugate to the cellular receptors [45].

2.2. Targeting at organ/tissue level

A typical classification of nanoparticle tumor-targeting strategies distinguishes between passive and active targeting, the first aiming to use generic tumor characteristics, such as leaky vasculature, higher rates of metabolism or levels of oxygenation of the tissue, to indirectly achieve a higher accumulation at the target, and the latter using specific ligands to actively deliver the drug through biorecognition. However, in vivo, both targeted and non-targeted nanoparticles arrive to the tumor vicinity via the so called passive targeting or enhanced permeation and retention (EPR) effect [46], after which the mechanism of tumor cell internalization could be enhanced by the presence of surface ligands for active targeting [29]. The ligand should have the right conformation, and a high affinity for the corresponding receptor, and be able to exhibit high rates of cellular internalization. However, the utilization of a targeting ligand for specific tumor cells does not guarantee higher nanoparticle accumulation in the tumor, in comparison to the equivalent non-targeted nanoparticle [26,36]. Targeting tumor vessels could ensure higher nanoparticle accumulation locally in the tumor, after which the entry of the nanoparticles into the tumor tissue could be passively achieved due to EPR effects, as discussed earlier. Higher targeting efficiency should be obtained by combining these two strategies, and developing nanoparticles that are functionalized to target both the tumor vasculature and the tumor cells [36].

Perhaps the potentially most confusing, and uncertain, aspect of targeting is the degree to which conjugation of bioactive targeting moieties allows for the retention of their full and necessary functionality. Comments on this topic have been made above in relation to the use of conjugated antibodies, but the issue is much deeper. In essence, the almost unexpressed premise is that a targeting moiety attached to an engineered nanoparticle surface retains a sufficient and varied function to succeed. On the contrary, extensive experience from many disciplines from chemical and physical sciences suggests the opposite, and that grafting onto an engineered surface involves the loss, or perhaps even less desirable, the potential for inappropriate expression of biological signals. This may occur because the targeting ligand has a disrupted structure (as in proteins for example) or because its optimal orientation is screened from the target, or indeed, because of additional non-specific biomolecular adsorption from the environment (the 'protein corona effect'). The grafting of any species to a nearby nanoparticulate mass presupposes very large new interactions, which certainly lead to some additional and poorly controlled binding. For those trained in the colloidal sciences, the capacity of biological fluids with their massive variety of molecules, often with different charges, hydrophobic patches and so forth to remain colloidally stable always seems truly remarkable. On the other hand these systems have evolved to achieve just this (apart from active coagulation systems of course, such as in blood). The involvement of a new nanoparticle surface constitutes a very disruptive element in this mixture.

Conventional thinking suggests that, in order to maximize nanoparticle uptake at the target site, the nanoparticles must be able to evade detection by the immune system, avoiding opsonization (enhancement of binding to phagocytes) which results in the removal of particles from circulation, through macrophage recognition and clearance [47]. Macrophages recognize and clear bacteria, (recognizing specific protein patterns on bacterial surface coatings) and as such nanoparticle interfaces should be designed so as not to bind and present at their surface known opsonins, such as fibrinogen, IgG and IgA, or components of the complement system (especially C3b, C4b, and iC3b). Surface modification to reduce non-specific protein binding can, in general, also help in reducing opsonin-independent phagocytosis. A common strategy utilized to achieve this is to coat the particles with poly(ethylene glycol), which is known to reduce non-specific protein binding due to a combination of steric hindrance, related to the polymer flexibility, and hydrophilicity [48]. This approach was investigated where PEG coating was applied to polymeric nanoparticles in an attempt to reduce serum adsorption which leads to complement activation [49]. When PEG layers were added, thus reducing serum adsorption and in particular IgG and complement adsorption, NP-induced serum dependent complement activation via both the classical and alternative pathways was reduced [49]. This reduced protein binding property has been exploited for "stealthing" of nanomaterials in vivo, enabling particles to become long-circulating through recognition evasion [50,51]. Another potential approach includes the use of dysopsonins such as Human Serum Albumin (HSA) or apolipoproteins, to promote prolonged circulation in the bloodstream [6]. Even though it is true that the addition of low affinity polymers or coating such as albumin greatly lowers the amount of non-specific adsorption and improves circulation times in the blood, it is more recently emerging, however, that considerable protein adsorption events remain also after these modifications [52,53]. The role of this in confounding the biological outcomes is still almost unstudied and may contribute to the difficulty in achieving efficient targeting in vivo.

