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Peptide and protein nanoparticle conjugates: versatile platforms for biomedical applications

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Abstract

Peptide- and protein-nanoparticle conjugates have emerged as powerful tools for biomedical applications, enabling the treatment, diagnosis, and prevention of disease. In this review, we focus on the key *roles* played by peptides and proteins in improving, controlling, and defining the performance of nanotechnologies. Within this framework, we provide a comprehensive overview of the key sequences and structures utilised to provide biological and physical stability to nano-constructs, direct particles to their target and influence their cellular and tissue distribution, induce and control biological responses, and form polypeptide self-assembled nanoparticles. In doing so, we highlight the great advances made by the field, as well as the challenges still faced in achieving the clinical translation of peptide- and protein-functionalised nano-drug delivery vehicles, imaging species, and active therapeutics.

1 Introduction

The use of nanoparticles (NPs) provides new opportunities for the development of more effective, safe, and commercially-viable biomedical technologies. Whether as drug-delivery vehicles, high-contrast imaging agents, or active therapeutics, NPs enable new approaches to be taken for the treatment, diagnosis, or monitoring of disease within the human body. Unfunctionalised, naïve NP constructs are often able to fulfil their desired function under controlled *in vitro* settings. However, in the more complex environment of the human body many questions still remain - How can the particle be targeted to the site of disease? Can clearance be avoided to ensure a suitable therapeutic lifetime? Will the accumulation of a biomolecular corona diminish activity?

In recent decades, the formation of peptide- or protein-NP conjugates has emerged as a vital tool for addressing many of the difficulties that arise as a result of these considerations. These hybrid materials enable the favourable characteristics of nano-sized structures to be combined with the biological activity, biocompatibility, and versatility of both naturally derived and synthetic polypeptides.

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In this review, we provide a comprehensive overview of the development and use of peptide/protein-NP conjugates in biomedicine. While many reviews have previously been published on methods to *create* NP conjugates, or their subsequent *applications* within the body, the specific features imparted by the peptide or protein on the NP conjugate have been less widely discussed. We will therefore focus on the distinct *roles* played by the peptide/protein in improving, controlling, or defining the performance of nano-technologies (Figure 1). In doing so, we will deliver a detailed reference for both experts and those new to the field of NP technologies alike. Furthermore, we hope to stimulate discussion and innovation within the field, in order to overcome many of the difficulties that continue to hinder the clinical translation of these potentially powerful tools.

2 The benefits of peptide/protein-NP conjugates in biomedicine

The unique properties and size regime of NPs offer many benefits over small molecules and larger micrometre sized particles. These have been widely exploited within the biomedical field and reviewed extensively elsewhere.1–6 By way of context for this review, we will briefly summarise here some of the key factors that make NP-based technologies particularly attractive, and the roles in which they have predominantly been applied.

One of the most prominent realms in which NP systems have found utility is as vehicles for drug delivery. Small molecule drugs often suffer from poor pharmacokinetics, exhibiting rapid clearance and difficulties reaching the desired site-of-action *in vivo*.1 As a result, severe side-effects may accompany any therapeutic benefit, while it is common for *in vitro* efficacy to be poorly translated to a clinical setting.7 The ability of NPs to solubilise therapeutic molecules, enhance retention and circulation, and promote accumulation in the target tissue makes them attractive vehicles for overcoming these limitations, which hinder the drug discovery process.8 These effects can be further enhanced through the incorporation of peptide or protein coatings, as will be the focus of this review, by further improving pharmacokinetics, enabling tissue targeting, and promoting cell and tissue penetration.9 Similarly, NP drug delivery vehicles offer several benefits over the use of protein- and antibody-drug conjugates (ADCs), which have emerged over the last 10 years as promising clinical tools for the treatment of a range of diseases.10 In particular, NP platforms offer the possibility to incorporate multiple functionalities within a single construct. As a result, problems such as the poor tumour penetration often exhibited by ADCs can be overcome, as described in Section 5.10,11 Furthermore, the encapsulation of the therapeutic agent allows the need for a cleavable linkage to be avoided, and enables high levels of drug to be delivered for every recognition event.12

The benefits of both bare NPs and those decorated by polypeptides, have also been widely exploited in the field of *in vivo* imaging. When compared to small molecule imaging agents, NPs often offer the advantages of greatly improved signal-to-noise ratios, stable signal generation, high spectral resolution for multiplexed detection, and the ability to display multimodal signal generation.13,14 As a result, NPs have found increasing utility for imaging in a range of modalities, including near-infrared (NIR) fluorescence,15 magnetic resonance imaging (MRI),16 and positron emission tomography (PET)17. Indeed, many NP-based imaging technologies are now routinely used in a clinical setting.18

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Stimuli responsive NPs are also finding increasing utility within the biomedical field, often leading to the localised destruction of pathological tissue.19 Whether as a result of magnetic or light induced hyperthermia, or NP-mediated photoablation, such technologies are strongly reliant on adequate accumulation at the site of treatment. Many systems rely on inorganic cores, from which it is vital to reduce the leaching of metal components due to their associated toxicity. As a result, peptide or protein coatings that can direct, stabilise, and eventually lead to the clearance of therapeutic NPs are a key component of these emerging technologies as they approach clinical application.20

3 Commonly utilised nanoparticles

NPs have found widespread use across the material, physical, engineering, and biological sciences, with the number of new technologies increasing at an exponential rate.21 The particle structures which enable these applications are equally diverse, with precise control over material, architecture, and design enabling a broad spectrum of tunable properties dependent on the end application.4 Despite this large variation, a number of NP formats have found particular utility in the biomedical field. Much of the research to be discussed within this review has focussed on the use of 6 main categories of NPs (though many others have also been utilised in more niche settings, such as peptide-based NPs as discussed in section 8). Each will be briefly introduced in order to contextualise the discussion that follows (Table 1):

i) Gold nanoparticles (AuNPs). AuNPs are attractive structures for biomedical applications.22 As one of the most stable and least toxic metal NP formulations, AuNPs offer a safe and effective diagnostic and therapeutic tool.23 The ability of thiols to form stable linkages with the surface of AuNPs allows versatile surface chemistry to be achieved.24 Decoration of the particle surface with a wide range of biomolecules enables the biodistribution, physical properties, and intracellular fate to be modulated. Furthermore, AuNPs exhibit unique optical and electronic properties, with concerted electron oscillation following excitation leading to strong light emission via localised surface plasmon resonance (LSPR).25 Their interaction with light is strongly dependent on size, shape, surface chemistry, aggregation and environment. These properties enable the use of AuNPs in a range of imaging modalities, including NIR fluorescence, photoacoustic imaging, and x-ray computational tomography, as well as finding utility in photoablation and hyperthermic therapies.22,26

ii) Magnetic nanoparticles (MNPs). MNPs are composed predominantly of magnetically-responsive elements, such as iron, nickel, and cobalt in various different forms, and are finding increasing utility in the biomedical field.27,28 Iron oxide particles have found most widespread use, particularly as high contrast imaging agents for MRI.29 The application of alternating magnetic fields can also be utilised as a minimally invasive stimuli for generating hyperthermia as described above.30 Finally, the inherent magnetism of MNPs enables their spatial distribution to be easily manipulated, allowing the guided delivery of drug-containing vehicles.31

iii) Semi-conducting NPs and quantum dots (QDs). In order to undertake fluorescence imaging *in vivo* it is advantageous for emission to be in the NIR to allow tissue penetration.

32 Conventional small molecule NIR fluorophores suffer from poor photo-stability, high hydrophobicity which hinders distribution, and weak signal-to-noise generation. In contrast, semi-conducting NPs (and in particular QDs) possess narrow, size-tunable, and high quantum yield emission.33 Furthermore, they can be excited with broad wavelength light and display greatly improved resistance to photobleaching and chemical degradation. They are therefore highly attractive structures for undertaking biomedical imaging.34 However, the high toxicity of cadmium metal, common in many QD formulations, necessitates the use of stable coatings able to prevent leaching into the biological milieu.

iv) Mesoporous silica nanoparticles (MSNPs). MSNPs possess tunable pore sizes, large surface areas, and high pore volume. They are therefore attractive drug-delivery vehicles, with the ability to encapsulate a large payload of cargo ranging from small molecules up to large proteins.35,36 The ease of surface modification, and cost-effective and scalable production adds to the attractiveness of MSNPs. Furthermore, the classification of silica as a ‘Generally Recognized as Safe’ substance by the United States Food and Drug Administration (FDA) greatly facilitates regulatory approval of MSNP technologies, though questions on the long-term effects, biodegradability, and biocompatibility of many silica nanotechnologies remain unanswered.37 In addition to their applications in drug delivery, MSNPs have also found widespread use as imaging agents, as a consequence of their ability to encapsulate and concentrate contrast agents for a wide range of modalities.38

v) Liposomes. Liposomes are spherical vesicles, most commonly composed of at least one bilayer of self-assembled phospholipids. The similarity of the liposome bilayer to that of the cell membrane is particularly attractive for drug delivery applications, and indeed liposomes are perhaps the mostly widely used and investigated NP carriers for drug delivery, and were amongst the first NP-based technologies to enter the clinic.2 Liposomes are able to encapsulate both hydrophilic cargo, within the aqueous interior, and hydrophobics, within the membrane bilayer. Furthermore, liposomes can be readily functionalised with lipid, or hydrocarbon functionalised ligands, making them a stable, cost-effective, and attractive tool for biomedical applications.

vi) Polymer nanoparticles. The versatility of synthetic polymers enables a wide range of particle architectures and end-applications to be explored. Polymer vesicles (commonly referred to as polymersomes) and micelles, composed of self-assembled amphiphilic block copolymers, are particularly attractive due to their ability to encapsulate cargoes in an analogous fashion to their corresponding lipidic analogues.39,40 The NP size, membrane thickness, porosity, and many other factors can be tuned by adjusting block length and pendant group functionalisation. Furthermore, the design flexibility enabled by synthetic polymer chemistry enables the introduction of stimuli-responsive or biologically active functionalities, which are able to modulate particle properties in a smart, predictable manner. 41 As such, polymer NPs are finding increasing use across a number of biomedical disciplines.

4 Enhancing stability

It is striking that the number of NP systems that have found successful application in biomedical applications pales in insignificance when compared to the vast body of literature on the use of NP constructs under controlled model settings.42 This is predominantly due to the significant challenges associated with translating NP stability under idealised conditions to the complex environments of biologically relevant scenarios.43 Peptide and protein coatings play a major role in enabling NP based technologies, and their ability to provide both biological and physical stability will be summarised in this section (Figure 2, Table 2, and Table 3). Importantly, these two factors are not necessarily independent or complementary. As will be discussed, a coating which prevents NP aggregation may also impact on the rate at which the construct is cleared from the circulation. It is therefore important to carefully consider design criteria in order to ensure that the end-application can be achieved.

4.1 Biological stability

In order to reach the desired site-of-action, NPs must navigate their way around an ensemble of biological obstacles. These include defined biological surfaces, endothelial and cell membrane barriers to permeation, and circulating monocytes and macrophages of the body's immune system.44 To achieve their function, NPs must circulate in the bloodstream for long enough to reach their target cells, tissues, or organs, whilst avoiding removal by active phagocytosis or renal clearance.44,45 The nanoscale dimensions in themselves can lead to greatly altered pharmacokinetic properties when compared to small molecules *in vivo*. For example, for 'hard' inorganic particles, renal clearance is greatly reduced above a diameter of ~5.5 nm due to the size cut-off of the kidneys for urinary excretion (Figure 3).46 This ability of NPs to modulate pharmacokinetics has been widely utilised as a means to improve the retention, biodistribution, or *in vivo* stability of an encapsulated small molecule.47 However, these differences do not come without their own challenges - dependent on the size, structure, or functionalisation of a NP, detrimental tissue accumulation can occur, while the uptake of particles by macrophages can not only lead to rapid removal from the circulation, but also the induction of an unfavourable inflammatory response.48

As soon as NPs are introduced into a biological environment further complications arise, as a mixture of biomacromolecules interacts with the particle surface, often masking the effect of the particle and strongly influencing the pharmacokinetic properties.49–51 In protein-rich media, such as blood, this new coating is referred to as the protein corona. The 'hard' corona is made up of tightly-bound proteins, forming thermodynamically favoured interactions with the NP surface, followed by a 'soft' corona of rapidly exchanging proteins (Figure 4).52,53 The nature of this biomolecule coating is highly dependent on the environment and thus difficult to predict, often resulting in a dramatic loss of activity or function and strongly influencing the nanomaterial fate *in vivo*.45,54,55

Peptide and protein coatings can be used to improve upon the biological stability of delivered NPs and address these difficulties. By altering the interactions with host cells and circulating biomolecules, peptide/protein coatings can modulate and control tissue accumulation to ensure NP activity (Figure 2). In order to understand how this can be

achieved, it is important to first understand the factors which induce clearance. The adsorption of opsonins (plasma proteins such as Immunoglobulin G, complement factors, and fibrinogen) to the NP surface plays a prominent role in triggering clearance, by inducing macrophage recognition and subsequent elimination via phagocytosis.56,57 By inhibiting this process, so too can the recognition and removal of NPs by the immune system be reduced.56 In contrast, when the protein corona is enriched with serum albumins or lipoproteins (often referred to as dysopsonins) recognition is blocked and circulation times are increased.53,58,59 Amongst the other NP properties known to promote clearance, surface charge and hydrophobicity are particularly important. Although conflicting reports exist on the relative clearance rates of positively or negatively charged particles, it has become apparent that a near-neutral charge may in fact be most favourable.60 At the same time, a reduction in hydrophobicity has also shown to be a key parameter in reducing the uptake of NPs by macrophages.45,60 While beneficial from a biological perspective, it is important to note that particles which exhibit neutral or low surface charge often exhibit reduced colloidal stability, as discussed in the subsequent section.61 It is therefore important to consider the delicate balance that determines NP fate when designing peptide/protein coatings.

One widely utilised method to limit opsonisation and NP clearance from the circulation is the use of stealth polymers, such as poly(ethylene glycol) (PEG), which form a protective layer around the NP, neutralizing surface charge, conferring hydrophilicity, and providing a steric barrier to adsorption.56,62 However, there is a fine line between providing a coating which improves biodistribution and diminishing interactions with target cells and tissues.61 The coating of NPs with PEG has been associated with reduced cellular uptake and thus therapeutic efficacy,63,64 the generation of PEG-specific antibodies which accelerate clearance from the blood,65,66 and preferential accumulation in the liver and spleen67. The versatility of peptide design thus offers an attractive alternative to such coatings (Figure 2a). The use of non-ionic peptides, or those offering zwitterionic balanced charge may be particularly useful for reducing clearance. Following this strategy, Guerrero *et al* conjugated the amphipathic peptide CLPFFD, previously shown to mediate transport across the blood-brain barrier, to AuNPs with the aim of increasing particle delivery to the central nervous system.68 By reducing the negative charge relative to citrate-capped AuNPs, not only was delivery to the brain improved but accumulation in the spleen was also reduced. However, this approach is not generally applicable. In a similar study, AuNPs were coated with the weakly negatively charged cell-penetrating peptide VG-21.69 Although enabling improved cell delivery due to the presence of VG-21, preferential accumulation was also observed within the spleen.69 Similarly, Morais *et al* reported that the coating of AuNPs with CALNN led to increased clearance and liver accumulation compared to citrate-capped particles.70 These results highlight the difficulty in predicting pharmacokinetic properties - often apparently similar peptide sequences can have drastically different effects on clearance. This conclusion is strongly supported by the research of Poon *et al*, who revealed the complexity of designing peptide-NP conjugates for escaping the body's reticuloendothelial system.71 By coating AuNPs with a mixture of PEG and either the negatively charged therapeutic peptide Myx or the positively charged cRGD targeting peptide, it was found that the effect of the PEG/peptide coating could act independently or

synergistically, depending on the sequence employed. As such, it is important to evaluate NP pharmacokinetics on a case-by-case basis.

An alternative approach to avoiding clearance is to rely on biological signalling to enhance retention. This has been most prominently achieved by using the membrane protein CD47. By interacting with the signal-regulatory protein alpha (SIRPα) and triggering downstream anti-phagocytic processes, CD47 acts in effect as a ‘marker of self’, sending out a ‘do not eat me signal’ (Figure 2b).72–75 Peptides derived to mimic the effect of CD47 have thus been designed and shown to reduce phagocytosis.76 Importantly, peptide coated NPs displayed greatly reduced accumulation in the liver and spleen, and enhanced accumulation in cancerous tissues following intravenous injection (Figure 5). Similarly, Qie *et al* recently demonstrated that NPs functionalised with full-length CD47 were also able to modulate clearance time, enabling the evasion of different macrophage populations.77 However, this work also highlighted the need to better understand the interaction of nanomaterials with macrophages displaying distinct phenotypes, in order to pave for the way for truly immunologically inert nanomaterials.77

The adsorption of dysopsonins is known to enhance retention. A third approach to improve NP pharmacokinetics is therefore to promote the formation of a favourable dysopsonin-protein corona (Figure 2c). This can be achieved either through the pre-coating of NPs prior to *in vivo* application,79 or by tuning the NP surface properties to promote the enrichment of dysopsonins over opsonins *in situ*.53,80 Serum albumin, the most abundant protein in blood, plays a wide range of roles including acting as a molecular and protein transporter, maintaining oncotic pressure, and buffering blood pH. Pre-formation of an albumin corona is also able to act to prevent the attachment of alternative proteins and improve NP stability. 53 Peng *et al* showed that such a strategy was able to limit phagocytosis and prolong circulation time *in vivo*, through simple NP pre-incubation in a solution of bovine serum albumin (BSA).58,81 Hydrophobin, an exogenous protein expressed by fungi has high affinity for NP surfaces and can also alter the composition of the protein corona when NPs are exposed to bodily fluids.52 The high affinity of hydrophobin enables the protein to remain strongly associated with the NP surface, even when in competition with other plasma proteins. Apolipoprotein coatings have also been shown to increase NP circulation time. A study of polystyrene-NP coatings demonstrated that amino and sulfonate functionalised particle coatings led to the accumulation of high levels of apolipoprotein.59 These results highlight the importance of surface charge and functional groups on influencing protein corona formation. Notably, the binding of specific apolipoprotein subtypes was also shown to strongly influence subsequent cell uptake *in vitro*.59 Schöttler *et al* demonstrated that the apolipoprotein clusterin (also known as ApoJ) reduced the non-specific cellular uptake of sterically protected polymer-NPs.80 A recent study highlighted the effect of initial polymer coverage on the downstream effects of clusterin, which was able to shield NPs from opsonisation at low PEG densities but did not effect NP clearance at higher PEG coverages. 82 The downstream effects of protein corona formation must also therefore be considered.