If we consider again the case of TfR for in vivo targeting, several studies suggest that the in vivo distribution of transferrin conjugated PEG-modified gold nanoparticles (AuNP-PEG-Tf) at organ level is independent of transferrin conjugation. Nevertheless, evidence is given that the Tf coating influences nanoparticle localization *within* a particular organ, with tumor cells over-expressing TfR showing higher nanoparticle uptake [26,54] when compared to non-targeted NPs. A dependence of the uptake on the number of grafted Tf per NP has also been shown. This example highlights the difference between targeting at organ level and tissue level. As discussed above, functionalizing nanoparticle surfaces with ligands for receptors over-expressed in cancer cells does not necessarily result in higher accumulation inside specific organs. Indeed, these observations might suggest that even our basic understanding of the underlying mechanisms of targeting is still somewhat limited. In analogy with the dual strategy mentioned above (targeting tumor vessels and then tumor cells), nanoparticles designed to first target the desired organ and then the desired cells may be more successful.

As we noted above, for in vivo studies, there are also many new, potentially confounding, circumstances and again caution is necessary in the interpretation of the data and its significance for targeting. Examples include the fact that differences in amounts of grafted ligand, linker composition and stability, and other details affect the conformation and presentation of the ligand at the nanoparticle surface. Additionally, the inherent non-specific adhesion of proteins from the surrounding environment can mask targeting or introduce competing biological signals. For example, the nature of these captured proteins can affect the clearance processes (e.g. if the particles show an affinity for opsonins or dysopsonins). In fact, most nanoparticle delivery systems seem to localize in the liver and spleen, where often as much as 30–40% of the injected dose accumulates. This constitutes a far greater fraction than that which reaches other organs, including the targeted tumor, where in contrast often only 1–2% of the injected dose arrives. This suggests that undesirable protein binding [55] or other factors promoting liver and spleen localization may dominate.

3. The protein (biomolecule) corona

The biomolecule corona that forms on the surface of nanoparticles once they are exposed to a biological environment is a dynamic entity in continuous exchange with the surrounding medium: the relevant kinetic processes include exchange of proteins between the nanoparticle surface and the plasma, between the nanoparticle surface and the cell surface (including any specific receptors of interest) and between the nanoparticles and any high affinity free protein molecules in the medium that could compete for the cell surface [17], as shown schematically in Fig. 2. Where chemical modification of the nanoparticle surface has been carried out, either to graft a targeting ligand, or indeed to template the surface to promote specific protein binding, then additional adsorption from the environment eventually produces a composite interface that we hypothesize to be the key determining element of the outcome of a targeting strategy. In this, it is important to understand the issues of the relative affinities of all elements of the system (or their exchange times) and those species that reside on the nanoparticle surface for time scales that exceed the processing times (for example receptor recognition) of the biological target of interest may play a role in directing the fate of the nanoparticles. As noted, this may include those molecules adsorbed as well as grafted.

For simple unmodified nanoparticles, as discussed earlier, nanoparticle–biomolecule complexes are composed of the core nanoparticle surrounded by a 'hard' corona of slowly exchanging proteins, and an outer "soft" collection of weakly interacting, and rapidly exchanging proteins [11,16,17,56].

This biomolecule "corona" represents the bio–nano interface that the cell "sees", and thus it becomes important to understand the

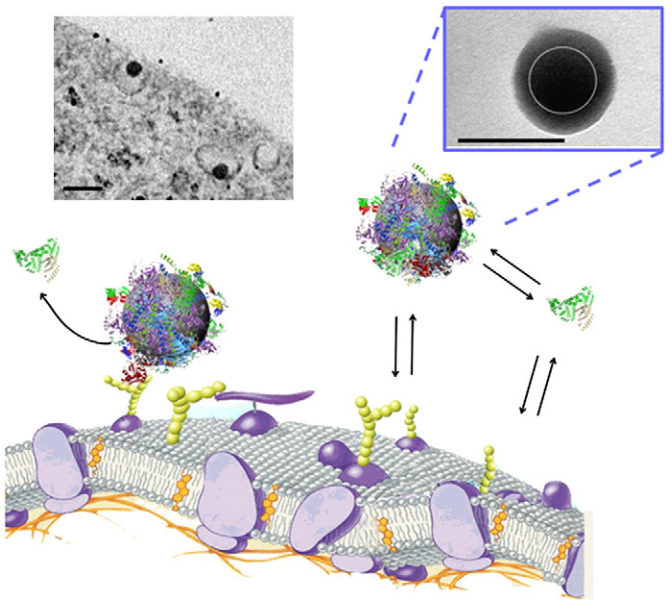

Fig. 2. The nanoparticle protein corona in situ: equilibrium between free proteins in solution and proteins on the cell membrane. Inset (upper left) 50 nm silica particles entering an A549 cell (From Ref. Shapero [44]). Inset (upper right) for illustration only, electron microscopy image of 100 nm polystyrene nanoparticle and (outside white boundary) its biomolecule corona from human plasma. Scale bars 100 nm. Redrawn from Refs. [7,17,44].