Fibrinogen is a circulating glycoprotein in the bloodstream and a well known opsonin able to inhibit cell adhesion at suitable concentrations. When low fibrinogen concentrations are adsorbed on NP surfaces, a single highly adhesive monolayer is formed.83 In contrast, dense

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coatings at high concentrations reduce cell adhesion under both static and flow conditions, via the formation of a nanoscale multilayer matrix.84 However, translating fibrinogen to designed NP coatings for increased circulation remains challenging, as fibrinogen can also lead to NP aggregation as a consequence of the formation of inter-particle bridges.85

4.2 Physical stability

The physical stability of a NP construct is an important consideration when designing a biomedical technology. Not only are suitable stabilities required to ensure that the particle is able to fulfil its function, but the leaching of small molecules from the particle structure or the formation and accumulation of larger aggregates must also be controlled to minimise toxicity. In order to demonstrate the ability of NPs to be applied both *in vitro* and *in vivo* they must form a dispersed, stable suspension under physiological conditions. Several reports have demonstrated that agglomerated NPs have drastically altered effects on properties, in particular toxicity, which can lead to large discrepancies in experimental output.86–90 Indeed, aggregated particles display a greatly decreased surface area and reduced cellular uptake when compared to dispersed particles, often leading to an underestimation of toxicity prior to application *in vivo*. In contrast, sedimentation and decreased diffusion can significantly impact the 'effective dose' experienced by cells *in vitro*, leading to large discrepancies in experimental readout and significantly decreased efficacy upon *in vivo* translation.91,92 NP aggregation can also trigger opsonisation in the bloodstream, as discussed above, making particles more visible to the phagocytotic system. 63,64 Thus, physical stability and biological stability are intimately linked.

The flocculation of particles in aqueous solution is governed by the Derjaguin-Landau-Verwey-Overbeek theory (DLVO theory).93,94 The stability of the dispersion depends on the balance between attractive and repulsive forces - a particle becomes unstable and starts to agglomerate when the repulsive energy is not sufficient to counteract the van der Waals attractive energy.88 At biological salt concentrations the situation becomes more complicated, however the basics for stability remain similar.95 Sufficient electrostatic and steric stabilization is required to provide the repulsive energy to prevent agglomeration.88 For biomedical applications, coatings must not only provide colloidal stability, but also aqueous solubility, while conserving the NP functionality. The formation of a protein corona upon application *in vivo* may either enhance or compromise NP colloidal stability. Most commonly, the steric bulk provided by the protein coating can provide significant stabilisation, limiting inter-particle attractive forces.49,96,97 This phenomena is able to offset the low contribution of stabilising electrostatic repulsion forces which are largely negated in the presence of the high electrolyte concentrations of physiological conditions. 97,98

Peptides have been particularly widely used to provide physical stabilisation to metal and semi-conductor particles, for which the high susceptibility to aggregation must be carefully mitigated. For example, depending on the capping layer utilised, unfunctionalised AuNPs are incredibly sensitive to environmental changes, with small changes in pH, salt concentration, temperature, or the presence of biomolecules often leading to rapid and irreversible aggregation.26,33,99–103 Through combinatorial design, peptide sequences

have been identified that are able to find a careful balance of charge, polarity, functionality, hydrophobicity, and length.104 In 2004, Lévy *et al* identified the pentapeptide CALNN through such libraries, as a water-soluble peptide able to provide extremely stable AuNPs following surface functionalisation.105 The presence of the N-terminal cysteine enables facile functionalisation of the particle surface, with the rest of the sequence providing a densely packed, negatively charged peptide corona which is able to withstand aggregation.

The ability of thiols to bind to metal surfaces has led to cysteine capped peptides being widely utilised as capping agents for metal NPs.106–109 Indeed, peptides such as CALNN can be used as a core peptide to which additional biological functionality can be attached, providing a stable peptide-functionalised inner corona bearing pendant peptides which can then direct and influence NP activity.110–112 However, although cysteine-metal binding is sufficient to provide stabilised NPs *in vitro*, the reversibility of thiol-metal bond formation means that ligand exchange can occur.113–115 Although the concentration of free thiols in the blood is far lower than that observed intracellularly, it remains high enough to induce loss of monothiol coatings during circulation.116 This is in part due to the possibility for dissociative ligand exchange (SN1-like pathway), in which ligands are rapidly diluted within serum upon dissociation, and replaced by circulating small molecule thiols.117 In addition, the high levels of serum albumin in the blood, containing a single reduced cysteine residue, have been shown to mediate ligand exchange in biological samples.118,119 This loss of NP coating can lead to two, often concurrent outcomes in the dilute regime provided by *in vivo* settings: i) Replacement of the initial thiol coating by thiolated biomolecules, leading to a loss of any function imparted by the capping layer, as discussed throughout this review; and ii) Loss of any stabilization effects provided by the capping layer as ligands are gradually lost.120 As a result, increasingly stabilised peptide coatings have been explored in recent years. This is often achieved through the use of multi-dentate binding sites - that is, to use multiple cysteine residues or unnatural amino acids bearing dithiol motifs, to provide multiple anchoring points to the metal surface for each individual peptide (Figure 6).121–124 In doing so, even with reversible desorption of one thiol group from the metal surface, the other is still attached maintaining stability. Due to the proximity of the unbound thiol, this will then simply reattach and reform the stabilised NP construct.

An alternative approach, particularly widely employed for the functionalisation of QD surfaces, is the utilisation of poly-histidine sequences, able to form multi-dentate NP interactions. The ability of histidine residues to bind a variety of metals is well established throughout biology, and the hexa-histidine motif in particular has been found to form stable metal coordinates (Figure 6).126–128 Hexahistidine tagged peptides and proteins have therefore been widely used to provide stable biomolecule AuNP and QD coatings which are resistant to desorption for a wide variety of applications.129,130,125,131,132

5 Promoting cell and tissue penetration

As discussed above, for a NP to be useful in biomedical applications, it is vital that the particle is able to reach the necessary site of action – the ability of a NP to fulfil its specified purpose is not sufficient. This is particularly true for particles that are delivered systemically, rather than being applied directly at the desired site of action. Even once a particle has

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overcome the significant challenge of avoiding rapid clearance, the body places formidable barriers in the way of subsequent distribution and delivery of NPs at both the tissue and cellular level.1 These restrictions are the body's way of protecting and maintaining homeostasis, preventing the uncontrolled transport of material into and out of sensitive environments. Penetrating these barriers remains one of the biggest challenges in biomedicine, both for small molecule therapeutics and the NPs that are the focus of this review. The decoration of particles with peptides or proteins able to mediate transport or penetration is at the forefront of efforts to enable NP technologies to reach the areas they need to perform their function. In this section, we will discuss each barrier in turn and give a critical overview of the key sequences that have been utilised to modulate NP transport across them (Table 4 and Table 5).

5.1 Cell penetration

The cell membrane is a selectively permeable barrier, which efficiently protects the cell interior from its environment. Some specific small molecules may be efficiently transported across the membrane, however the transfer of NPs into the intracellular space is far more challenging.133 Although in some cases the uptake of unfunctionalised particles is possible, cell membrane impermeability often results in a highly detrimental lack of activity.134 Furthermore, even when NPs *are* taken up by cells, for example via endocytosis, the challenges are not over – up to 99 % of particles may be sequestered from the cytosol or nucleus in endosomes and organelles, depending on their exact properties, the target cell type, and the uptake mechanism.135,136 There is therefore a pressing need to develop NP constructs that can be directed to the desired site-of-action, in order to maintain functionality.

In recent years the decoration of NP surfaces with peptides able to promote uptake has come to prominence, with many so-called 'cell-penetrating peptides' (CPPs) and sequences able to trigger receptor-mediated endocytosis being studied in detail.137,138 Furthermore, sequences able to efficiently promote endosomal escape following internalisation have also been identified and used to great effect. We will here first briefly discuss the key mechanisms of peptide-mediated NP uptake, before highlighting the main sequences that have been most widely utilised in biomedical applications. As a word of caution, it is important to note that the efficacy of both penetration and subsequent endosomal escape is highly dependent on the experimental conditions.139,140 While a particular sequence may successfully mediate the transport of one NP cargo, it may fail to enable penetration of another.141 Similarly, while uptake may be efficient with a cell line *in vitro*, such results may not be readily replicated in the corresponding tissue upon *in vivo* translation. It is therefore paramount to test and characterise a newly designed NP-CPP construct under biologically relevant conditions, rather than relying on literature precedence for the transport of related, but essentially distinct NP cargoes.

Mechanism of uptake—In general, penetration mechanisms can be split into two major categories: i) direct, energy-independent pathways; and ii) those relying on active uptake via endocytosis (Figure 7). Often, multiple uptake pathways will be exploited in parallel, with the exact environmental conditions determining which process dominates.142 As such, it

can prove complicated to deduce the exact mechanism of internalisation and conflicting reports are therefore commonplace.143

Direct uptake mechanisms are typically initiated by the interaction of the peptide-NP conjugate with the cell membrane, most commonly via electrostatic interactions of positively charged surfaces with negatively charged phospholipids.110,144 This is followed by permanent or transient destabilization of the cell membrane, for example through pore or inverted micelle formation.142,145 Although many CPPs were at first thought to be uptaken via such means, subsequent reports have demonstrated that experimental artefacts in fact led to initial discrepancies and it is no longer thought that direct uptake is the major contributing factor in the penetration of CPP-coated NPs.145,146 In contrast, during endocytotic uptake, interactions with cell membrane components lead to the engulfment of the peptide-NP construct, which is then transported intracellularly in endosomes. Endocytosis occurs predominantly via macropinocytosis, or clathrin- or caveolin-dependent receptor-mediated uptake, but in all cases subsequent escape of the cargo from their resultant endosomal location is a key step, as described in the subsequent section.

The exact mechanism by which a particular NP is internalised is a vast area of research, and one which often remains contentious. Exact details are therefore outside the scope of this review and readers are instead directed to a number of excellent reviews on the topic. 133,147,139,148 It has become increasingly evident that it is difficult to draw generalisations. Importantly, the precise means by which a particular construct is taken up is highly dependent on not only the peptide sequence used to promote uptake, but also on the target cell type, and perhaps most strongly the exact nature of the NP cargo, including size, structure, and surface properties.

Key peptide sequences—By some estimates, in excess of 800 unique peptides have been identified which are able to promote cell penetration in *in vitro* experiments.150 Of these, only a handful of prominent sequences or closely related analogues have been translated to biomedical applications. Although there are many possible explanations for the paucity of peptides that have been used in such settings, 2 factors are of particular importance. Firstly, many sequences induce toxicity, particularly amphipathic peptides which often induce membrane disruption in order to promote uptake.151 Secondly, and perhaps more importantly, is the efficiency with which the best CPP peptides are able to deliver their cargo - it is difficult to improve upon the penetration efficiency, even if other properties leave a lot to be desired, as described below.

The most commonly utilised method to mediate internalisation is to rely on highly cationic peptide sequences, able to first strongly bind negatively charged cell membranes and then induce transport via either a direct or endocytic pathway. This group includes the now ubiquitous human immunodeficiency virus (HIV) derived TAT peptide, as well as synthetic polyarginine and penetratin (derived from the Drosophila Antennapedia homeodomain) sequences. Indeed, the CPPs utilised for the *in vivo* delivery of NP substrates fall almost exclusively into this category. In 1999 Schwarze *et al* demonstrated that the conjugation of TAT to a protein substrate could be used to deliver the cargo to all parts of the body.152 Since this initial report, a number of NP cargoes have been systemically delivered to all cells

indiscriminately, with preferential localisation determined merely by the NP pharmacokinetics.153,154 The passive accumulation of NPs in tumours has also been exploited to allow the preferential delivery of TAT-labelled, drug-loaded micelles155 and chitosan NPs156, as well as anti-tumour silver NPs157 to tumour cells in animal *in vivo* models (Figure 8). Similarly, arginine-rich peptides158,159 and penetratin160,161 have been used to both penetrate tumour cells, following passive accumulation, and to target the brain as covered in Section 5.3. Interestingly, the non-natural D-enantiomer of TAT has also been shown to mediate cell penetration. This strongly suggests that NP uptake as a result of TAT decoration is not due to a specific biological interaction - rather it is the density or distribution of positive charges along the peptide backbone that are responsible for internalisation.154,162 Importantly, despite the ability of cationic peptides to increase uptake efficiency, cytosolic delivery of the NP cargo often remains low.163 The strong affinity of positively charged peptides towards negatively charged endosomal membrane components may hinder endosomal escape. As a result, additional factors able to mediate this process may need to be incorporated during NP design, as discussed in section 5.2.

Increasing cell and tissue specificity—In all of the cases noted above, the low specificity of CPPs for a particular cell type or target organ leads to widespread tissue delivery. CPPs cross cell membranes in a largely indiscriminate manner, leading to the uptake of NPs by almost all cells that are encountered. As such, technologies relying on these sequences are often associated with significant side-effects, particularly when they are used to deliver a therapeutic payload. In order to address this limitation, a number of methods for CPP ‘screening’ have been reported that enable NP targeting prior to unmasking of the penetrative sequence. In an important early demonstration of this principle the Tsien group attached a complementary polyanionic peptide, able to electrostatically bind and thus block the activity of a polyarginine CPP, via a matrix metalloproteinase (MMP) cleavable linker region.164 In the presence of the corresponding protease, typically upregulated within the tumour environment, peptide cleavage resulted in the unmasking of the cationic CPP and intracellular payload delivery. Such systems have been subsequently utilised for the intracellular delivery of QDs165 and more recently for the selective *in vivo* delivery of PEG-polycaprolactone (PCL) NPs to tumours166 (Figure 9).

A related approach is to screen the activity of the CPP using steric bulk. Harris *et al* reported the coating of TAT-functionalised iron oxide NPs with a shield of PEG, via a MMP-2 cleavable peptide linker.167 Upon action of the protease within the tumour environment, the TAT peptide was unmasked enabling cell uptake. Alternatively, ultra-violet (UV) light-cleavable groups can be used to trigger cleavage of appended lipids with spatial precision. 168 Finally, hydrazone-linked PEGs that are cleaved in the mildly acidic tumour environment have also been reported for the selective unblocking of TAT peptides at the desired site-of-action.169,170 Importantly, hydrazone structure has been shown to play a vital role in the rate of hydrolysis, and thus the ability of the NP construct to undergo acid-mediated cleavage. By suitable choice of coupling partners, hydrazone half-life can be tuned from a matter of minutes, all the way up to months at neutral pH.171,172 This therefore represents an important consideration during construct design. For example, aliphatic

hydrazones may display insufficient stability to allow application *in vivo*,170 while diaryl linkages may prove too stable to allow acid responsive behaviour to be displayed.173

Role of peptide/protein density—The density of CPP surface coverage is an important determinant of NP behaviour. Sufficient ligands must be presented to enable efficient penetration, while also avoiding overcrowding which can diminish bioactivity and prevent the presentation of dual-functionalities.174 In the early 2000s, Zhao *et al* demonstrated that the efficiency of MNP uptake was highly dependent on the number of conjugated TAT peptides.175 Penetration was greatly enhanced when more than 10 peptides were displayed on the particle surface, with a non-linear response suggesting multi-valent interactions were at least partially responsible for the increase in efficiency. This has been validated by a number of subsequent reports – a lower coverage of peptides bearing multiple CPP arms often results in a higher cell uptake than that observed for particles coated with a high density of mono-valent peptides, though the effect is not universal.176,177 Indeed, in a recent report Breger *et al* demonstrated that QDs decorated with a single ligand could be efficiently uptaken if a multivalent dendrimeric CPP was presented.177 A number of hypotheses have been made to explain the origins of these effects, and, as is often the case, the exact answer is probably dependent on the precise cell type, NP cargo, and construct design. While receptor clustering and crosslinking is known to play a major role in many cell-surface recognition events and may also be important in mediating cell uptake, an increase in membrane curvature and pore formation as a result of greater localised electrostatic binding has also been proposed to play a role.177,178 Intriguingly, the Jana group has recently demonstrated that the valency itself is a key determinant in not only uptake mechanism, but also subsequent sub-cellular location, and indeed the rate at which particles are subsequently ejected by exocytosis.179 A high valency of TAT peptides on the surface of QDs was found to lead to an increased rate of initial cell uptake. However, the same particles were then rapidly processed and exocytosed, leading to an overall drop in penetration efficiency.180