corona evolution, in terms of the kinetics of nanoparticle–protein association/dissociation, and the nanoparticle–protein complex composition and structure, as these nanoparticle–biomolecule complexes engage with cells. Indeed, nanoparticle behavior both in vivo and in vitro suggests that the interaction between nanoparticles and plasma proteins, as well as other blood components, is one of the main determining factors in the fate of the particles: the adsorbed protein layer influences not only cell uptake and trafficking [41], but also the specific binding of proteins affects particle internalization and biodistribution in vivo [57].

Thus, for most cases it is more likely that the biologically relevant unit is not the nanoparticle per se, but a nano-object of specified size, shape, and protein corona structure. Indeed, protein adsorption on the nanoparticle surface can affect the protein native structure and activity, and it has recently been shown that these conformational changes can be responsible for triggering specific signals within the cell, such as inflammatory signaling [58,59]. Constituents of the long-lived protein corona may expose relevant (cell-facing) interactive sites activating the cellular machinery [16]. Our findings with a range of different nanoparticle–biomolecule complexes suggest that, in comparison to typical cell-membrane-biology events, the nanoparticle corona is likely to be a defining property of the particle, whether it activates cellular machinery or not [17,45].

Multiple factors such as size, surface curvature and detailed chemical nature determine how biomolecules assemble at the nanoparticle surface [10,60–65]. In fact, the determination of binding rates, affinities, and stoichiometries of protein association with, and dissociation from, nanoparticles in biological fluids is particularly complex, since a great variety of proteins at different concentrations coexist and compete for binding to the NP's surface: it may be that conventional mechanistic understandings for adsorption at solid surfaces [66] are relevant also for nanoparticles. At the beginning the most plentiful associating proteins will occupy the NP surface, but eventually they may be replaced by the less abundant proteins with higher binding affinity which will be the main constituents of the hard protein corona on "longer" timescales. What the cell actually 'sees' during (for example) nanoparticle uptake into the cell, or other biological processes,

requires us to know if those bound proteins reside long enough to be of biological significance. For those with sufficiently slow exchange kinetics, their corona itself would constitute the primary biological interface [17].

3.1. Implications for targeting

In the context of designing the nanoparticle surface interface to enhance in vitro cellular uptake through specific ligand–receptor interactions, or to control the in vivo biodistribution of nano-objects, these "corona" issues represent a fundamental factor. In considering the implications of the corona or bio–nano interface phenomenon for nanoparticle targeting and drug-delivery two counter approaches suggest themselves. One approach, the surface engineered approach, would be to design an interface which exhibits minimal interaction with the surrounding environment except for displaying the specific recognition/targeting desired i.e. a type of adsorption-proof nanoparticle. A second approach involves exploitation of the "corona" phenomenon itself for targeting, by understanding which proteins effectively deliver particles to which location. One key set of studies shows that having a specific surfactant coating (Tween 80) that spontaneously bound Apolipoprotein E (ApoE) from the dispersion medium was *as effective* at delivering a drug to the brain as the specifically surface-engineered particle where ApoE was chemically grafted to the ENP to target the relevant receptor [67,68].

Given the previous discussion it can come as no major surprise that the efficiency of surface engineered nanoparticles must be assessed in the context of other biomolecules that they interact with on-route to the target site, as these could mask the effect of the targeting molecule, or reduce its efficiency as a result of steric hindrance and other effects, as discussed further below.

In contemplating the second approach, that of using the bio–nano interface itself to effect the targeting, the role of the adsorbed protein layer(s) in determining nanoparticle uptake, transport and sub-cellular localization is currently being elucidated. The effect of protein adsorption to the surface of polystyrene nanoparticles on the subsequent uptake of the nanoparticles by hepatic cells has been investigated [69]. When nanoparticles were administrated in the absence of serum in the culture medium, significant uptake was obtained, linked to higher adhesion levels at the plasma membrane. In the presence of serum on the other hand, adsorption of proteins to the nanoparticle surface caused a decrease in the surface energy and consequently a lower uptake of the nanoparticles. Albumin pre-coating of the polystyrene nanoparticles and subsequent exposure under serum-free conditions showed a decreased uptake, suggesting that the presence of proteins screens the non-specific binding of the nanoparticles to the cell surface. Similar work in our own research group confirms that very different uptake rates are observed in the absence and presence of serum proteins in the medium, and indeed that differences in the composition (e.g. heat inactivation to remove complement proteins versus non-heat inactivation) or source of the serum proteins can also have significant effects on nanoparticle uptake, as shown in Fig. 3.