5.2 Endosomal escape

As described above, the endocytosis of NPs (both with and without peptide functionalisation) leads to internalisation within endosomes. Unless for tissue imaging purposes, this location is typically not the end target, with delivery to the cytosol or nucleus usually necessary for activity. As such, the endosomal escape of the NP cargo is an essential yet often overlooked factor in enabling function. While a number of small molecule additives such as chloroquine may act as transduction enhancers, their use is not a plausible solution for cell penetration *in vivo*. As an alternative, a number of peptide sequences have been identified that are able to promote escape, typically through the formation of disruptive α-helices, which can be triggered selectively within the acidic environment of endosomal compartments.181 The N-terminal domain of influenza virus hemagglutinin HA2 was one of the earliest sequences identified, with Plank *et al* demonstrating that HA2 functionalisation of DNA-based NPs greatly enhanced cell transduction.146,181,182 Other sequences, such as oligomers of the tetrapeptide GALA183 and Penetration Accelerating Sequence (Pas)184,185 have also been utilised to promote the endosomal escape of functionalised liposomes and QDs respectively. An alternative approach to enhance escape

has recently been reported by Morshed and co-workers. By attaching TAT peptides to AuNPs via an acid labile hydrazone linkage, they were able to promote endosomal escape through cleavage of the CPP. Using a non-cleavable linkage resulted in the strong binding of the peptide to the negatively charged endosomal membrane being retained and thus hindered particle escape.186

5.3 Crossing the blood-brain barrier

The blood-brain barrier (BBB) is a formidable and restrictive obstacle, able to exclude over 98 % of small molecule drugs and almost all nanoscale objects in order to maintain brain homeostasis.187,188 The endothelial cells of the brain capillaries form continuous tight junctions that preclude paracellular transport.188,189 While it is possible to disrupt this barrier and allow passage between cells, more commonly strategies which aim to overcome the BBB rely on transport *through* the cells that make up the barrier (Figure 10). However, the downregulation of receptors that mediate vesicular transport, and the presence of highly active efflux pumps makes even such transcellular movement difficult.188

Four main strategies have been exploited in order to allow BBB penetration, each of which can be exploited by peptide or protein decorated NPs: i) Receptor-mediated transport, whereby NP interactions with over-expressed receptors at the BBB trigger internalisation; ii) Transporter-mediated movement, hijacking the natural uptake of nutrients such as glutathione by the brain; iii) Adsorptive transport, as a result of the strong binding of positively charged particles to the negatively charged BBB; and iv) The exploitation of CPPs, which have also been demonstrated to mediate transport across the BBB (Figure 10). For an in depth discussion on these processes and the mechanisms by which NPs can be transported across the BBB the reader is referred to excellent recent overviews by the groups of Teixidó, Gao, and Tosi.187,188,190

Importantly, the ‘leakiness’ of the BBB is often increased during pathogenesis. This is particularly true for tumours originating in the brain, more commonly referred to as gliomas. 191 Gliomas are therefore commonly used as a model system on which to demonstrate the ability of a particular NP construct to penetrate the BBB. While this is not in itself problematic, it is important to have in mind the possible effects of impaired cellular junctions on NP transport in such systems.

Key peptide sequences—The key CPP sequences utilised for the *in vivo* delivery of NPs, as outlined in section 5.1, have all also been utilised to deliver NP cargoes across the BBB. Their ability to penetrate the cells of the brain endothelium enables transcellular transport into brain tissue. QDs,192 peptide self-assemblies,193 and AuNPs186,194 have all been shown to penetrate the brain following TAT functionalisation. Similarly, the delivery of polyarginine-functionalised liposomes195 and penetratin-labelled PEG-PLA particles160 has also been reported. However, as described above, the promiscuity of CPPs limits any specificity for brain targeting and these reports are also associated with systemic delivery to other organs and tissues. As a result, strategies improving specificity for brain targeting represent a more attractive solution.

The most common technique to promote BBB specific transport is to target receptors at the blood-brain interface, which are able to induce receptor-mediated endocytosis. The transferrin-receptor (TfR) is overexpressed on brain endothelial cells, playing a crucial role in the transport of iron across the BBB by mediating the endocytosis and transport of the iron-binding protein transferrin (Tf). The attachment of Tf itself to NPs has therefore been widely used as a means to mediate BBB penetration, transporting a range of structures including drug-loaded serum albumin NPs,196 liposomes,158,197,198 polymersomes,199 and polymer dendrimer particles,200 as well as RNA-based structures201. Alternatively, anti-TfR antibodies, such as OX26 can be utilised to mediate transport.202–205 However, possible difficulties with the species-specificity of antibodies may partially hinder such technologies. For example, since OX26 is targeted to the rat TfR, NPs coated with this antibody are unable to be readily translated into human systems.206

The use of the TfR as a target for BBB penetration has significant limitations in spite of its attractiveness as a target. Firstly, the TfR is commonly expressed on the surface of many tissues, in particular the liver and spleen, lowering organ specificity.158,207 Secondly, and more significantly, due to the importance of transporting iron to all areas of the body the physiological levels of Tf circulating in the blood are high, leading to virtual saturation of the TfR and preventing the binding of exogenous protein.208 This greatly limits the ability of Tf-labelled NPs to cross the BBB.

Receptors for the closely related iron-binding protein lactoferrin (Lf) are also commonly over-expressed at the BBB.187 Since the endogenous circulating level of Lf is far lower than that of Tf, receptor targeting is greatly facilitated.209 As a result, a wide range of Lf-conjugated NPs have been reported, and demonstrated to cross the BBB.208,210–214 An alternative solution to the high physiological levels of circulating Tf has been offered by Lee *et al*.215 Through phage display, they were able to identify a short peptide sequence capable of binding the TfR in a non-competitive manner with Tf. As a result, efficient receptor mediated endocytosis can be instigated even at saturating levels of Tf.216 Recently Prades *et al* demonstrated that N-methylated, enantio, and retro-enantio derivatives of this peptide maintained or even *enhanced* the ability to transport NPs across the BBB, while decreasing susceptibility to protease mediated-degradation particle clearance (Figure 11).217 This work represents an impressive demonstration of the synthetic versatility of peptides and their ability to greatly improve the characteristics of NP cargoes.

The short peptides Angiopep-2 and glutathione (GSH) represent two of the most promising candidates for the clinical translation of BBB-penetrating NP technologies. Both peptides have the ability to target and be internalized by receptors overexpressed on brain endothelium, specifically low-density lipoprotein receptor-related protein (LRP) and the GSH receptor respectively. These two peptide sequences have been widely studied for their ability to mediate transport. Importantly, they both display extremely low associated toxicities and are therefore key components of a number of technologies currently in clinical trials.188,218 Angiopep-2 was first identified by Demeule *et al* from the LRP binding Kunitz domain of a number of proteins able to cross the BBB.219 In the subsequent 10 years, Angiopep-2 has been utilised to deliver a wide range of NP cargoes across the BBB, including liposomes, polymer-, upconverting-, and gold-NPs (Figure 12).166,220–229

Similarly, GSH decoration has been used extensively to deliver NPs, particularly drug-loaded liposomes, across the BBB.230–232 Indeed, technologies based on these systems for the delivery of doxorubicin to gliomas have recently completed Phase I/IIa clinical trials using the tradename G-technology® formulation.188

In addition to these prominent examples, a number of other peptide sequences have been identified which are able to mediate transport across the BBB. Many of these are derived from pathogens or toxins, mimicking the ability of the parent protein/peptide to elicit damage within the brain by either disrupting the endothelial membrane or by receptor-mediated endocytosis. The snake venom-based peptides CDX233 and chlorotoxin234,235, bee venom-derived MiniAp-4,236 opioid glycopeptide g7,237,238 and rabies virus-derived peptide RV29239,240 have all been shown to enable the delivery of NP cargoes across the BBB. Finally, the attachment of cationic proteins has also proved to be an effective method for promoting NP transport. In particular, the cationization of serum albumin proteins and subsequent NP decoration has been shown to promote BBB penetration. Initial binding is induced by electrostatic interactions with the highly negatively charged brain endothelium, and is followed by subsequent adsorptive-mediated transcytosis.241–244

5.4 Tumour penetration

The structural and functional abnormality of tumour tissues offers many opportunities for NP delivery and penetration, but also many challenges which must be overcome. Early studies in rodents suggested that the endothelial barriers lining the vasculature displayed increased permeability to large species, while reduced lymphatic drainage enhanced tumour retention.245,246 These two factors combined form the basis for the enhanced permeability and retention (EPR) effect.247 However, the impact of the EPR effect on nanomedicine remains highly controversial.248–251 While studied extensively in rodents, the clinical relevance of the EPR effect in human patients remains uncertain. Even within a single model species the porosity of the vasculature can vary dramatically depending on the tumour type, growth stage, microenvironment, and even individual.252 Furthermore, even with accumulation, the subsequent transport of particles across the blood-tumour barrier and penetration of the poorly vascularised, hypoxic environment of densely cellularised solid tumours remains problematic.

A number of peptide sequences have been identified that are able to first target NPs to the tumour site, and then also lead to their distribution across the entire mass. Amongst these, the C-end rule (CendR) class of peptides identified and pioneered by the Ruoslahti group are particularly prominent. CendR peptides were first isolated from phage display libraries by Teesalu *et al.*253 By screening peptides against cancer cells they were able to identify a consensus (R/K)XX(R/K) sequence which promoted cell uptake. This sequence shares homology with the neuropilin-1 binding domain of vascular endothelial growth factor (VEGF) (with which CendR peptides are thought to compete for uptake).253 Importantly, this motif must be present at the C-terminus of the peptide in order to cause penetration, hence the name CendR. Internal or reversed sequences have no biological effect.

Importantly, the requirement for the CendR sequence to be placed at the peptide C-terminus enables it to be used as a cryptic internal sequence, blocked by a protease-sensitive directing

motif for specific delivery to a target tissue. This was exploited by Sugahara *et al* to produce the peptide iRGD, able to both target a variety of NP substrates and promote their uptake after protease processing.254,255 This is achieved through a multi-step mechanism, in which binding is first induced by interaction of a terminal RGD motif with integrins overexpressed on the surfaces of many tumours (see section 6.1). This targeting moiety is then proteolytically cleaved exposing a CendR peptide, which induces cell penetration following interaction with nueropilin-1. Combining the RGD targeting and CendR penetration motifs within a single modular peptide construct enabled greatly enhanced tumour penetration of lipid micelles, iron oxide nanoworms, and albumin NPs, when compared to particles bearing either motif in isolation (Figure 13).254 iRGD NP decoration has subsequently been utilised to deliver polyethylenimine (PEI)-PEG NP gene therapies to glioblastomas,256 iron oxide imaging agents to detect breast cancer metastasis,257 and doxorubicin coated AuNPs to deep tumour sites258.

As will be discussed in section 6.1, a number of peptide sequences which target NPs to cancerous regions have been identified and used to home particles to the desired site. Most sequences often merely lead to NP distribution in the periphery of the tumour vasculature, rather than actively enabling penetration into the deeper tissue. However, a smaller number of peptide sequences have been identified that are able to promote both processes, first targeting NP delivery to the tumour, then penetrating away from the vasculature via receptor mediated transcytosis. One such peptide sequence is IL-13p, a truncated derivative of the inflammatory cytokine interleukin-13 (IL-13). IL-13 has a promiscuous receptor activating profile throughout the body, yet IL-13p has been found to be selective for the receptor IL-13Rα2, which is strongly overexpressed on the surface of many tumour cells and thus represents an interesting target for tumour-directed therapies.259 In addition to providing receptor specificity, IL-13p overcomes the low stability and high immunogenicity of the parent protein. Furthermore, it has been found to subsequently promote cell and tumour penetration, making it an effective tool for NP delivery.260 In a series of papers, Gao and co-workers demonstrated that IL-13p decorated PEG-PCL NPs could be used to efficiently deliver anti-cancer therapeutics and suppress glioma growth *in vivo*.259,261,262 Wang *et al* have also demonstrated that the targeting of IL-13Rα2 with a phage-display derived binding peptide enables glioma targeting and penetration, allowing the delivery of poly(lactic-co-glycolic acid) (PLGA)-PEG NPs.263

In a similar manner, the cyclic peptide Lyp-1, first identified from phage-display libraries by Laakkonen *et al*, is a tumour-homing peptide targeting the over-expressed p32 receptor.264 Karmali *et al* demonstrated that delivery of the clinically approved paclitaxel NP delivery vehicle Abraxane® could be significantly improved through Lyp-1 decoration.265 Subsequent investigations have further developed this peptide, by combining Lyp-1 with a cryptic CendR motif, greatly increasing cell penetration as well as distribution throughout the tumour mass, and thus increasing the efficacy of therapeutic delivery.266,267

6 Targeting particles for therapeutic and diagnostic purposes

Directing a NP construct to the organ or tissue in which its action is required is crucial in ensuring the successful function of both therapeutic and diagnostic tools. The ability of

peptides and protein coatings to modulate distribution within the body has been widely exploited. When combined with motifs that reduce clearance by the reticuloendothelial system and successfully deliver particles intra-cellularly, targeted delivery can lead to highly potent nano-technologies.

Targeting approaches can be broadly classified into two modes, 'passive' and 'active', though these names can be misleading. They imply the rational targeting of particles through design rather than natural distribution, and active guiding to the target site rather than localisation through chance encounters respectively.247 Passive targeting exploits the normal NP biodistribution, as typified by the observed accumulation of particles in the vasculature of some tumours via the EPR effect, as discussed above.268 While this may also enable targeting of the liver and spleen, in which NPs are naturally deposited following clearance from the blood, other organs and tissues require more sophisticated 'active' means of directing delivery.269 This is most commonly achieved through the introduction of a recognition motif around the corona of the NP, that can preferentially bind cell-surface receptors and other biomolecules exposed within the target area. Small molecules and vitamins, carbohydrates, peptides, proteins, and aptamers can all be utilised to target delivery.270 In this section, we will discuss the most common polypeptide sequences utilised to direct NP transport, and their impact on biomedical technologies.

6.1 Peptide targeting motifs

Short peptides have emerged as the preferred agent for influencing NP distribution due to a number of advantageous properties (Table 6). Firstly, when compared to full size protein targeting agents peptides are able to form compact NP coatings due to their small size, limiting disruption of the NP hydrodynamic diameter. Furthermore, their surface loading can be controlled, enabling the simultaneous presentation of multiple targeting sequences, providing high affinity and synergistic binding. Peptides of less than 30 amino acids can also be accessed in a straightforward manner via solid-phase peptide synthesis, enabling the facile introduction of functional chemical handles and non-natural residues. This may provide both ease of conjugation, and sequences with increased binding and thus targeting efficiencies. Finally, peptides offer the possibilities of lowered immunogenicity, increased stability of presentation, and reduced binding to physiological biomolecules when compared to full length proteins.271

Many of the peptide-targeting ligands utilised in biomedical applications were first identified from phage display panning experiments against a target tissue, receptor, or cell type.272–275 First reported in 1985 by Smith, phage display relies on the presentation of short amino acid sequences on the protein coating of filamentous phage.276 Random peptide libraries of a defined length can be expressed, and then screened for binding against the target. Following several rounds of increasingly stringent screening, coupled with the amplification of binding phage, peptides with high target affinity can be identified.277 Phage display has been most widely used to screen binding to a target protein or cell-surface receptor. However it is also possible to systemically deliver phage libraries *in vivo*. Using this technique, phage binding to a target tissue or organ have been isolated and peptide sequences mediating cargo transport to the desired location identified.274,278–280

The Ruoslahti group have utilised *in vivo* bio-panning to identify a number of sequences able to target NP delivery, particularly for cancerous tissue.264,281–283 Åkerman *et al* demonstrated that 3 of these sequences, GFE (targeting membrane dipeptidase on the endothelial cells of lung vasculature),281 F3 (binding blood vessels and various tumour cell types),282 and Lyp-1 (targeting tumour lymphatic vessels, as discussed in section 5.4),264 could be used to selectively deliver QDs *in vivo*.284 Interestingly, F3 and Lyp-1 labelled particles can be used for the multiplexed imaging of both the blood and lymphatic vessels within the same tumour (Figure 14). The exquisite sensitivity and target specificity offered by these sequences is in part due to their multivalent presentation on the QD surface. This highlights the power of combining peptide display with nanomaterials for biomedical imaging. In a similar manner, the tumour-homing pentapeptide CREKA was also identified from phage libraries, and found to target the clotted plasma proteins that accumulate within the interstitial tissue and vessel walls in cancerous masses (Figure 15).285,286 This peptide was able to deliver both iron oxide NPs and liposomes to the desired site, where they were observed to induce additional clotting and thus amplified accumulation.

Poor perfusion of the tumour vasculature has been shown to limit the penetration of NPs into the tumour tissue and presents a major obstacle to effective tumour treatment. In section 5 we introduced a number of sequences which address these difficulties, mediating transcellular transport deep into the tumour mass. The Lyp-1 peptide described above is one such sequence, playing a dual-functional role in mediating both targeting and penetration. As discussed in section 5.4, Karmali *et al* demonstrated that combining the CREKA and Lyp-1 peptide sequences within a single NP construct enabled both the targeted delivery and tumour penetration of paclitaxel containing Abraxane® particles.265 Accumulation and tumour penetration were greatly enhanced when both peptides were present, compared to NPs functionalised with a single species, demonstrating the ability of CREKA and Lyp-1 to act in a synergistic fashion to minimise tumour growth. The phage-display derived CendR family of peptides, introduced in section 5.4 as tumour-penetrating sequences, can also be combined with targeting motifs to provide dual-functional linear peptides. Indeed, as discussed previously the internalizing iRGD sequence makes use of one of the most widely studied and utilised targeting groups, RGD, to direct cargo to cells overexpressing α_v integrin.253–255 iRGD coated NPs have been shown to be efficient vehicles for drug-delivery and imaging applications.256–258 In contrast, RGD peptides lacking the proteolytic sites required to expose the CendR sequence, do not enable the penetration of deep tumour tissue *in vivo*, despite their ability to instigate tumour delivery.254,287,288 A number of other peptide sequences able to target NPs to tumour tissue have been identified, including cyclic- and linear-NGR (binding the tumour vasculature receptor CD-13).289–293

Many peptides have been identified that target a wide range of tumour types. Sequences also exist for directing particles to a specific cancer, through binding to over-expressed tissue-specific receptors. For example, the bladder cancer binding peptide Bld-1 was identified from whole cell phage-panning by Lee *et al*.294 This peptide has subsequently been shown to mediate the directed delivery of doxorubicin-loaded MSNPs for the treatment of bladder cancer.295 Similarly, peptides able to bind human epidermal growth factor receptor-2 (HER2) have been utilised to selectively deliver NPs to breast cancer tissue, mimicking the activity of anti-HER2 antibodies used in a number of clinical breast cancer treatments.296–

298 Additional imaging and therapeutic NP-peptide conjugates have been developed for specifically targeting prostate cancer,299,300 predominantly using sequences identified through biopanning against cancerous cells *in vivo*.

Although peptides targeting tumours have been most widely studied, other sequences specific for healthy tissue have also been identified and used to deliver NP cargoes. Adipose tissue has been particularly widely targeted as a consequence of its important role in obesity and related pathologies. Kolonin *et al* first identified the white fat directing peptide CKGGRAKDC from phage-display libraries and demonstrated its ability to carry pro-apoptotic peptides for the reversal of obesity (Figure 16).301,302 Hossen *et al* subsequently demonstrated the delivery of drug-loaded PEGylated NPs for the control of adipose behaviour and weight gain.303 This peptide has also been reported to mediate the delivery of proangiogenic agents, able to stimulate the conversion to brown adipose tissue which is more readily expended during normal energy usage.304

The heart and brain are major organs of interest for the delivery of NPs for therapeutic and diagnostic purposes. Following myocardial infarction, the leakiness of blood vessels in the left ventricle promotes passive NP accumulation.268 Particles can also be selectively delivered by targeting the angiotensin II type 1 receptor, commonly overexpressed in infarcted tissue. Dvir *et al* demonstrated that a truncated 9 amino acid peptide mimicking the behaviour of angiotensin II was thus able to direct the delivery of therapeutic liposomes.305 Alternatively, Nguyen *et al* reported that the overexpression of MMPs within the heart following infarction could be used to direct the aggregation of drug-loaded NPs, following proteolytic degradation of hydrophilic surface peptides and thus accumulation at the infarct site.306 In order to mediate NP delivery to the brain, Mann *et al* recently developed the short tetrapeptide CAQK, which despite its relatively short length, is remarkably able to target the delivery of both small molecule and NP cargoes.307 Importantly, this peptide displayed affinity for the extravascular tissue, rather than the blood vessels themselves. As a result, delivery was enabled throughout the site of acute brain injury for the distribution of therapeutic payloads.

In addition to peptides which can direct the delivery of particles to certain parts of the body, several sequences able to drive NP accumulation within specific subcellular locations following cell uptake have also been identified. Distinct from peptides that induce cell penetration or endosomal escape, these sequences enable intracellular trafficking to the site at which their action is required.271 Although such sequences have been most commonly utilised for the *in vitro* study or targeting of intracellular processes, more recent studies have also demonstrated their application in a biomedical setting.271 Agemy *et al* combined the mitochondrial targeting peptide $(KLAKLAK)_2$ with tumour-targeting CGKRK and penetrating iRGD sequences, to form a tri-functional iron oxide NP coating, able to efficiently deliver particles within mouse glioblastoma models. In addition to its targeting role, $(KLAKLAK)_2$ also promoted apoptosis through disruption of the mitochondrial membrane, increasing the therapeutic effect.308 In another example, the endoplasmic reticulum (ER) retention signal ‘Eriss’ (derived from the adenovirus E13-19K protein) has been used to direct NPs to the ER of lymphocytes, improving the processing and presentation of NP-displayed antigens. By doing so, the efficiency of synthetic vaccines can

be greatly enhanced *in vivo* as discussed in section 7, leading to an improvement in the generation of immunity.309,310

6.2 Protein targeting motifs

The recognition of protein binding partners by cell-surface receptors is one of the most important interactions in biology. The vast number of hormones, growth factors, cytokines, cell adhesion proteins, and cell-signalling structures are involved in virtually all intracellular interactions that enable the formation and function of complex tissues.311 It is therefore not surprising that protein-NP coatings have become an important means to drive targeted delivery to a certain area of the body where a particular receptor is likely to be overexpressed (Table 7).270,275

As described in section 5.3, the iron-binding proteins Tf and Lf mediate the transport of this key nutrient around the body. NPs coated with these structures can therefore be targeted to sites where their relevant receptors are expressed, as epitomised by their ability to deliver particles to, and indeed induce transport across, the BBB.187 The TfR has a particularly widespread distribution throughout the body – almost all cells are thought to express cell surface TfR, albeit at significantly varying levels.312 However, the overexpression of TfR in certain cancers as a result of rapid cell growth kinetics, and thus a demanding need for iron, enables the use of Tf as a targeting agent when combined with potential particle accumulation within the tumour vasculature via the EPR effect.313 Interestingly, a number of papers have demonstrated that TfR targeting does not occur as a result of altered biodistribution, with similar accumulation kinetics being observed for both functionalised and non-functionalised NPs. Instead, it is the ability of the targeting group to induce penetration and uptake within the target tissue that leads to an enhancement of the therapeutic efficiency of NP drug-delivery vehicles.314,315

The degree of Tf modification plays an important role in determining the level of NP uptake, whether through multi-valent interactions or increased chance of recognition.315,316 Salvati *et al* have also highlighted the importance of carefully designing NP coatings in order to maintain maximal activity *in vivo*. Following the incubation of Tf-functionalised silica NPs in serum, they showed that rapid loss of TfR targeting occurred due to the accumulation of a blocking protein corona.317 Despite this sensitivity, Tf functionalisation has been the most widely utilised strategy for directing tumour delivery. Such systems have been shown to mediate the transport of a wide range of NP cargoes, as well as being at the core of several liposomal based therapeutic technologies currently in clinical trials (Figure 17). 313,314,316,318–321

Many other protein ligands have also found widespread use for the selective targeting of NPs to cell surface receptors within a specific tissue or organ. An important consideration is the ability of many such ligands to trigger cellular responses and downstream effects. While this may be favourable in certain applications, in such cases the protein substrate is not merely acting as a targeting motif but is also controlling or inducing a biological effect.322 For example, growth factors such as epidermal growth factor (EGF) and VEGF have been used to target NPs to their relevant receptors, when overexpressed on the surface of tumour cells and tissues.270,322–327 However, the potency of growth factors as cell signalling moieties

and their often promiscuous activation profiles must be carefully controlled, particularly following systemic delivery. Furthermore, cell-surface adhesion motifs can also be targeted. Chen *et al* demonstrated that iron oxide NPs could be functionalised with lymphocyte function-associated antigen (LFA)-1 proteins, able to target intercellular adhesion molecule-1 (ICAM-1) on the surface of both tumour cells and inflamed tissue *in vivo*.328

High density lipoproteins (HDLPs) have also found utility for selective NP delivery. These lipid-protein hybrids naturally form spherical particles in order to mediate the transport of lipids around the body. The interaction of HDLPs with a number of receptors commonly implicated in pathological conditions can be exploited to deliver NP based therapeutic agents and imaging tools.270 The scavenger receptor type B-1 (SR-B1) has been most widely targeted. For example, Yang *et al* demonstrated that AuNP templated HDLP particles could be used to selectively sequester cholesterol *in vivo* (Figure 18). These structures could be targeted to B-cell lymphoma cells, due to their need for cholesterol and thus upregulated expression of SR-B1.329 Similarly, apolipoprotein A-I, the major component of HDLPs, has been demonstrated to enable the selective delivery of fluorescent NPs to cancerous tissue *in vivo*.330

Although cell surface receptors are the most commonly utilised target for NP delivery, other cell or tissue specific markers can also be utilised to mediate NP targeting. Most prominently, lectins, or carbohydrate-binding proteins, can direct NPs to tissues displaying particular cell-surface glycans.270 Glycoproteins and glycolipids play a vital role in cell biology, providing stability to the cell membrane, facilitating cellular recognition, and adding an extra level of complexity and functionality to the proteins that determine cell behaviour. With rapidly improving tools for studying the role of glycans in many normal and diseased states, so their biological importance is being increasingly appreciated. As a result, specific glycoforms have now been identified to be more prominently displayed during certain pathologies, making them attractive targets for NP delivery. Lectins commonly display exquisite sensitivity for a particular sugar structure and conformation, often analogous to that shown by antibodies. Wheat germ agglutinin (WGA) binds specifically to *N*-acetylglucosamine residues commonly found on the alveolar epithelium, and has thus been utilised by Surti and Misra to deliver corticosteroid loaded NPs to lung tissue.331 A number of groups have subsequently used WGA to induce the intra-nasal transport of functionalised NPs, due to its ability to both bind and promote transcellular delivery across the epithelial barriers of the nasal passageway.332–334 *Ulex europaeus* agglutinin I (UEA-1) can also be used to selectively bind α-fucose residues on the surface of microfold cells (M-cells) within the small intestine.335 Oral delivery of UEA-1 labelled antigen containing NPs enables the initiation of a mucosal immune response and vaccination of the recipient.336,337 Mucosal M-cells can also be targeted via intranasal delivery, providing a facile means to undertake needle-free NP immunization.338

6.3 Antibody targeting motifs

The ability of antibodies (Abs) to bind with high specificity and affinity for their target antigen makes them ideal directing groups for the delivery of NP cargoes. It is therefore unsurprising that Ab-NP conjugates have found widespread utility in the biomedical field.

275,339,12,340–342 The ability of Abs to neutralise the effect of their target or to induce a favourable biological response allows their use as therapeutic agents in their own right. They have also commonly been exploited to deliver a therapeutic cargo, in the form of an Ab-drug conjugate (ADC).343,344,10,345 At the start of 2017, 52 monoclonal Ab technologies were in clinical trials, with a further 16 awaiting or having been granted marketing approval.346 The importance of tumour delivery is highlighted by the fact that 40 % of these systems were targeted towards cancer therapy. The generation of Abs for a target protein, cell type or tissue of interest is outside the scope of this review, and the reader is directed instead to a number of comprehensive reviews for further details.275,347,348

Most Abs are composed of 4 chains (2 light and 2 heavy) each of which has a constant and a variable region. Specificity towards the desired target is generated predominantly by the variable region. Ab fragments may therefore retain binding affinity of the full length construct, though in some cases bisantigen binding is necessary to maintain full affinity. These smaller targeting groups can be produced via genetic engineering or careful protease-mediated Ab digestion, generating motifs which overcome the disruptive size, potential immunogenicity, and high cost of the parent Ab.342 Both full length and fragment Abs have been extensively used to target the delivery of NP cargoes, as have naturally occurring single domain camelid Abs.349 Tumours have been particularly widely targeted due to the frequency with which cell-surface receptors are overexpressed and can thus be used as a site for selective delivery.12 The most common realisations of Ab-NP strategies deliver a therapeutic payload via a liposomal drugdelivery vehicle, though other NP formats for both therapeutic and diagnostic purposes have also been the subject of significant interest.342

Overexpression of HER2 is implicated in over 30 % of breast cancers and thus represents an important target for delivery.350 The HER2 selective Ab trastuzumab (sold under the tradename Herceptin) and other related structures are probably the most widely studied and utilised targeting groups for NP delivery. In a series of early papers, Park and co-workers demonstrated that the labelling of liposomes with an anti-HER2 Ab resulted in an efficient delivery vehicle for targeting breast cancer xenografts with chemotherapeutics or DNA.351–353 HER2 targeted iron oxide NPs have also been widely reported as a means to induce directed hyperthermia and for MRI imaging (Figure 19).354–360 NDong *et al* recently reported that a single antigen-binding fragment of trastuzumab could also be used for targeting *in vivo*, leading to effective delivery of iron oxide particles to tumour sites.359

The epidermal growth factor receptor (EGFR) is another popular target for Ab-mediated delivery. EGFR overexpression is implicated in many cancers, especially those with epithelial origins, and thus serves as a useful target for immunoliposome targeting.361–363 Indeed, a single antigen-binding fragment of the monoclonal Ab cetuximab conjugated to doxorubicin loaded liposomes has been tested in phase I clinical trials for the treatment of patients with solid malignancies.362 A number of other cancer markers have been widely targeted via Ab-NP conjugates (both for therapeutic and diagnostic purposes) including the TfR,204–206,364,365 B-lymphocyte associated antigen CD19 for the treatment of lymphoma, 366,367 the melanoma and carcinoma marker endoglin,368–370 nucleosomes released by proximal apoptotic tumour cells,364,371 death-receptor 5 using the commercial Ab Conatumumab,372 and the adhesion receptor VCAM-1373.

The choice of Ab, the use of full length protein or fragment, and the NP cargo are all important considerations when designing an Ab-labelled technology. However, the widespread interest in Ab-NP conjugates has led to the identification of a number of other key design criteria. The orientation of the Ab on the NP surface is an important consideration – non-specific protein modification techniques can lead to Ab conjugates with many different NP binding points, some of which may result in an antigen-binding domain that is positioned in a hindered or blocked orientation. Indeed, a recent study by Herda *et al* demonstrated that as little as 5 % grafted protein possessed an accessible epitope following non-specific conjugation to silica NPs.374 The use of site-specific modification strategies able to selectively form Ab-NP conjugates away from the active recognition site are likely to improve the efficiency of NP targeting *in vivo*.342,375 To this extent, Greene *et al* recently reported a strategy to functionally re-bridge distal disulphide bonds within a trastuzumab fragment-Ab, enabling orientated Ab display on the surface of PLGA-PEG NPs and a corresponding increase in binding efficiency when compared to non-specific Ab conjugation.376

In a related manner, the Ab loading density on the NP surface can play an important role in targeting efficiency. At high levels, hindered antigen-binding may result leading to an initially counter-intuitive drop in targeting efficiency. Indeed, Jiang *et al* demonstrated that the size of the NP template plays an important role in determining hindrance and thus binding efficiency, with increasing curvature leading to an increase in separation and thus decrease in steric inhibition of antigen recognition.377 Although multivalent Ab display may be desirable in some cases in order to promote receptor recognition, recent papers by the groups of Davis and Prosperi have highlighted that this may not always be the case. 378,379 In both cases, a singly Ab labelled NP was found to be sufficient, and in the case of Colombo *et al* superior to multivalent Ab display for effective tumour targeting.

7 Mimicking biological species

Many biological entities fall within the nanometre size regime, displaying multi-valent peptide or protein motifs on their surface. NP-polypeptide conjugates are able to effectively mimic the behaviour of these structures, stimulating signalling pathways and eliciting cellular responses. The interactions induced by these particles are responsible for many of the applications discussed in this review. The promotion of receptor clustering is often implicated in receptor-mediated endocytosis and cell or tissue penetration,380,381 and NP targeting may be enhanced by multi-valent interactions at the nanoscale. As these topics have already been covered in detail above, in this section we will focus on the ability of NP-polypeptide constructs to mimic the multi-valent display of antigens present on the surfaces of pathogens and tumour cells. By doing so these conjugates are able to induce a controlled immune response and thus generate immunity in the recipient. Technologies which rely on the encapsulation, rather than surface presentation of antigens, are outside the scope of this review and the reader is instead directed to a number of comprehensive reviews on this topic. 382–384 Furthermore, the use of self-assembling peptides/protein NPs as vaccine candidates will be detailed in section 8 below and so will also not be discussed in detail here.

NP vaccines can offer the benefits of increasing antigen stability over soluble delivery. Furthermore, their nanoscale size may promote scavenging by dendritic cells and therefore improve T-cell presentation, minimising the activation of alternative immune response pathways.382,385 Reddy *et al* demonstrated that when NPs were of a sufficiently small size (< 100 nm) preferential drainage and accumulation in the lymph nodes was enabled.386 However, other reports have suggested that particles up to 1 μm in diameter may also preferentially accumulate under certain delivery conditions, highlighting the need for further investigation into this phenomena.387 Preferential exploitation of the lymphatic system brings particles into closer contact with the residing dendritic cells and leads to an enhanced activation of the immune system maximising vaccine efficiency. However, the major advantage of NP-based vaccines lies in their ability to enable the multivalent display of antigens, promoting the interaction of multiple ligand-receptor pairs. Such processes often play an essential role during T-cell activation, rather than relying on individual recognition events.388 As such, the presentation of multiple antigens on the NP surface can play a far more effective role in modulating the immune response than presentation of free peptide or protein motifs. Interestingly, it has been shown that the antigen patterning plays a crucial role during activation. As a result, antigen grafting is a key design feature during the production of NP vaccines. The Yu group demonstrated that enhanced antigen clustering elicits a stronger immune response than uniformly distributed ligands at both the micron and nano scale.388,389 Similarly, particle surfaces that closely mimic the natural context of the antigen, such as liposomes able to mimic cell membranes, have been found to improve activation efficiency.390,391

Amongst the NPs used for synthetic vaccines, ‘hard’ NPs have generally been more widely applied for the surface *presentation* of biomimetic peptide/protein antigens. In contrast, ‘soft’ particles able to *encapsulate* a payload have commonly been utilised for the delivery of antigen following particle recognition and subsequent release.384 AuNPs in particular have found widespread use as NP antigen carriers due to their ease of surface functionalisation, high surface-area, biocompatibility, and tunable size.392 Importantly, recent research suggests that AuNPs may also act as size- and shape-dependent adjuvants, stimulating the immune system and enhancing antigen recognition.393,394 As a result, the attachment of peptide and protein ligands to AuNPs has emerged as an effective means to stimulate the production of Abs against a wide range of pathologies and pathogens, including malaria,395 foot and mouth disease,396,397 the *Yersinia pestis* bacteria responsible for plague,398 cancers via the mucin-1 (MUC-1) glycoprotein,399,400 influenza A virus,401 *Streptococcus pneumoniae*,402 respiratory syncytial virus,403 encephalitis causing viruses,404 and HIV via gp120 derived peptides405.