So far, significant effort has been dedicated to identifying those proteins with the highest affinity for nanoparticle surfaces of different compositions or surface characteristics, in order to begin to understand how protein binding to nanoparticles can mediate their interactions with the biological environment: for example, adsorption of opsonins (fibrinogen, IgG, complement factor, etc.) together with large particle sizes (>200 nm) is known to promote phagocytosis with removal of the nanoparticles [70,71], while binding of dysopsonins (HSA, apolipoproteins, etc.) and small sizes promotes prolonged circulation time in blood [69]. Similarly, it has been shown that apolipoprotein enrichment on nanoparticles' surface promotes interaction with LDL receptors resulting in enhanced transport across the blood brain barrier [67,68]. A further challenge arises in assessing the structural evolution of the nanoparticle corona over time as the nanoparticle interacts with living organisms, such as for example,

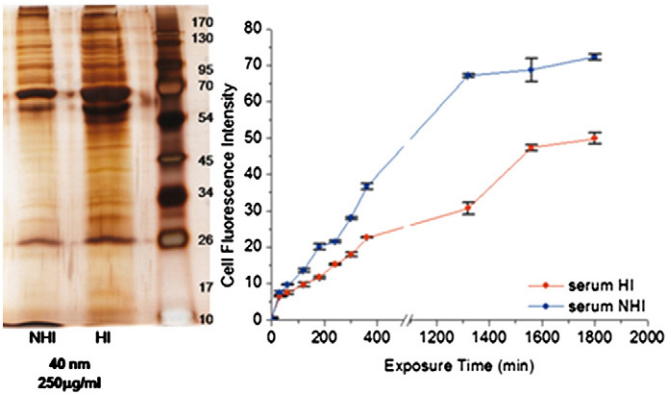

Fig. 3. Effect of protein corona composition on uptake of polystyrene nanoparticles by A549 cells. Right: Silver stained SDS PAGE of the coronas recovered from 250 µg/mL 40 nm PS nanoparticles incubated in cell culture medium in which the serum proteins were either heat inactivated (HI) or not (NHI). The gel shows that different coronas formed when serum of different compositions is used. Left: Kinetics of uptake of 40 nm polystyrene nanoparticles by A549 cells, as determined by flow cytometry. 25 µg/mL nanoparticles were exposed to A549 cells in medium with serum heat inactivated — HI (red line) and not heat inactivated — NHI (blue line). The data show that different uptake kinetics are observed when particles are exposed to cells in the different sera, probably as a consequence of the different protein coronas formed in the two conditions.
Data are reproduced from Ref. [45], where full details of experimental procedures can be found.

how the corona changes as nanoparticles pass through cellular membranes and are transported to their final sub-cellular location [14]. This may be of interest in the context of evolutive or sequential targeting (e.g. first vasculature then cancer cells, first organ then cells, first biological barrier then target cells) given that corona adaption to changing medium has already been observed [72].

Another question still to be answered is whether the long-lived protein layer on the nanoparticle surface mediates the binding to cells through a specific active mechanism or via non-specific interactions with the biomolecules, behaving as a simple coating which reduces the surface energy of the nanoparticles. Recently, Ehrenberg et al. have reported that although different surface modified 100 nm polystyrene NPs have different specific protein coronas, this does not result in differential association of the particles to endothelial cells, suggesting that binding and cellular uptake may not be triggered by interaction of the protein with specific receptors [73]. In this study, many of the most abundant proteins were removed from culture media prior to formation of the protein corona, which altered the profile of adsorbed proteins on the nanoparticles but did not affect the level of cellular association. The authors suggested that an assessment of the adsorptive capacity of nanoparticles could be useful in order to predict the magnitude of nanoparticle cellular interactions.

4. Surface functionalization in engineered nanoparticle design

Significant research effort has been devoted to decorating nanoparticle surfaces with a range of biological moieties to target specific cellular receptors, using a range of methods including adsorption, covalent coupling and specific interactions, resulting in biologically active composite nanoparticles [74,75]. Functional nanoparticles can offer a route to enhanced affinity delivery vehicles where multivalent binding possibilities can result from enthalpic gains through multiple receptor binding, and decreased entropic costs [76–80].

In the following section an overview of surface chemistry applied in bioconjugation to nanoparticles is given, followed by a discussion of problems and drawbacks encountered with such an approach in order to try aid in design strategies to overcome current deficits in nanoparticle functionalization for targeting.

4.1. Synthetic routes to bioconjugation

Biofunctionalization has been demonstrated for a variety of nanomaterials, including silica [81], polystyrene [82], gold [83] and biodegradable polymer nanoparticles [84]. The choice of biofunctionalization procedure may initially be informed by the scaffold material (nanoparticle composition) but in creating bionanomaterials of controlled functionality (i.e. with controlled bio–nano interfaces) there is an array of other factors to take into account in the design of optimally active bionanoarchitectures. Such factors include biomolecule compatibility with the reaction conditions, impact of the structural interface on biomolecule conformation, final product dispersibility, and product stability [85].

Covalent conjugation is attractive as it can provide a route to an irreversibly bound biofunctional layer, which would be expected to be stable across the biological conditions it is to encounter in its ultimate in vivo role. Typically, conjugation protocols consider the available functional groups on both the protein and the nanomaterial to be decorated. The most common procedures utilize carbodiimide chemistry, whereby the amine of the biomolecule forms an amide bond to a carboxylated nanoparticle. This so-called "EDAC" chemistry is a biocompatible coupling procedure which can be performed in one step with EDAC forming an active intermediate with carboxylic acids susceptible to amine substitution. This chemistry has been applied to functionalization of a range of nanoparticle types including silica [86], polystyrene [87,88] and gold [18]. N-hydroxysuccinimide or sulfo-N-hydroxysuccinimide can also be used in parallel to form an activated ester as has been successfully demonstrated for gold [81,89,90], silica [91] and polystyrene [92] nanoparticles.

For amino functionalized particles a common functionalization approach is to use the bifunctional glutaraldehyde as a cross-linking agent between the particle and protein amino groups with the reversible imine groups formed being reduced to fixed secondary amines. This method has been commonly applied to the amino expressing silica or silica shell nanoparticles, which result from aminopropylsilane functionalization [93,94].

In an approach similar to that of using the primary amine presenting amino acids on protein surfaces for coupling, the thiol presenting cysteine residues can also be used in chemoselective conjugations [95,96]. Thiol–maleimide coupling forms covalent bonds in a highly specific manner [97,98]. Other biocompatible reactions have also been applied. The Huisgen cycloaddition "Click" reaction is considered especially attractive given its chemoselectivity and stability [99,100]. A drawback is that the biomacromolecule requires modification to express azide or alkyne groups, but this approach has been successfully applied in some cases recently, such as iron oxide NP-oligonucleotide conjugation [101], iron oxide–HSA conjugation [102], gold nanoparticle-targeting peptide [103,104] and gold nanoparticle–enzyme assemblies [104]. It has been noted as an improvement on carbodiimide chemistry in terms of the extent and efficiency of labeling and even in terms of the binding to target receptors of the resultant conjugates [105]. Terminal epoxide presenting materials have also become of interest given their propensity toward nucleophilic addition of amines in water [106,107]. This allows direct coupling of amino presenting biomolecules with no other reagent necessary. This chemistry has been applied to silica nanomaterials where the commercial availability of the epoxysilane glycidopropylsilane makes it readily accessible [108,109].

Gold readily forms Au – S and Au – N bonds, thus gold nanoparticles can be described as somewhat ready-made for bioconjugation and have been used, as such, for direct coupling of proteins on their surface through binding with free amino and cysteine presenting residues which may be present on the targeting moiety. Protein coupling to gold has been used in this manner to stabilize colloidal solutions, while also demonstrating biological activity, with examples such as immunogold (antibody stabilized gold), as well as more recent ones [41]. However, conformational changes induced on binding have been observed which could be expected given the range of gold–protein interactions which are likely to occur [110–112]. Such changes may limit the functionality of these bioconjugates, thus more sophisticated architectures, which generally employ the use of a PEG spacer between the gold surface and the targeting moiety are increasingly proposed for gold nanoparticle systems [26,113].

Biomolecules may also be conjugated to nanostructures using supramolecular interactions, with the obvious example being the streptavidin–biotin interaction which has been widely applied across the biosciences in conjugation protocols [114]. Other examples which use supramolecular interaction to fix and direct biomacromolecules onto nanosurfaces include DNA–DNA on gold [115] and affinity peptides [116].

Choice of conjugation approach as a component of surface architecture is thus vital regarding successful receptor recognition.