Both single- and multi-walled carbon nanotubes (CNTs) have also been widely explored as antigen carriers due to their largely biologically inert nature, facile surface modification, and ability to induce cell penetration. However, recent reports on their toxicity may limit further development towards applications in patients.406 Since being first introduced by Pantarotto *et al*,407 peptide- and protein-modified CNTs have been utilised to generate immune responses against a range of antigens including tumour lysate,408 *Plasmodium vivax* apical membrane antigen-1 derived peptide,409 *Mycobacterium tuberculosis* protein derivatives, 410 Wilm’s tumour antigen,411 and foot and mouth virus derived antigens412. ‘Hard’ NPs

such as polystyrene nano-beads,413,414 polyacrylate dendrimers,415 and calcium phosphate particles416,417 have all also been shown to enable Ab generation *in vivo*. The Baneyx group have recently reported that a calcium phosphate binding peptide epitope can be attached to the termini of the desired antigen, inducing biomineralization and particle assembly at a late stage of the formulation process.416,417 By doing so, the low stability of calcium phosphate particles can be mitigated, allowing 'on-demand' production and application to take place.

Although 'soft' organic NPs have been more rarely used for the formulation of vaccines, a number of examples exist and in many cases they reveal interesting facets of vaccine design that should be carefully considered, regardless of the particle type. For example, liposome formulations have been commonly utilised for the encapsulation of peptide or protein antigens, enabling delivery to immune cells typically following targeted delivery. Virosomes, liposomes functionalised with virus components (often influenza virus derived) able to mimic viral envelopes, have been particularly widely used as safe, self-adjuvanted, and stable antigen delivery vehicles.418,419 However, Guan *et al* have demonstrated that the surface-presentation of ligands within liposome formulations is able to mediate activation of alternative branches of the immune system *in vivo*, when compared to encapsulated antigen.420 Careful NP design is therefore key in ensuring that the desired response is triggered. Similarly, peptide-lipid amphiphiles have also been studied, as discussed in section 8, for their ability to self-assemble into peptide-decorated micelles. The use of peptides as a key structural component results in an extremely high density of surface antigen coverage, enhancing recognition and activation efficiency.421 Importantly, the crowded environment provided by such a set-up has been found to induce peptides to adopt a secondary structure more akin to their natural presentation within the parent protein.415,422 As a result, the Abs generated downstream are better able to produce an effective response when subsequently challenged.

While applications using peptide- and protein-NP conjugates as vaccines to stimulate a protective immune response to previously unencountered antigens have been most widely studied, technologies which modulate the immune system, and in particular mitigate auto-immunity, have begun to emerge. In a healthy individual, tolerance of self-antigens is maintained by the activity of regulatory T cells (Tregs). Modulation of Treg activity and addressing deficiencies is thus an attractive target for the treatment of autoimmune diseases.423 Tsai *et al* demonstrated that the presentation of recombinant major histocompatibility complexes bearing type 1-diabetes associated peptides on the surface of iron oxide NPs resulted in the *in vivo* expansion of Tregs, and ultimately the restoration of normoglycemia in diabetic mice.424 Furthermore, the co-delivery of myelin peptides, known to be targeted by auto-immune responses in patients suffering from multiple sclerosis, with tolerogenic small molecules that stimulate Treg proliferation on the surface of AuNPs, has subsequently been shown to suppress symptoms within mouse models of the disease.425 More recently, Hess *et al* utilised QDs as a scaffold for the display of myelin peptides allowing the authors to monitor the distribution and activated pathways of the NP-peptide complexes *in vivo*.426

8 Playing a structural role

In much of this review, we have focussed on systems in which the peptide or protein defines or modulates NP function or performance. In addition to these roles, peptide/proteins have emerged as key structural motifs, which are integral to the formation as well as eventual end application of the NP. Indeed, in many of the scenarios to be discussed in this section the particle is composed entirely of peptide or protein components.

Peptide self-assembly into complex nano-architectures can be instigated by a combination of intra- and inter-molecular non-covalent interactions, including hydrogen bonding, electrostatic or hydrophobic interactions, and π-π stacking.427 Although individually these interactions may be weak, cumulatively they are able to define the secondary and tertiary structures of complex native protein architectures, and can be exploited to produce a vast array of self-assembled structures on both the nano- and micro-scale. The formation of architectures, ranging from fibres and tubes, through to vesicles and micelles, and on to more elaborate structures such as crystals and donuts can all be instigated.427–431 While the fundamental driving forces behind the formation of a particular architecture are becoming increasingly well understood, in many cases the route of assembly remains a dynamic process which can be affected by seemingly minor modifications of peptide primary structure or growth conditions.432–436

The formation of self-assembled peptide architectures has found widespread application across the biomedical field, and further afield in the wider materials research community. Here, we will focus on the formation of 3D spherical NPs, particularly peptide/protein micelles and vesicles. The conditions for the formation of these structures are often strict, requiring precise control over composition, assembly conditions, and handling. For an in depth overview of the wider field, and in particular the use of self-assembling peptides in the formation of 2D nano-fibres, hydrogels, and nanotubes, the reader is referred to excellent recent reviews on the subject.427,429,430,437–440 It should be noted that we will not cover nanosized aggregates of globular proteins in this review, such as those formed by serum albumins or gelatin, which have found increasing clinical use in recent years. Such systems do not rely on the specific self-assembly of polypeptide components, and thus fall outside the scope of this review. A number of reviews focussed on these topics have recently been published.441–444

8.1 Dipeptides

Dipeptides represent the simplest self-assembling peptide motif. Since the breakthrough discovery by Reches and Gazit that di-phenylalanine assembled into nanotubes at high concentrations,445 a wide-range of nano-architectures have been reported, making use of both natural and non-natural amino acids.446 Often, the presence of a di-aromatic motif is vital, providing the driving force for assembly via π-π stacking. The ease with which dipeptides can be accessed synthetically makes them particularly attractive structures for biomedical applications, with researchers from diverse backgrounds able to exploit their use.

The first dipeptide shown to form spherical particles was the unnatural structure di-phenyl*glycine*, in stark contrast to the nanotubes formed by the closely related di-

phenyl*alanine*.447 The significance of seemingly minor differences and subtle changes in structure highlight the surprising versatility of such simple structures. The situation is further complicated by the dynamic nature of dipeptide nanostructures, which have been observed to result in reversible transitions between architectures in response to stimuli or incubation conditions.446,448,449 Despite this apparent plasticity, dipeptide NPs exhibit remarkable stability. Indeed, di-phenylglycine particles were shown to be stable to both acid and base treatment with no observed change in particle number.447

Despite the simplicity of dipeptides assemblies, their application in biomedicine has been limited to just a few reports. In an early example, Alam *et al* demonstrated that H_2N-methionine-dehydrophenylalanine-CO_2H NPs could be loaded with the anti-cancer drug curcumin and used to induce tumour regression in a mouse melanoma model.450 Importantly the unnatural amino acid dehydrophenylalanine not only increased packing efficiency, and thus enhanced physical stability through increased π-π stacking, but also promoted biological stability by providing protease resistance. More recently, Fan *et al* reported the assembly of H_2N-tryptophan-phenylalanine-CO_2H, to produce fluorescent NPs.451 By combining π-π stacking with peptide-zinc interactions they were able to produce particles with visible light emission, mimicking the red-shifted emission exhibited by fluorescent proteins upon metal-binding. Subsequent modification of the particle surface with a MUC-1 binding aptamer enabled biocomptability to be enhanced when compared to other fluorescent NPs such as quantum dots.451 Although this promising system has only so far been demonstrated *in vitro*, it offers an attractive means through which to produce simple, photo-stable, biocompatible NPs for *in vivo* imaging in the future.

8.2 Peptide amphiphiles

Amphiphilic peptides, containing both a hydrophobic and hydrophilic domain, can be broadly split into three categories: i) Those composed only of native amino acids (with or without minor modifications at the termini); ii) Those containing unnatural amino acids; and iii) Lipid- or polymer-conjugated peptide hybrids. In all three groups, self-assembly into spherical particles is typically driven by a mixture of hydrophobic and electrostatic interactions, with the formation of secondary structures possible in some cases.427,431

The formation of nano-vesicles composed entirely of native amino acids, referred to in some instances as ‘peptosomes’, was first reported by Vauthey *et al*.452 1 or 2 C-terminal aspartic acid residues, bearing 2 or 3 negative charges respectively, were found to be sufficient to drive the assembly of an N-capped hydrophobic peptide chain of 6 alanine, valine, or leucine residues. Dynamic heterogenous mixtures of nano-tubes, vesicles, and micelles were observed, depending on the exact peptide sequence. Subsequent reports on the formation of vesicles from glycine-tail anionic peptides453 or cationic peptides bearing lysine or histidine termini432,433 validated this approach, with the hydrophobic ‘tail’ and charged ‘head’ group in effect mimicking the structure of lipid surfactants. Interestingly, it has recently been reported that rather than forming tail-to-tail arrangements analogous to those observed in lipid membranes, the hydrophobic regions of amphiphilic peptides form interdigitated β-strand-like assemblies, leading to greatly reduced membrane thickness.454

Despite these important fundamental studies on the ability of short amphiphilic native-peptides to form vesicles, biomedical applications have to date been limited by the relative instability of self-assembled constructs. Linear peptides typically exhibit high critical aggregation concentrations (CACs) in aqueous solution, below which particle formation does not occur, and a dynamic equilibrium with free peptide therefore often exists. This situation is further complicated in complex biological fluid.433–435 A number of different approaches have been taken to address this issue, yet often CACs or dissociation constants are not reported, obscuring the analysis of particle stability. Gudlar *et al* showed that branched peptides derived from natural transmembrane helices can enhance vesicle stability as a result of enhanced hydrophobic interactions.455 These branched structure were found to be a key driving force for the preferential formation of vesicles over fibres, closely mimicking the di-hydrophobic tail of native lipids and enabling the delivery of cargoes to cells *in vitro*. In order to stabilise vesicles post-formation, van Hell *et al* incorporated cysteine residues into the primary structure of the amphiphilic peptide SA2.456,457 SA2 is a rationally-designed amphiphilic sequence containing sequential hydrophobic residues of decreasing bulk, leading to a cone-shaped monomer that promotes the formation of spherical architectures, with a CAC of 0.5 μM prior to crosslinking.434 The formation of interchain disulphide linkages between adjacent cysteines led to the production of stable vesicles, and enabled cellular delivery of encapsulated photosensitizers.457

An alternative approach to stabilise self-assembled peptide architectures is to incorporate non-native functionalities. In its simplest form, this can involve the incorporation of unnatural amino acids, as reported by Tanisaka *et al*, providing increased hydrophobic interactions as well as resistance to proteases *in vivo*.458 Stable vesicles composed of hydrophilic sarcosine and hydrophobic methyl glutamate residues were shown to accumulate in cancer tissue as a result of the EPR effect in animal xenografts. Taking this concept further, the formation of lipid- or polymer-hybrid peptides has become an attractive means by which to drive peptide self-assembly.459,460 Liu *et al* demonstrated that addition of cholesterol to the end of a hydrophilic hexaarginine-TAT peptide block drove micelle formation (CAC = 10.1 μM).193 These structures were able to preferentially disrupt bacterial cell membranes and thus act as antimicrobial agents *in vivo*, though it is important to note that host haemolysis is a likely side-effect at higher concentrations. Similarly, Lv *et al* have shown that triblock PEG-polyphenylalanine-polyglutamic acid polymer-peptide hybrids are able to form stable stealth nanoparticles, for the delivery of encapsulated doxorubicin to tumours (CAC = 2.6 μM).461 Upon glutamic acid protonation in the increasingly acidic environment of maturing endosomes, disassembly and subsequent cargo release led to tumour apoptosis.

8.3 Dendrimers

As an extension to the use of linear peptide amphiphiles, higher order, repetitively branched peptide dendrimers with increased bulk and a unique globular architecture have also found utility. On their own, dendrimers do not typically interact sufficiently to form stable self-assembled structures. In a series of papers the Gu group have developed an elegant solution to this barrier, through the use of a cooperative self-assembly process. Hydrophilic polylysine dendrimers are first electrostatically bound to a linear hydrophobic peptide

bearing a negatively charged C-terminal glutamate residue. This amphiphilic, supramolecular dendrimer can then further self-assemble into nano-sized micelles, capable of encapsulating a hydrophobic drug or DNA cargo.462 Under weakly acidic endosomal conditions following uptake, glutamate protonation leads to disassembly of the nanostructure, and release of the cargo.463 These dendritic systems have been used to successfully deliver the anti-tumour drug doxorubicin *in vivo* through passive targeting,464 and more recently to allow directed delivery by exploiting the modularity of dendrimer assembly to enable surface functionalisation.465

8.4 Coiled-coil peptides

Along with β-sheets, α-helices are the most common secondary structures adopted by polypeptides. Hydrogen bonding between the backbone amide oxygens and the N-H bond 4 residues away induces a right-handed helical structure in which the amino acid sidechains are directed away from the core. Further stabilisation can be achieved through the subsequent assembly of multiple peptides to form coiled-coil motifs, whereby multiple helices wrap around each other into a superhelical bundle.466 Assembly is driven first by the exclusion of hydrophobic residues form the aqueous exterior, and then stabilised and enhanced by inter-strand electrostatic interactions between polar and charged amino acids.467 The resultant assemblies offer a powerful means to produce complex bioactive materials that have found use across a range of disciplines.466,468–470

Coiled-coil peptides were first used in the nanotechnology field by Stevens *et al* to drive the assembly of AuNPs into higher order aggregates.471 The formation and application of NPs composed entirely of coiled-coil peptide structures was subsequently driven by the group of Burkhard. In 2006 they reported the rational structure-based design of a linear peptide, composed of the pentameric repeat forming coiled-coil domain of cartilage oligomeric matrix protein (COMP)472, and a *de novo* designed peptide that formed a self-assembled trimer. This peptide was able to undergo self-assembly into regular, polyhedral NPs, and display multiple copies of a bioactive species on the particle surface.473 As described in section 7, such a display is capable of effectively mimicking pathogen antigen presentation. Coiled-coil NPs have subsequently therefore been demonstrated to act as novel immunogens for the production of vaccines, for diseases including severe acute respiratory syndrome (SARS),474 malaria,475 and HIV476,477 (Figure 20). Further developments which enhance NP stability, including the addition of lipid tails,477,478 and the formation of elastin-like peptide hybrids (as discussed below),327 have additionally been reported, bringing CACs into the low nM range. Importantly, the coiled-coil core has been shown to only be weakly immunogenic itself. As a result, Abs generated against these NPs are predominantly targeted towards the displayed antigen, though low titres against the particle have also reportedly been generated.476,478,479

8.5 Peptide-nucleic acid complexes

The non-viral delivery of DNA or RNA to cells is a powerful emerging technique for the treatment of disease. A number of treatments based around the delivery of plasmid DNA, or antisense, silencing, or micro RNAs are currently in the clinic, with an even greater number undergoing advanced clinical trials.480–483 Positively-charged peptides, able to self-

assemble into compact NPs with negatively charged nucleic acids, are particularly attractive. By exploiting the research discussed throughout this review, peptide vectors have been shown to protect the DNA/RNA cargo from damage, enable targeting of the desired cell type, and perhaps most importantly facilitate intracellular delivery and endosomal escape, as described in section 5.484 Indeed, the same cationic residues that enable NP formation, such as lysine and arginine, are also those able to promote cell penetration.

Wu and Wu were amongst the first to report peptide-nucleic acid NP mediated gene delivery. Complexes of plasmid DNA and polylysine were shown to efficiently deliver their cargo to cells *in vitro*.485,486 Many subsequent reports have focussed on the ability of peptide-nucleic acid complexes to transfect isolated cells, rather than being applied *in vivo*. This is in part due to the trade-off between the toxicity associated with longer cationic peptides, and the low particle stability when shorter sequences are used instead.487 Stabilisation through both dialdehyde crosslinking487 and disulfide formation488,489 has been reported, however the most common means of enabling *in vivo* application is the use of more complex peptide substrates capable of lowering CACs sufficiently to overcome these problems. In an early example, Rittner *et al* replaced acidic residues in the amphiphilic cell-penetrating peptide JTS1 with cationic lysines or arginines, producing a sequence able to both condense DNA and promote efficient systemic transfection *in vivo*.490 Other peptides, such as MPG,491 RALA (an arginine-rich analogue of the more commonly utilised endosomal escape peptide GALA),492 and amphiphilic arginine-containing triblock peptides493 have all found use for the *in vivo* delivery of nucleic acid cargos. More recently, the Kaplan494 and Zheng495 groups have reported the targeted delivery of peptide-nucleic acid NPs, via the introduction of a tumour-specific homing peptide (Figure 21). In both cases, preferential accumulation and subsequent nucleic acid delivery in the tumour tissue was achieved following intravenous injection. This was observed to prevent the potential side-effects that can occur as a result of systemic transfection.