4.2. Protein/antibody conformation/activity considerations

As the aim of biofunctionalization or design of the bio–nano interface is to impart highly specific biological function to nanomaterials, it is important that any potential detrimental, or indeed beneficial, effects of conjugation on the biomolecule functional properties be considered (e.g., physical effects on protein secondary and tertiary structure). Adsorption effects due to physical interaction between biomacromolecules and a range of materials are known to cause significant structural changes [117]. For example, nanoscale, surfaces have been shown to induce size dependent effects on protein conformation upon adsorption [61,118,119]. Besides size, as one would expect, hydrophobicity and electrostatics play important roles which may be controlled through surface chemistry modifications [120,121]. It has been postulated that the structural and functional effects on proteins caused by nanomaterial adsorption may be modulated in a controllable manner based solely on parameters of surface curvature and hydrophobicity, with lower hydrophobicity (e.g. PEG layers [122]) and diameters in a certain range conducive to preserving native structure [60,123–127]. Interestingly in some cases nanoparticle conjugation has been cited as beneficial to protein activity and stability [128].

Another effect of conjugation which must be considered is that on protein mobility. Since biomacromolecules are often bound to the nanoparticle at multiple points, as a result of the lack of specificity of many conjugation strategies, the ability of the bound biomolecules to take up an interaction-favorable conformation may be restricted. This detrimental effect of multiple conjugation sites on protein recognition activity has been clearly observed [129].

Besides the physical effects on the protein structure that may result from binding to nanoparticle surfaces, the actual directionality of the protein components becomes vital as regards biological efficacy, i.e., it is necessary to ensure the correct epitope presentation in order to engage with the target receptor. Attempts have been made recently to understand the effects of site specific conjugation on protein structure. With an ultimate goal of elucidating a single addressable site for protein attachment that results in optimal presentation of the functional part of the protein, studies have assessed the influence of attachment at different protein sites on the resultant conjugated protein 3-D structure [130–132], suggesting that the labeling site is a vital factor in not only alone directionality of coupling but also degree of structural influence/loss of function.

5. Advancement in targeting strategies

Based on the above assessment of the issues encountered in designing bio–nano interfaces for targeted drug delivery, we can summarize the challenges as follows:

1. The active site on the targeting ligand must be presented in the correct manner and 3-dimensional arrangement to optimize the fit to the receptor; optimal number, spacing, and orientation of the ligands must be considered;

2. The interfacial properties of the nano-scaffold must be designed such that the targeting moiety maintains functionality while also ensuring that adsorption of other biomolecules from the surrounding milieu does not screen or cover the targeting moieties, or provide steric hindrance preventing them from interacting with the target cellular receptors;
3. The particles must be able to evade the immune system, in order to increase their circulation time, and enhance the amount that reaches the target site.

This is represented schematically in Fig. 4.

Additionally, in order to optimize in vivo interaction, there is a need for real synthetic control at the molecular level, especially when considering molecular recognition events at the cell membrane [133]. At present there is still room for development of generic nanoparticle types which can be applied across in vitro and in vivo studies and which can provide the combination of colloidal stability, facile detection and highly specific biological efficiency while operating in the complex biological milieu.

The main limitations to the current approaches for targeting, from an interfacial science perspective, are the lack of orientation specificity in the linking chemistries used, and the lack of consideration of the influence of other proteins present that bind to the nanoparticles i.e., the corona influence, which could, for example, lead a particle to the lysosomal pathway, following cellular uptake. An illustration of the number of potential nanoparticle conjugation and binding sites in the previously discussed Tf (human transferrin) protein, is given in Fig. 5. Here, we have highlighted in Fig. 5(A) the interaction between transferrin and its receptor, and indicated the interaction region, which must be presented in the correct orientation to the receptor, in order to bind to it. In Fig. 5(C) and (d) all of the $-NH_2$ and –COOH sites, respectively, available for conjugation to a nanoparticle are shown, including those whose use would effectively block the active site for the transferrin–TfR interaction. Based on this approach, it is clear that using any of the commonly used chemistries (e.g. EDAC, reductive amination etc.) would not differentiate between these different available sites, meaning that the transferrin molecules would be conjugated to the nanoparticles in essentially random orientations, with any order present possibly resulting from supramolecular considerations (hydrophobicity, electrostatics). Clearly, this is not an optimal strategy to achieve targeting, and as such more selective approaches are needed.

Greater control of the protein orientation at the nanoparticle surface can be accomplished through careful attention to surface topography and chemistry properties on one hand, and protein structural details on the other. If the greatest biological efficacy is acquired through protein structure control, then surface curvature, biocompatible surface chemistry and linker architecture are the established means to control tertiary and secondary structural deformation, flexibility and conformational freedom. Controlled directionality can be attained through advanced protein modification/synthesis techniques to control the point of attachment and the consequent interface biological activity. Some advances have already been made in regioselective protein conjugation [104,130–132].