8.6 Elastin-like polypeptides

Elastin-like polypeptides (ELPs) are biosynthesised polymeric-peptide repeats, typically of the pentameric sequence $(VPG\underline{X}G)_n$, where X and n can be varied to ultimately determine the properties of a particular ELP construct. All ELPs possess an ‘inverse transition temperature’ (T_t), above which they undergo a sharp phase transition from a highly solvated peptide monomer to a desolvated aggregate.496,497 The exact properties are determined by the so-called ‘guest-residue’, with hydrophobic residues leading to a decrease in T_t, and conversely, hydrophilic residues leading to a corresponding increase.

The aggregation of ELPs can be exploited to form spherical, nano-sized cargo-delivery vehicles via a number of different strategies. The simplest realisation of such technologies relies on the passive accumulation of soluble ELP monomers within tumours via the EPR effect. Through suitable design of the sequence, T_t can be modulated to be slightly higher than body temperature. Peptide self-assembly can then be driven at the site of interest through the application of localised hyperthermia.498–500 During this process, NP assemblies are first formed which are able to promote cellular uptake, followed by the subsequent formation of larger aggregates which lead to retention at the site of heating.500

Importantly, such strategies require a precise control over the concentration of systemically-injected ELPs - too high, and off-target aggregation can result, too low, and no assembly will be observed upon heating.501 In spite of this sensitivity, the hyperthermia induced assembly of ELP NPs can still be used enhance the activity of attached drugs such as doxorubicin, promoting both retention and uptake at the site of heat application.502

The use of amphiphilic ELP structures that can form stable micelles above the T_t has more recently been reported, bringing CACs into the high nM-low μM range. In an early example, Dreher *et al* demonstrated that the attachment of hydrophobic doxorubicin (through an endosome cleavable hydrazone bond) could itself drive micelle formation.503 MacKay *et al* subsequently showed that by tuning T_t, stable structures with low CACs could be generated and used to effectively treat tumours *in vivo* (Figure 22).504,505 As an alternative, the formation of diblock ELP polymer can be used to drive assembly, with each block possessing a unique T_t. When the temperature is above the T_t of the first block but beneath that of the second, micelle formation will occur, which can be further stabilised by the introduction of disulphide crosslinkers.506

ELP-based NPs have been applied in a number of applications in recent years. In addition to early reports on drug-delivery, ELPs have subsequently been utilised as NIR fluorescent imaging agents,507 microPET contrast agents,508 and synthetic vaccines509. Furthermore, the attachment of multi-valent tumour targeting sequences,510 cell-penetrating peptides, 511,512 and pH-responsive ELP cores513 have all been seen to enhance the applicability of ELP-nanostructures in biomedicine.

8.7 Casein micelles

Casein is the collective term for a family of phosphorylated proteins commonly found in milk. These proteins have a well-defined, hydrophilic N-terminal domain, and a hydrophobic C-terminal domain, creating a structure that can in essence be viewed as an amphiphilic diblock copolymer.514 Unlike other proteins which may form globular aggregates, caseins are able to undergo controlled self-assembly to form stable micelle-like structures in aqueous solution with a CAC of 20-80 μM.515 This is particularly true, when individual casein family members, most notably β-casein, are used in isolation rather than as the naturally occurring mixture.515

Casein micelles have recently been the subject of increasing interest, as naturally occurring nano-delivery vehicles for a range of hydrophobic cargoes. The use of casein NPs to improve the bioavailability of supplemented vitamin D, prior to release following protease action in the stomach, has been the subject of human clinical trials.516 Similarly, the delivery of hydrophobic therapeutics, particularly for the treatment of stomach cancers, has been widely studied by the Livney group.515,517–519 Finally, the tumour accumulation of intravenously injected, cisplatin containing casein-micelles has been shown to result in improvements in therapeutic outcome *in vivo* (Figure 23).520 These promising early reports, coupled to the ease of use, inexpensive production, bio-degradable, non-toxic and non-immunogenic nature of casein micelles makes them particularly appealing as delivery-vehicles. It is likely that the coming years will see such structures finding increasing utility in biomedicine.

9 Sensing analytes and biomarkers

NP biosensing complexes offer a powerful means by which to detect and monitor disease, and to understand pathological conditions. This is usually achieved using optically active NPs, though other sensing modalities can also be utilised.33 The most common sensing platforms rely on a change in optical properties in the presence of the desired analyte or biomarker, or selective binding of a NP complex within a ‘detection-region’.521,522 Peptides and proteins are vital components of such systems, inducing NP binding or mediating a responsive output to the presence of the desired analyte. NP biosensors are most widely utilised for the *ex vivo* analysis of biofluids, as epitomised by the use of anti-human chorionic gonadotropin (HCG) antibody coated AuNPs in commercially available lateral flow pregnancy tests.523 The development of NP-based biosensors has become a vast field of research, and a detailed overview of *ex vivo* technologies is outside the scope of this review. The reader is instead referred to a number of excellent comprehensive reviews on the topic for further details.33,524–527 Instead, we will here focus on the far smaller body of literature that focusses on the application of NP-biosensors *in vivo*, and the challenges that have so far limited their widespread implementation. Such systems allow the true complexity of tissues to be captured in a way that *in vitro* testing of biofluids cannot provide.528

Sensing complexes provide a means to interrogate biological systems and probe differences in activity and temporal distributions of a desired analyte, as opposed to the spatial localisation provided by imaging modalities. Rather than being detected within a specific area, *in vivo* sensors offer a responsive platform to monitor small molecules, biomacromolecules, and diseased states in a continuous manner.528,529 Although implanted electrochemical sensors have been widely described, in particular for the monitoring of blood glucose levels, problems with induced fibrosis and foreign body responses can limit sensitivity and accuracy.528 NP based systems offer a viable alternative, but also have their own complications – in addition to the challenges of generating a signal that can be actively transduced for detection, NPs must overcome background signal generation within the complex environment of the body, be retained at the desired site of detection, and ideally provide a reversible and dynamic response. The use of peptide/protein-NP conjugates in particular must address the sensitivity of biological components to degradation, clearance, and unfavourable interactions.529 As a result, systems in which peptides and proteins play an active sensing role, rather than mediating imaging or targeting, have only recently begun to emerge.

In one realisation of such technologies, environmentally sensitive polymer NPs have been exploited to detect differences in analyte concentration as a result of conjugated enzyme activity. More specifically, polymer NPs incorporating platinum based fluorescent sensors have been shown to display phosphorescence dependent on environmental oxygen concentrations. Cash and Clark demonstrated that by conjugation of the histamine-metabolising, oxygen-consuming enzyme diamine oxidase to the NP surface, biologically relevant levels of this key inflammatory- and neuro-modulator could be detected by a change in phosphorence.530 This response was reversible upon diffusion of oxygen back into the biological milieu, with only a limited dropoff in polymer dynamic range observed upon

repeated applications. Similarly, Sun *et al* monitored glucose levels *in vivo* using a glucose oxidase-functionalised oxygen-polymer NP transducer.531 Subcutaneous implantation enabled retention of the particles for up to a month, enabling reproducible signal generation over this extended period.

As an alternative, the Bhatia group has pioneered the use of protease-sensitive NP coatings, which release peptide cleavage products into the urine upon enzymatic activity *in vivo*. In their original report, Kwong *et al* conjugated peptides sensitive to a range of common proteases onto the surface of iron oxide nanoworms.532 Due to their size, these particles were blocked from renal clearance and displayed accumulation in the liver. However, following protease activity, the peptide cleavage products were excreted in the urine, and could subsequently be detected by mass spectrometry, giving a panel-readout of *in vivo* protease levels. Subsequent iterations of this technology have demonstrated the detection of thrombosis and colorectal cancer using peptides terminated with recognition elements that can subsequently be detected in urine by enzyme-linked immunosorbent assays, or point-of-care lateral flow detection systems.533,534 In order to overcome the challenges presented by non-specific activation by circulating proteases, Dudani *et al* reported the use of photo-protected peptide-NP coatings which could be activated towards protease sensitivity with spatial and temporal control, following the application of UV or two-photon light.535 Furthermore, the sub-cutaneous implantation of 8 nm peptide-functionalised PEG-NPs, which enable gradual leaching of a particle reservoir into the blood stream, has recently been shown to allow the continuous monitoring of protease activity *in vivo*, although detection was still limited to a 24 hr time period.535

10 Outlook and conclusions

The past 20 years have seen a rapid increase in the development of nanotechnologies, and the exploitation of polypeptides in this context has also found increasing favour. The structural and functional versatility of peptides and proteins allows them to play an important role in modulating, instigating, and defining the activity of NP constructs. In this review we have outlined the key roles played by polypeptide coatings and structural components, and demonstrated how they can be utilised to improve the efficacy of NP tools in biomedical applications. In many cases, both peptides and proteins can fulfil the desired function. Each offers important advantages over the other (though exceptions to these generalisations exist) that should be carefully considered during NP-conjugate design (Figure 24). For example, the ease with which peptides can be produced via solid-phase peptide synthesis (SPPS) is highly beneficial. As well as offering cost-effective, quick, and scalable production, the versatility of SPPS enables unnatural chemical space to be explored. As such, the facile introduction of reactive handles and protease resistant residues, or structures able to promote biological interactions, is enabled (Figure 24b and c). Furthermore, the small size of peptides enables the disruption of NP hydrodynamic diameter and detrimental effects on activity to be minimised, while creating a dense, accessible, and flexible coverage of active sequences (Figure 24a and d). In contrast, the increased structural complexity offered by proteins enables NP-conjugates to typically attain increased activity and recognition, albeit potentially at the cost of decreased biological stability and increased recognition by the reticuloendothelial system (Figure 24e). In addition, small structural

modifications to proteins are usually well tolerated, without leading to major disruptions in activity. Corresponding changes in peptide sequence have a stronger influence on conformation and activity, potentially leading to a significant drop in the efficacy of the nanotechnology (Figure 24g). The situation is further complicated by the complex interplay between peptide/protein components and the particular NP core, cargo, application, and delivery route. As such, consideration of the precise polypeptide component is a vital step during NP-conjugate design.

Despite the advances that have been made in enabling effective and targeted delivery to the desired site of action, providing an integral means to maintain activity, and acting to hijack and exploit native biological pathways, there remains significant scope for the improvement of polypeptide-NP technologies. In a recent editorial, Leroux highlighted that while the number of papers reporting increasingly creative and complex NP systems increases exponentially, their therapeutic translation remains disappointing, highlighting the challenges faced by the nanotechnology field.536 Within the realm of peptide/protein-NP conjugates, we believe that addressing the following issues is vital to enable translation of these naïve systems to a biomedical setting:

i) The use of native amino acids brings with it the threat of protease sensitivity, and activity can often be rapidly lost in the complex environments faced *in vivo*. In this review, a number of approaches have been introduced towards addressing these difficulties. Unnatural amino acids are particularly effective, providing peptide bonds which are not recognised and processed by native enzymes. This is most easily achieved with synthetic peptides, as discussed above - relatively simple modifications such as N-methylation, enantio- or retro-enantio amino acids, or extended β- or γ- linkages can be introduced.217,537,538 While increased stability is harder to achieve with protein substrates, the advent of technologies which allow the introduction of non-natural stabilising residues,539,540 the addition of protective polymer coatings,541,542 or the formation of additional cross-links543,544 are now allowing such issues to be addressed. In order to fully facilitate the translation of NP-based systems, it is important that the research community begins to actively embrace such technologies.

ii) The small size of peptides allows them to form compact NP coatings, which lead to minimal disruption of desired function. They are therefore often the preferred choice when designing a NP-polypeptide conjugate, with the major drawback being reduced bioactivity (Figure 24a and e). Nature has had millions of years to slowly evolve proteins with complex 3D structures and potent activity, and it remains challenging for designed, screened, or derived peptides to fully recapitulate this. Nowhere is this more strongly felt than in the reduced ability of short synthetic peptides (< 30 amino acids) to target and bind a particular protein or cell type when compared to Abs or other binding proteins. Although strongly binding long peptides such as affibodies (> 50 amino acids) can be accessed synthetically, more typically they are produced recombinantly and still result in a bulky coating on the NP surface.545 There is a therefore a pressing need for technologies which can bring short peptide binding affinities down from the μM to the nM range.546 Recent advances in the screening, design, and exploitation of bicyclic peptides are enabling such levels to be

reached, and will surely be exploited in the near future to enhance the ability of NP-targeting strategies to achieve their goals.547–549

iii) The bioactivity of a peptide or protein-NP conjugate is highly dependent on achieving an appropriate and accessible orientation of the bioactive domain. Again, this can be easier to achieve using peptides rather than protein substrates - tethering residues can be easily introduced at the termini during solid phase peptide synthesis. Often protein substrates are attached either by non-specific conjugation techniques or simple adsorption, leading to a detrimental loss of structure, heterogenous coatings, and steric hindrance of the active site.375 It is essential that recent developments in the field of site-specific or selective protein modification are exploited to overcome these problems, to enable the controlled orientation of proteins on the NP surface. Strategies for genetically or chemically manipulating proteins to introduce and exploit uniquely reactive natural and non-natural amino acids, or to incorporate hexahistidine and other affinity tags, are becoming increasingly widespread and offer powerful tools to the biomedical research community.342,550,551

iv) Many of the applications for peptide/protein-NP conjugates discussed in this review rely on processes that are still poorly understood at a fundamental level. Controversy still exists about the precise mechanisms by which peptides mediate transport across biological barriers,145 coatings and particle components which were previously thought to be biologically inert are becoming increasingly found to induce an immunological response,66 and the design criteria required to produce stable self-assembled polypeptide NPs are still being developed436. In order to provide NP technologies that meet the challenging demands of biomedical applications it is important that these topics, and many others within the field, continue to be investigated and that a deeper understanding of the intertwined complexities that determine NP fate is provided.

v) Proper and standardised characterisation of NP-peptide/protein conjugates is essential to not only allow progression in the field, but to enable regulatory and clinical standards to be met.172 This challenge must be addressed by both the development of new tools which are able to reflect the true nature of NP complexes, as well as increased uptake by researchers of existing technologies, and a more thorough approach to NP characterisation being taken.552,553 At present, many systems reported in the literature are inadequately characterised, leading to large discrepancies in results, conflicting reports, and incorrect interpretations that subsequently propagate throughout the field. The inherent complexity of NP-bioconjugates makes them undoubtedly challenging to study – even what appears a simple measurement such as the concentration of particles in solution is often difficult.172 This becomes even more significant for more complex calculations such as the density or orientation of a protein on a NP surface, or the origins of biological effects arising from heterogeneous samples. However, novel tools that can answer these questions are beginning to emerge, and although often highly specialised, are beginning to be readily accessible to researchers, through national and international facilities for NP research and characterisation.553–556

A big leap towards enabling the clinical translation of many peptide/protein-NP conjugate technologies will be taken as the biomedical community addresses these issues. As a result,

our ability to treat, diagnose, and understand disease will be greatly enhanced, and allow nano-technology to fulfil its undoubted promise in the biomedical sciences.

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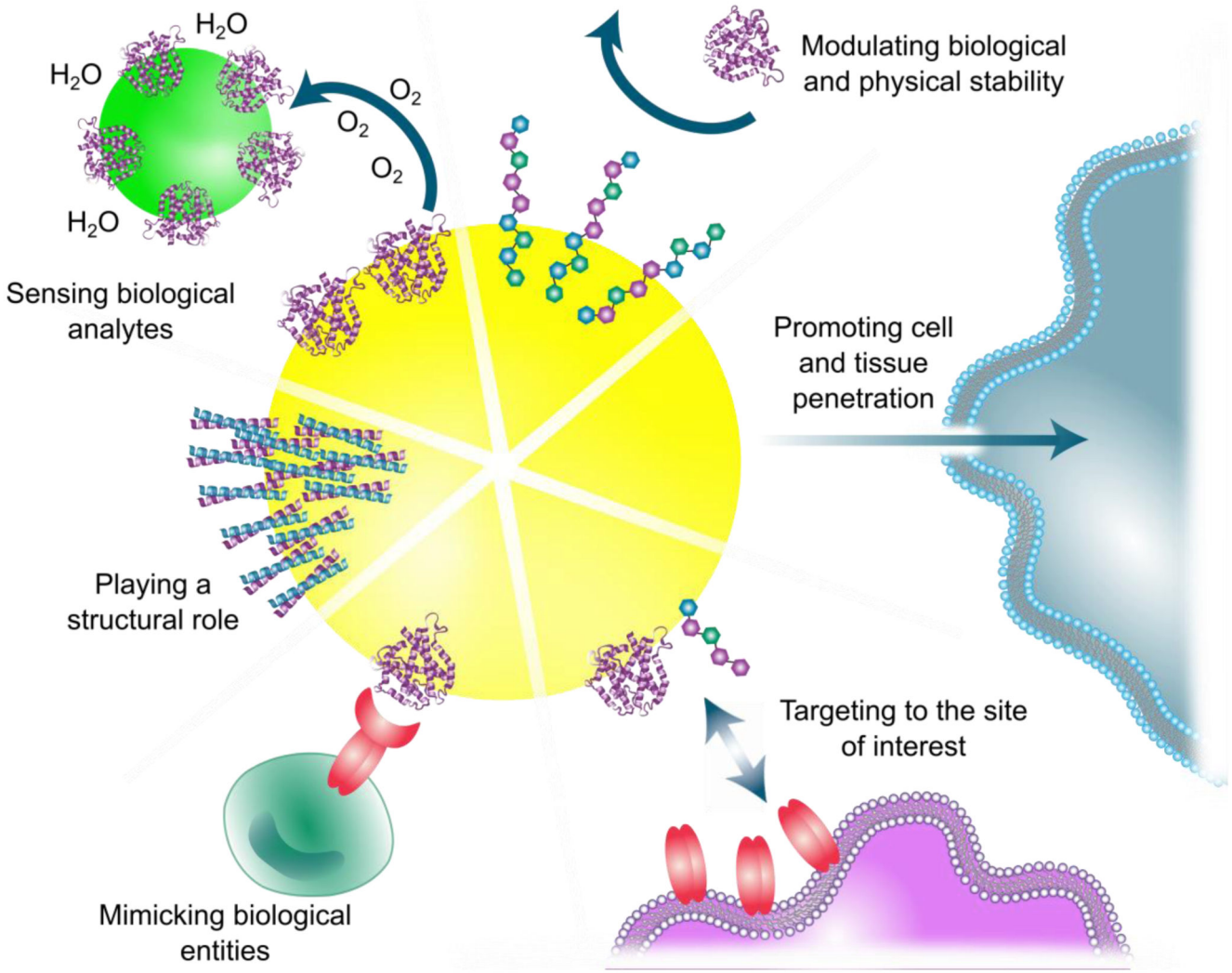

Figure 1.