Another challenge related to optimization of targeting efficiency is the fact that achievement of binding between the nanoparticle-bound ligand and its receptor does not imply that internalization of the nanoparticles will occur, either through the specific mechanism triggered by the recognized receptor (e.g. transferrin receptor triggers clathrin mediated endocytosis of transferrin molecules to regulate intracellular iron content) or through a different mechanism. Moreover, in systems where specific binding with cell receptors is achieved, uptake could still occur via multiple mechanisms, both specific (receptor mediated) and not. Thus, particle localization and signal activation inside the cells could also be different depending on the route of entrance and only a minimal fraction of the nanoparticles may reach the desired location. Considerable work is needed to address this issue, and there is already evidence emerging in the literature that nanoparticles utilize multiple uptake pathways even in a single cell type. In the case of

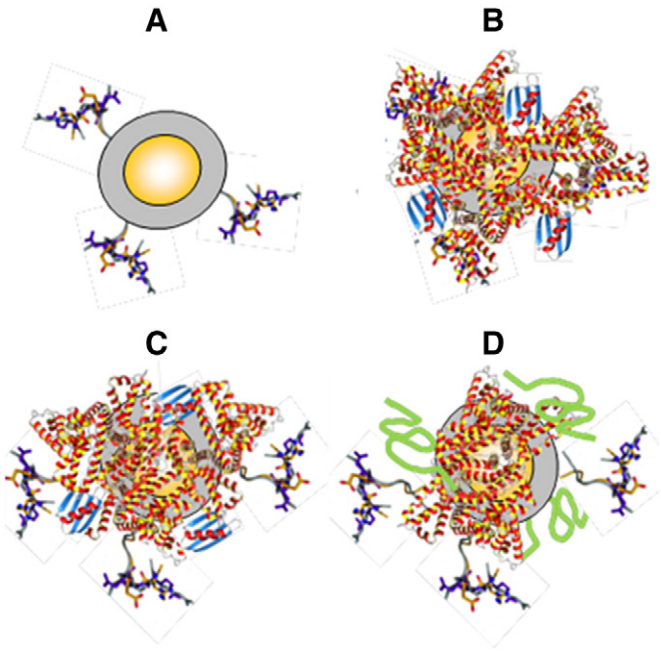

Fig. 4. Schematic representation of the challenges of developing targeted nanoparticles. (A) A 'pristine' targeted nanoparticle, with the targeting ligand grafted to the surface, before contact with a biological fluid. (B) The identical nanoparticle in a biological fluid, e.g. plasma. A dynamic layer of proteins and other biomolecules immediately coats the nanoparticles, with high abundance, low affinity proteins being gradually replaced by lower abundance, higher affinity proteins. (C) The use of spacers to locate the targeting moieties outside the biomolecule corona. However, in this case there may still be steric crowding effects, reducing the efficiency of the ligand–receptor interaction, and the ligand may have been conjugated to the nanoparticles in an incompatible orientation. (D) The use of PEG polymers can help to reduce protein binding to the particles, reducing the corona. However, the issue of ligand orientation remains, and PEG may not prevent all proteins from binding to the nanoparticles.

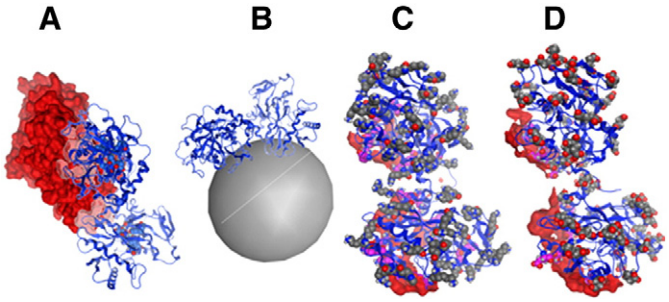

Fig. 5. (A) Transferrin interacting with the dimeric transferrin receptor. The interaction sires are highlighted in the paler red. (B) Schematic of transferrin conjugation to a nanoparticle. Depending on the orientation of the transferrin on the nanoparticle, it may or may not be able to engage with the TfR. (C) Transferrin protein 3D structure with the receptor binding domains highlighted as red patches. Amine (NH_2) side chains that can potentially randomly bind to NPs highlighted as red circles; Pink circles indicate NH_2 side chains whose use to bind to nanoparticles would mask the receptor binding site, thereby invalidating the targeting potential. (D) Same again, but this time showing the COOH side chains that can be used to link transferring to NPs as the red dots, there again being too many random possibilities. The COOH side chains that would interfere with receptor binding are shown as the pink dots. The authors thank Anthony Chubb for help with preparation of this figure.