Polypeptides can play an important role in determining NP functionality and fate. In this review, we will focus on the features imparted by the peptide/protein and their influence on NP behaviour.

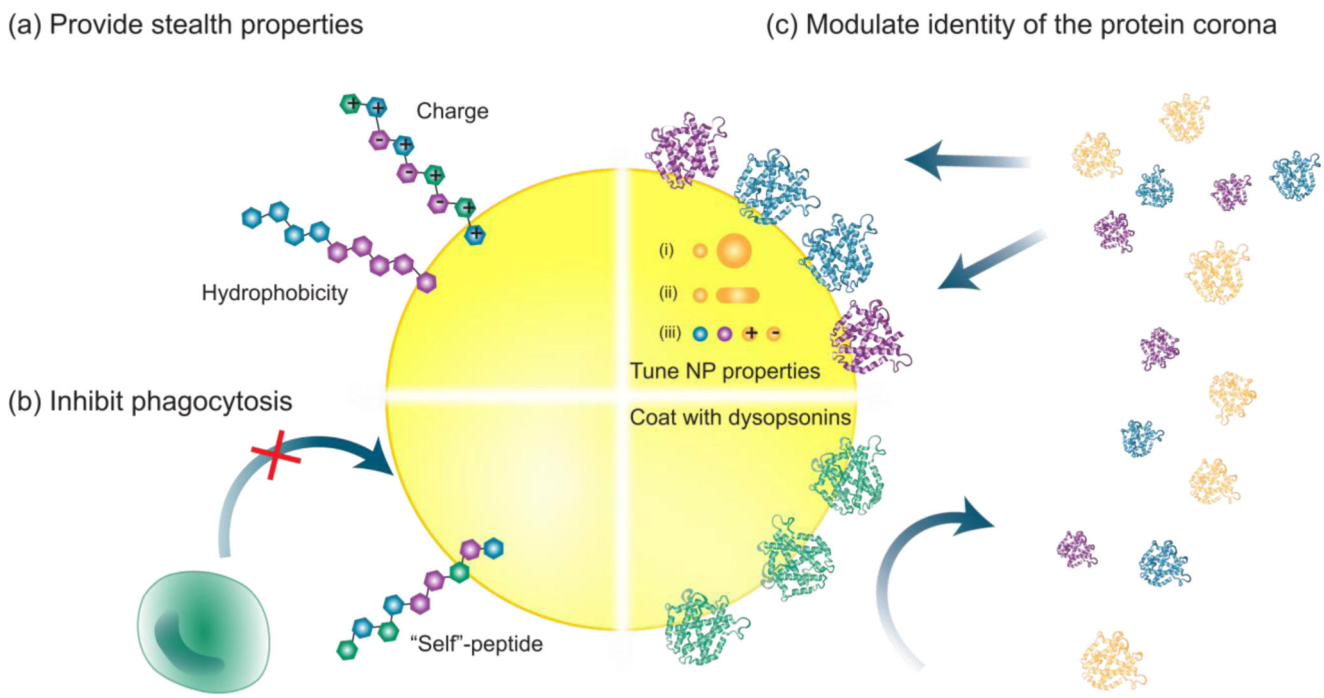

Figure 2.

Methods by which peptide/protein-NP coatings can be designed to influence biological stability and decrease susceptibility to clearance: a) Recognition and clearance can be limited by providing balanced charge or surface hydrophobicity; b) 'Self-peptides' can be recognised by macrophages, and used to inhibit phagocytosis; and c) The formation of a protein corona can be modulated by providing a stable peptide/protein coating, promoting the absorption of dysopsonins, or by tuning NP properties such as size, shape, or charge (i-iii).

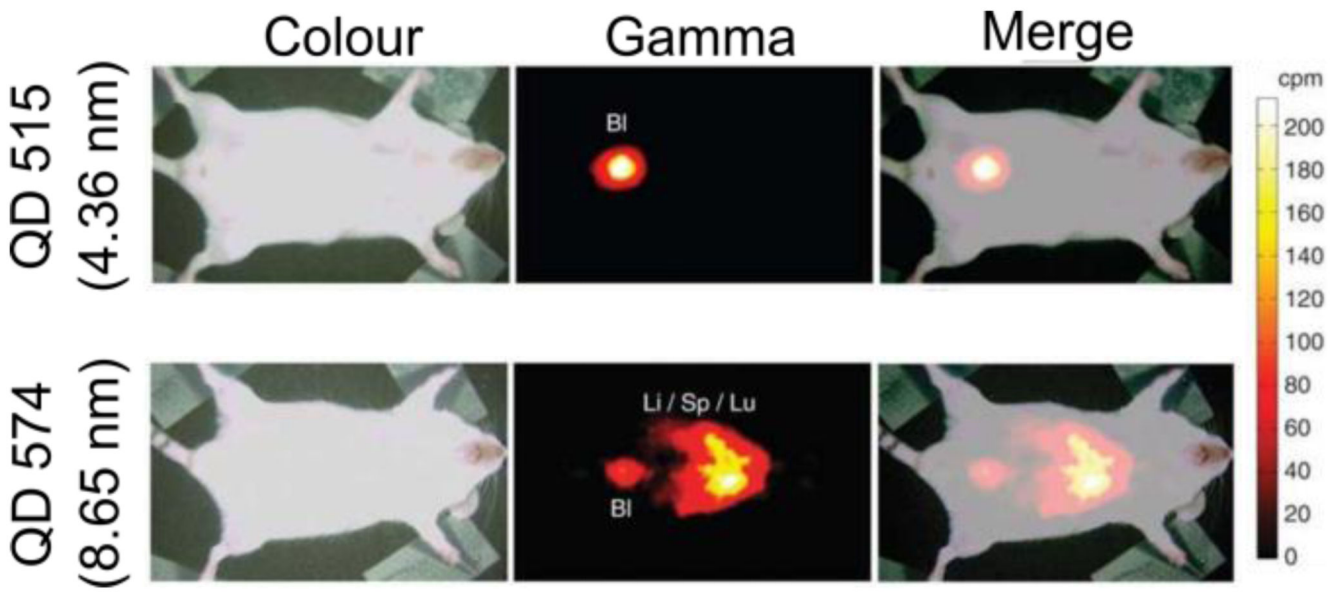

Figure 3.

Retention of different size QDs 4 hrs after intravenous injection. Particles of < 5nm diameter are rapidly cleared to the kidney, while larger particles are retained. Adapted from Soo Choi *et al* with permission from Nature Publishing Group.46

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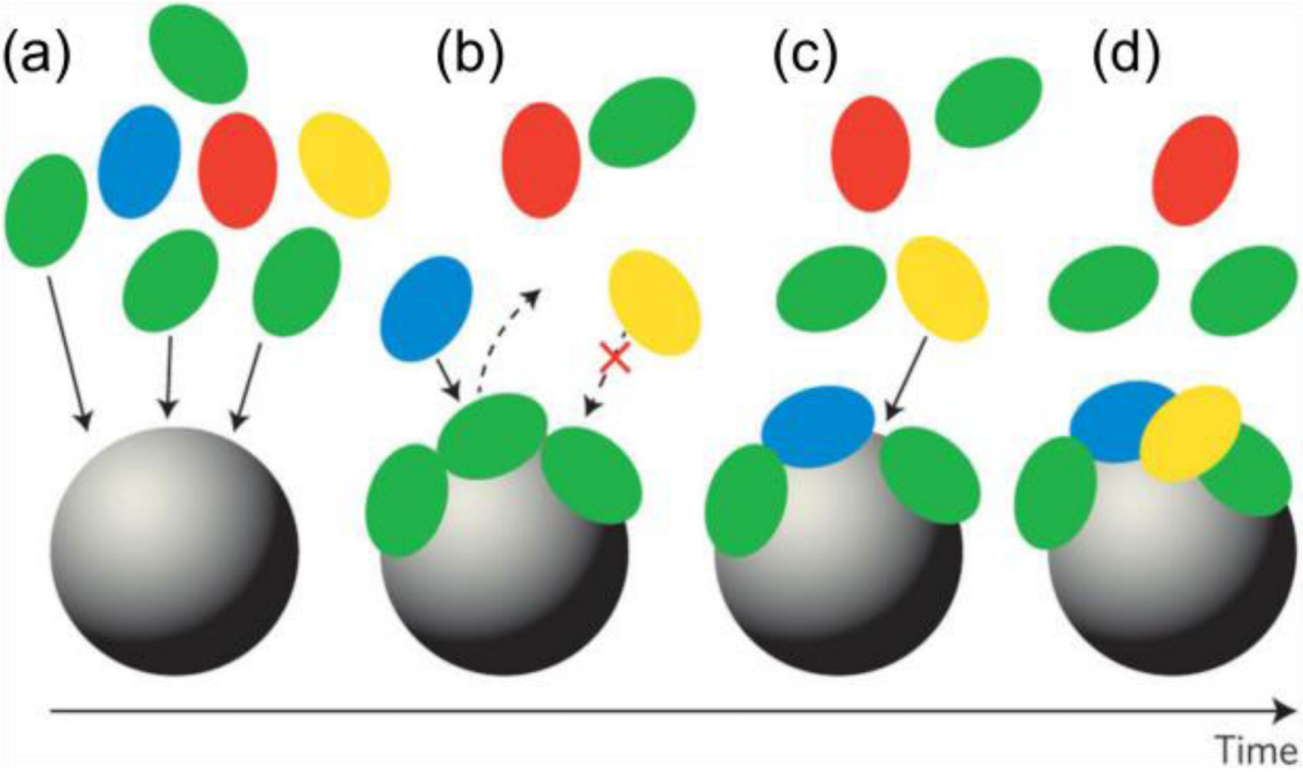

Figure 4.

Upon entering the body, NPs quickly acquire a protein corona. The exact composition of the corona is dynamic and highly dependent on the environment: a) An initial corona is formed of typically highly abundant proteins; b) Weakly bound proteins are gradually removed by proteins with a higher affinity for the NP surface; c) Adsorption of proteins can be dependent on the already existing coating, with inter-protein as well as protein-NP interactions determining affinity; and d) Gradually a stable coating of strongly adsorbed proteins is formed, creating a 'hard' protein corona. Reproduced from Monopoli *et al* with permission from Nature Publishing Group.49

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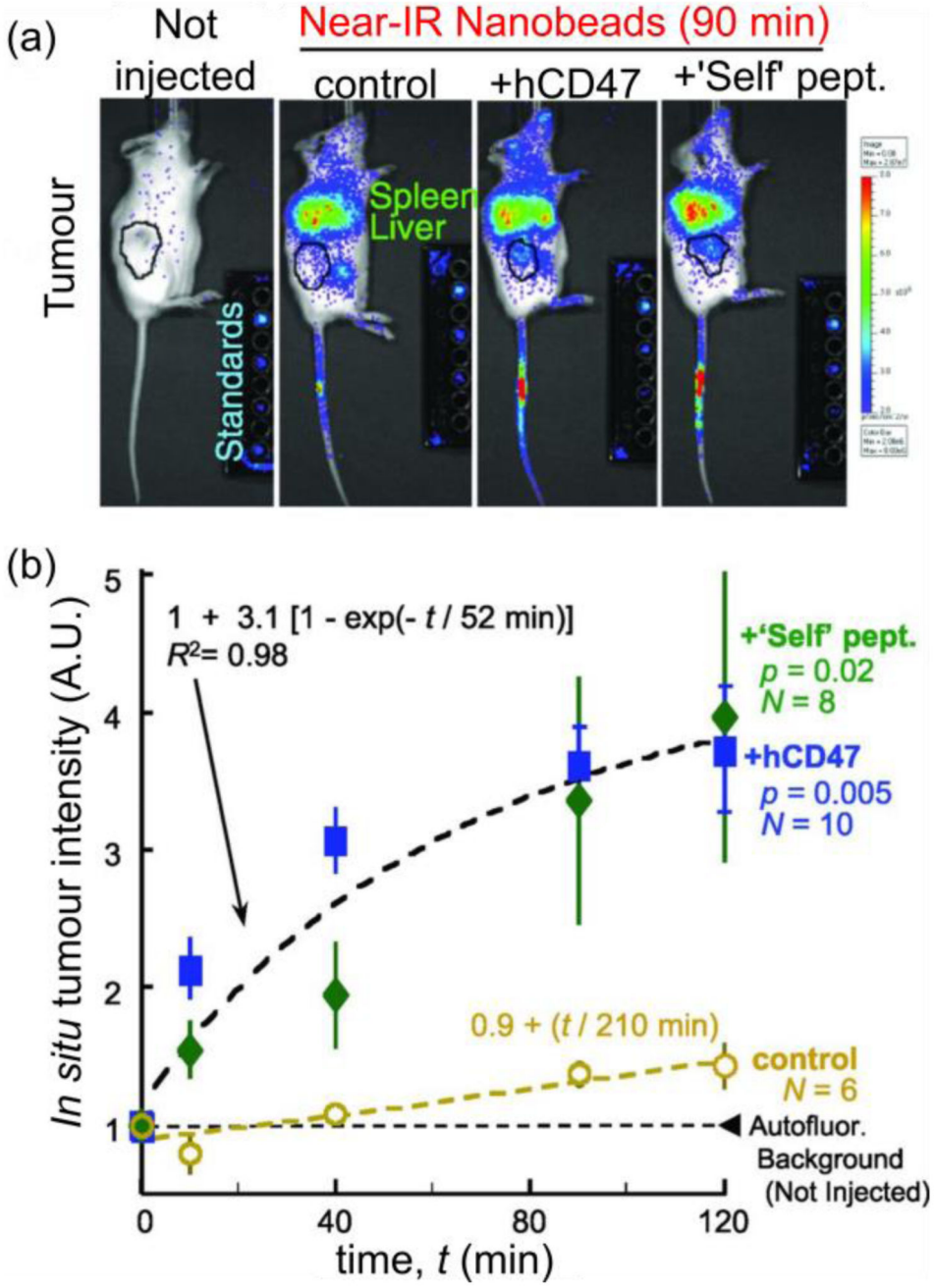

Figure 5.

a) Near-infrared imaging of mice injected with control NPs, or those coated with human CD47 or a CD47-derived peptide. An increase in tumour accumulation is observed as a result of reduced clearance; b) Quantification of tumour fluorescence intensity. Adapted from Rodriguez *et al* with permission from The American Association for the Advancement of Science.78

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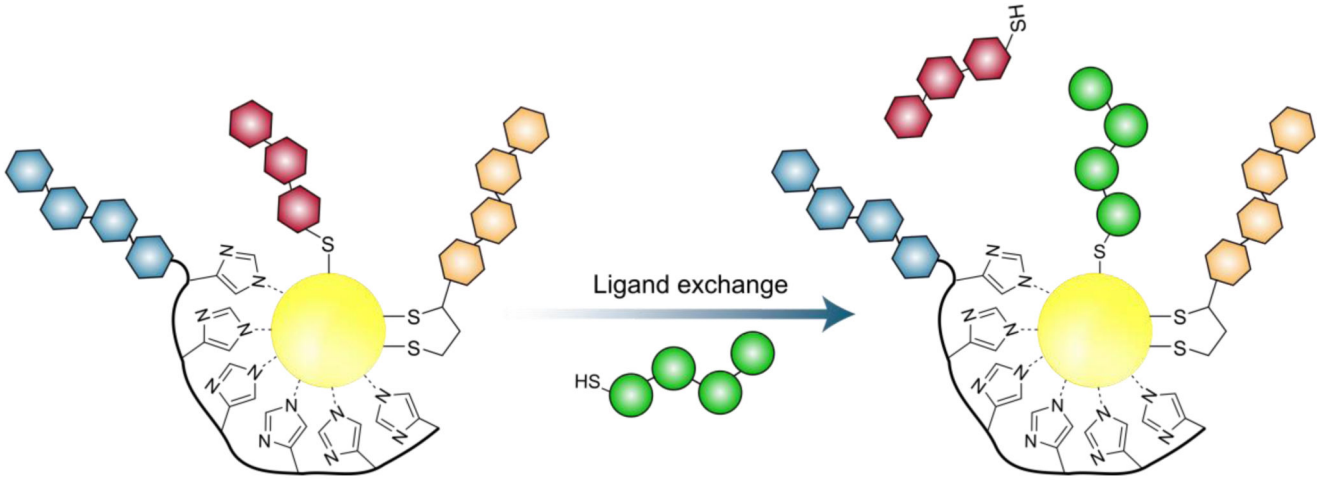

Figure 6.