untargeted nanoparticles, a study using pharmacological inhibitors of some of the major endocytic pathways to investigate nanoparticle uptake mechanisms in a range of representative human cell lines found that none of the inhibitors were able to significantly inhibit uptake of 40 nm carboxylated polystyrene nanoparticles, suggesting that the same nanoparticle might exploit different uptake mechanisms to enter different cell types [134]. Similar results were found in an assessment of the specificity of folate receptor (FR) targeting by heparin–folate–paclitaxel nanoparticles (HFT-T) [29]. In this work, FR-specific siRNA was used to reduce FR levels and uptake of the HFT-T nanoparticles was observed to be decreased to half of its value in the silenced cells, suggesting multiple pathways of uptake [29]. Thus, targeting in this sense is more challenging and still unresolved, as is the related issue of trafficking of the nanoparticles inside the cell to a specific sub-cellular localization, following uptake, especially in light of multiple uptake pathways. It is likely that the presence of other proteins in the nanoparticle–protein corona could be the source of the alternative uptake pathways.

Strategies to reduce the non-specific binding of additional proteins include, as discussed earlier, the use of protein resistant coatings such as PEG or polysaccharide, which gives prolonged circulation times in vivo, thus favoring extravasation of nanoparticles into tumor tissue, via the EPR effect, through the leaky vasculature of cancer cells. PEG can be used in vivo, as presented in Fig. 4D, to prevent non-specific binding of the proteins in the biological fluids, like serum etc. Depending on their length, PEG chains can arrange in different conformations which can confer different characteristics and effects. Surface coverage will also be a significant determinate of whether full or partial reduction of protein binding to the nanoparticles occurs.

As we discussed earlier, however, clearly protein adsorption, even though reduced, is present also on stealthed particles, thus the corona effect may still come into play, affecting biological outcomes. As such, research is needed in order to be able to design nanoparticle interfaces to avoid, or – on the contrary – exploit, coronas when directing nanoparticles to desired targets and sub-cellular locations. Brushed PEG not only can help reducing protein adsorption, but also can be used as a spacer to allow extension of the targeting ligands far from the nanoparticle surface (as shown in Fig. 4C), thus enhancing flexibility and increasing the potential for interaction with the corresponding receptors [135,136].

Even when binding to specific receptors is achieved, however, as discussed above, a different trafficking and final localization of the targeted nanoparticles is often obtained, compared to that of the free ligand, and that desired by the targeting procedure. Thus, in addition to cell membrane receptor targeting, it is desirable that surface functionalization of the nanoparticle or design of the bio–nano interface is tailored to subsequently direct nanoparticles out of the lysosomal pathway — this is a direction in our current research.

6. Conclusions

As it stands presently, the field of biospecific nanoparticle targeting requires much future research effort in order to deliver on its enormous promise. As presented here, there are multiple challenges to be met in order to design truly efficient targeted nanoparticles, and to be sure that the biological and in vivo outcomes are a consequence of that strategy. It may be that one needs to take a step back, and take a more measured view of these challenges. Thus, if we have confidence that it is one of the most major elements to ensuring such efficiency, then it could be that more fundamental advances are required in the engineering of nanoparticle interfaces with much greater precision, reproducibility, and control. Additionally, novel methods to characterize the success with which this has been achieved are necessary. Uncertainties in these capacities stem from the structural effects of conjugation on the targeting moiety functionality to understanding, controlling and exploiting the evolving biomolecular adsorption in response to changing environments, evading immune recognition etc. These and other factors outlined above can create many obstacles to the targeting conception.

Perhaps only by addressing all these challenges in parallel, and by fully understanding the role and evolution of the biomolecule corona on the nanoparticle surface in situ we will be able to control and exploit the bio–nano interface. Achieving such control will allow us to exploit all the advantages of nanoscale objects and their unique capabilities of interacting with the cellular machinery, crossing of biological barriers and encapsulation and delivery of therapeutic loads. In this way, we could move from a trial and error approach towards highly efficient purpose-designed targeted nanoparticles for optimized drug delivery with minimal side-effects. The benefits of a more focused strategy to address the bionano-composite interface could go far beyond targeting efficacy, to the gathering of much more reassurance for nanomedicine safety, thereby smoothing the pathway through the regulatory process for these new technologies. The question at the broader level must be if it is worth the investment. To some degree the answer to that depends on just how important one considers the future benefits that nanomedicine can confer. For those of us that believe that we have barely touched the potential, there can be little doubt. The potential is sufficient for a renewed and absolute commitment to developing scientific knowledge at the bionano-corona-interface.

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