Monothiol ligands are prone to ligand exchange on the surface of metal NPs, and coatings can therefore be rapidly lost in biological environments. In contrast, bidentate dithiol ligands offer increased stability, as two simultaneous exchange events are required in order to disrupt the coating. Alternatively, hexahistidine ligands offer strong and stable metal binding.121,125

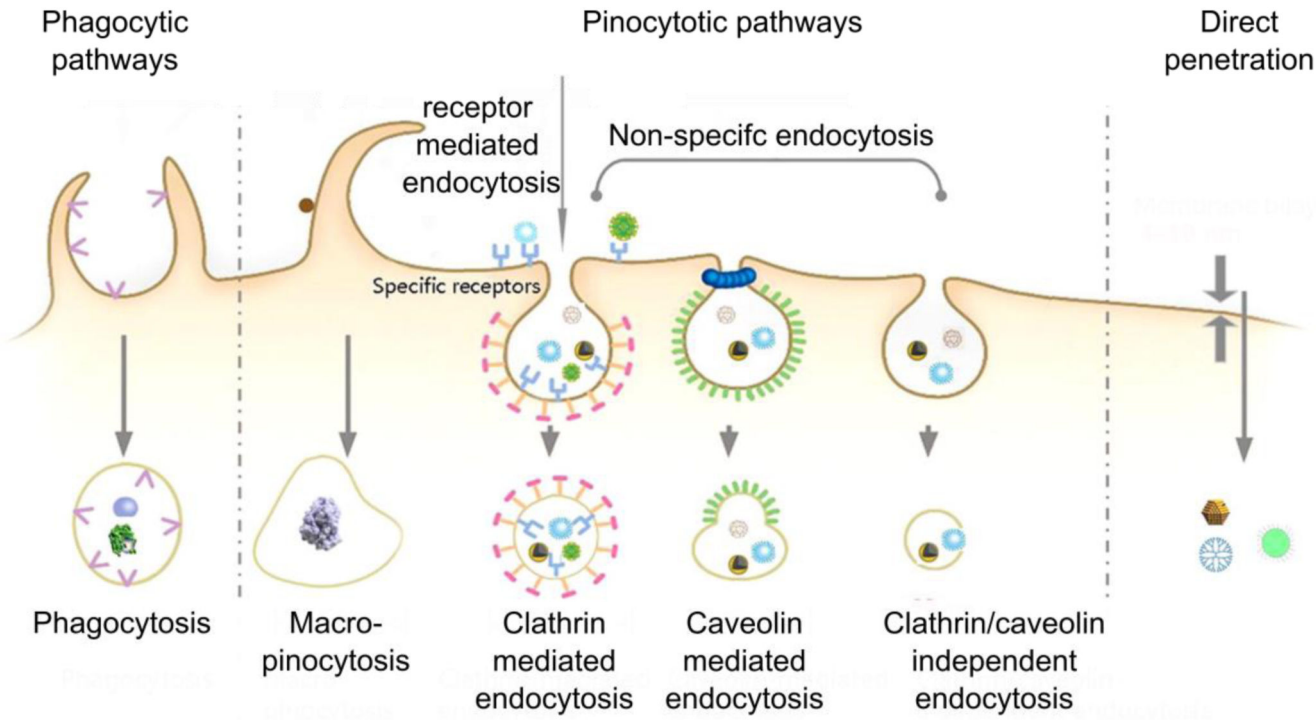

Figure 7.

Schematic demonstrating the main mechanisms by which NPs are uptaken and subsequently intracellularly processed by cells. The exact mechanism taken by a particular particle is highly dependent on the precise NP characteristics, polypeptide coating layers, target cell type, and environment. The situation is further complicated by the ability of particles to exploit multiple different uptake pathways in parallel. Adapted with permission from Zhu *et al.*149 Copyright 2013 American Chemical Society.

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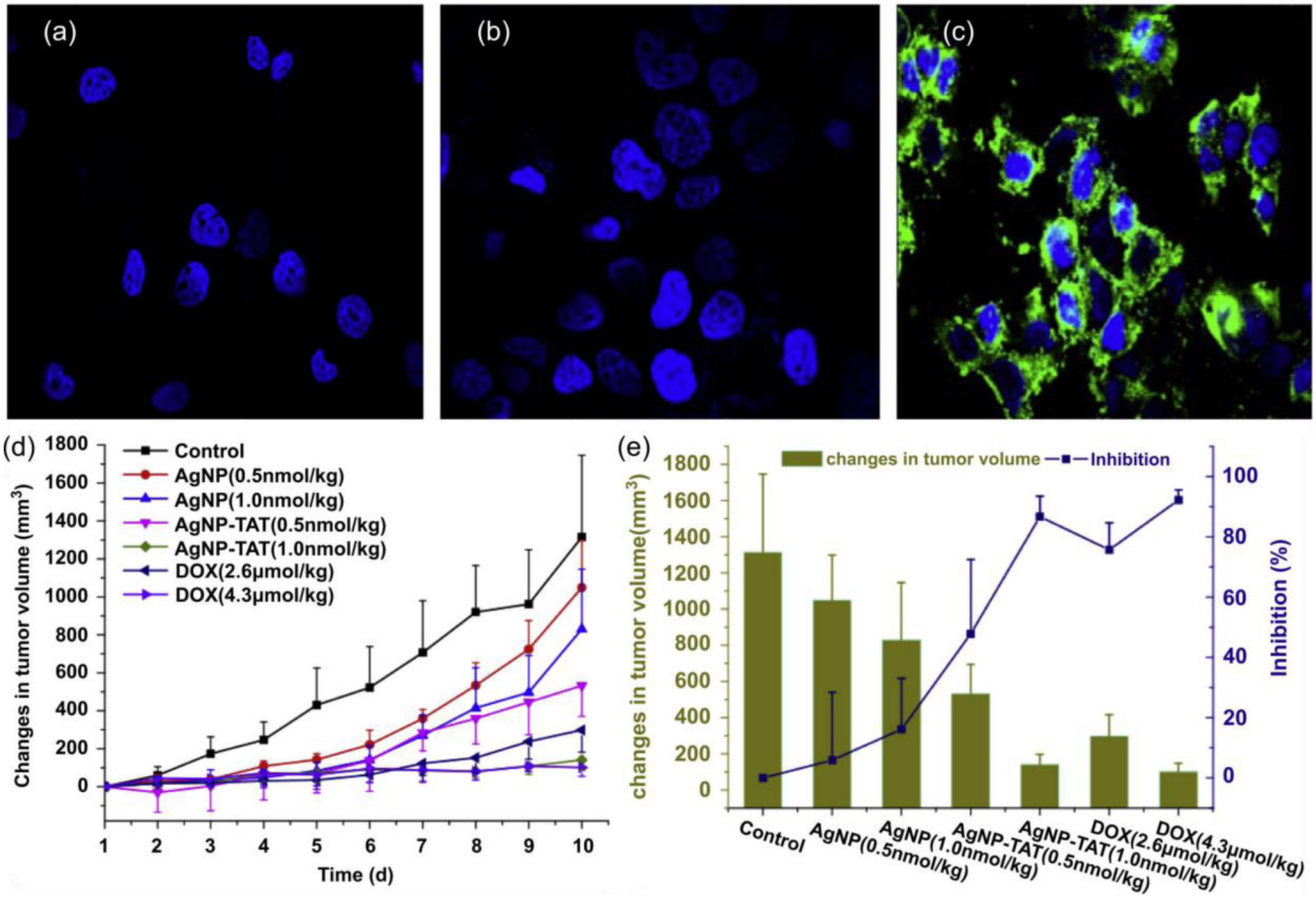

Figure 8.

Cellular uptake of: a) Phosphate-buffered saline (PBS); b) Bare silver NPs; and c) TAT-functionalised silver NPs. TAT-particle accumulation in tumours enables a reduction in tumour growth when compared to unfunctionalised particles, due to increased intracellular delivery. Adapted from Liu *et al* with permission from Elsevier.157

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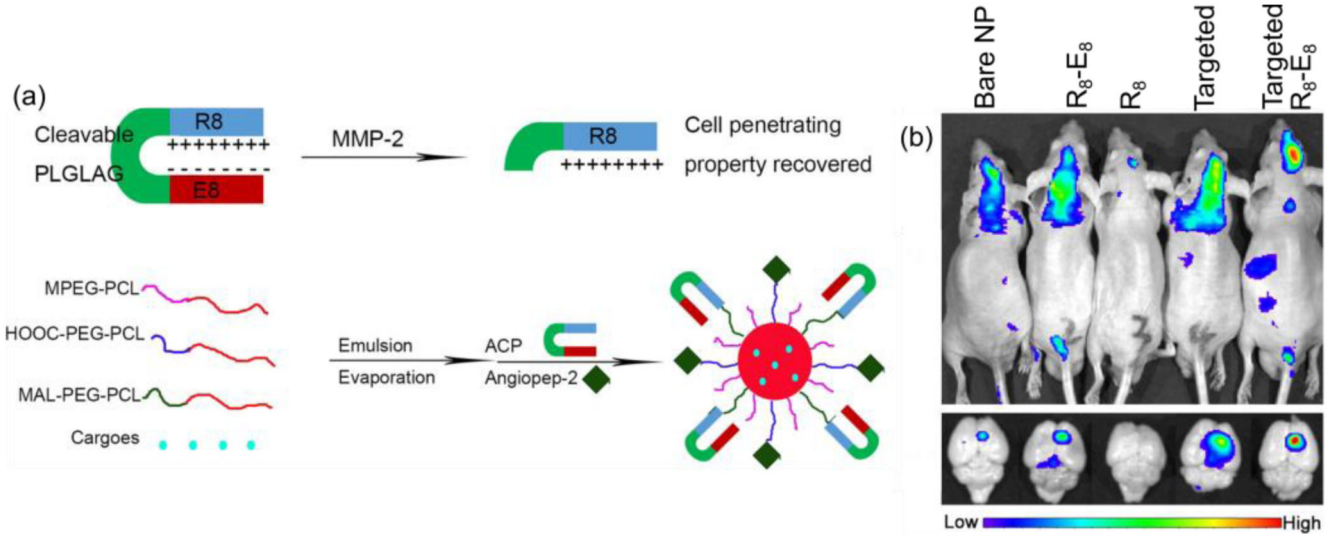

Figure 9.

a) Schematic demonstrating the electrostatic blocking of polyarginine cell-penetrating ability. An R8 peptide is blocked by electrostatic binding of an octa-glutamic acid peptide, connected via an MMP cleavable linker. Upon protease activity in the tumour environment, cleavage of the linker will lead to release of the glutamic acid blocking group, and activation of R8-mediated penetration. b) NIR imaging of mice injected with NPs coated with R8 or E8-R8 peptides, in the presence or absence of an angiopep-2 glioma targeting sequence (see section 5.4). Targeted R8-E8 functionalised NPs display the greatest enhancement at the tumour site. Adapted with permission from Gao *et al.*166 Copyright 2014 American Chemical Society

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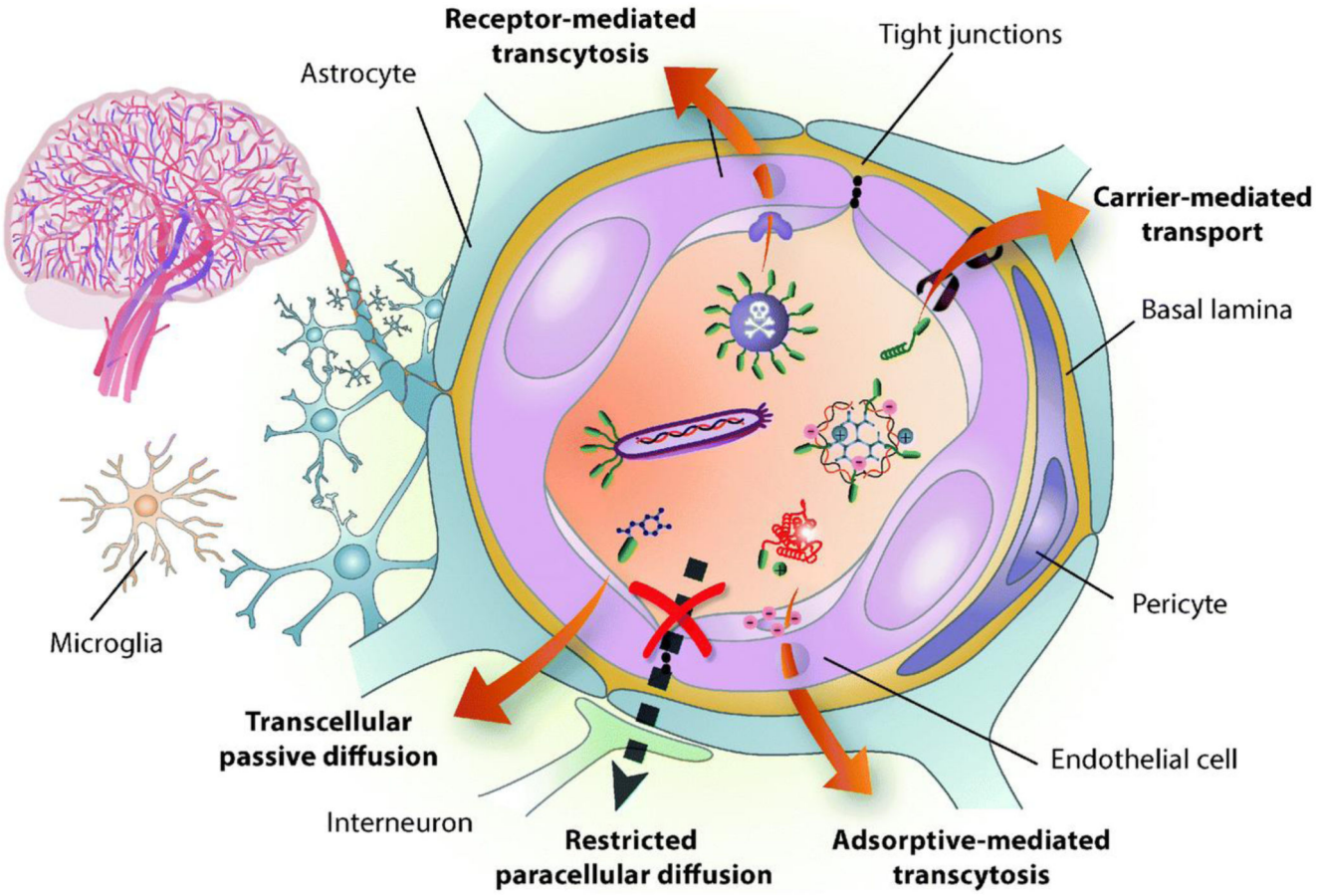

Figure 10.

Schematic demonstrating the key mechanisms by which peptides and proteins can mediate the transport of cargo across the BBB. NPs are most commonly transported via transcellular mechanisms, rather than passing through the tight endothelial cell junctions. For a detailed overview, readers are directed to the excellent recent review by Oller-Salvia *et al* from which this graphic is reproduced, published by The Royal Society of Chemistry.188

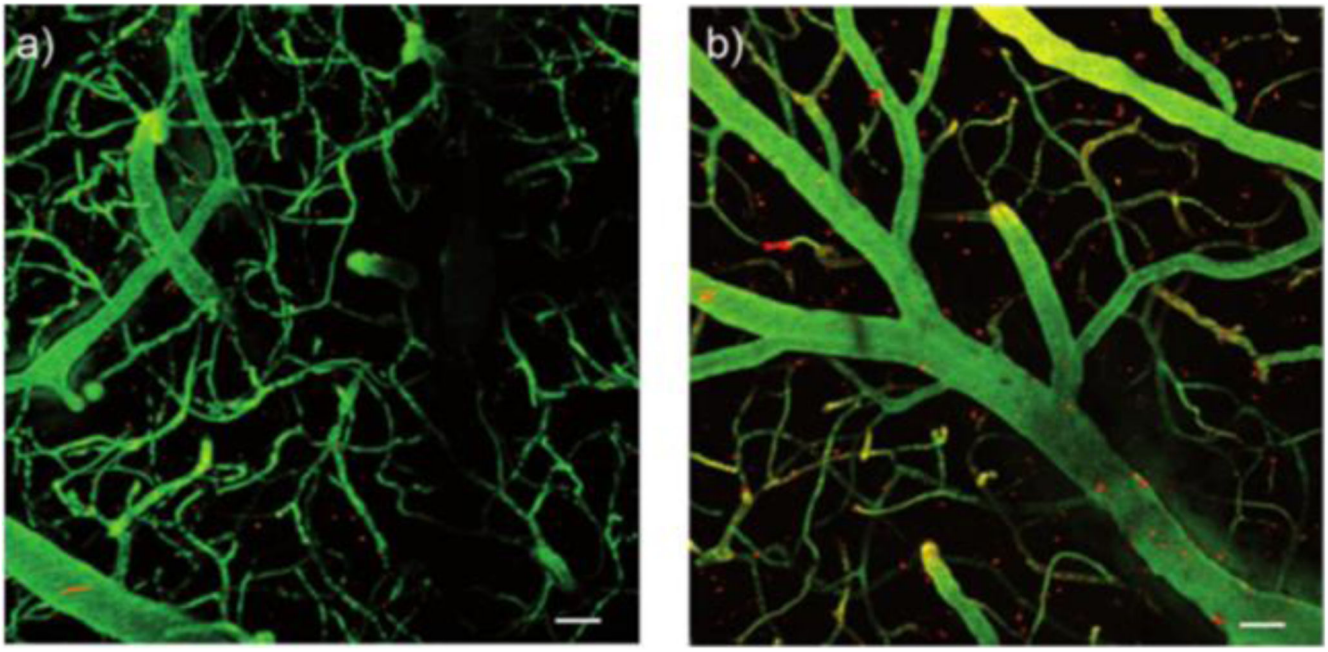

Figure 11.

Fluorescence microscopy images of brains from mice intravenously injected with QDs (red channel): a) Unfunctionalised QDs; b) QDs functionalised with a retro-enantio TfR binding peptide identified from phage display screening. Scale bar 30 μm. Reproduced from Prades *et al* with permission from John Wiley and Sons.217

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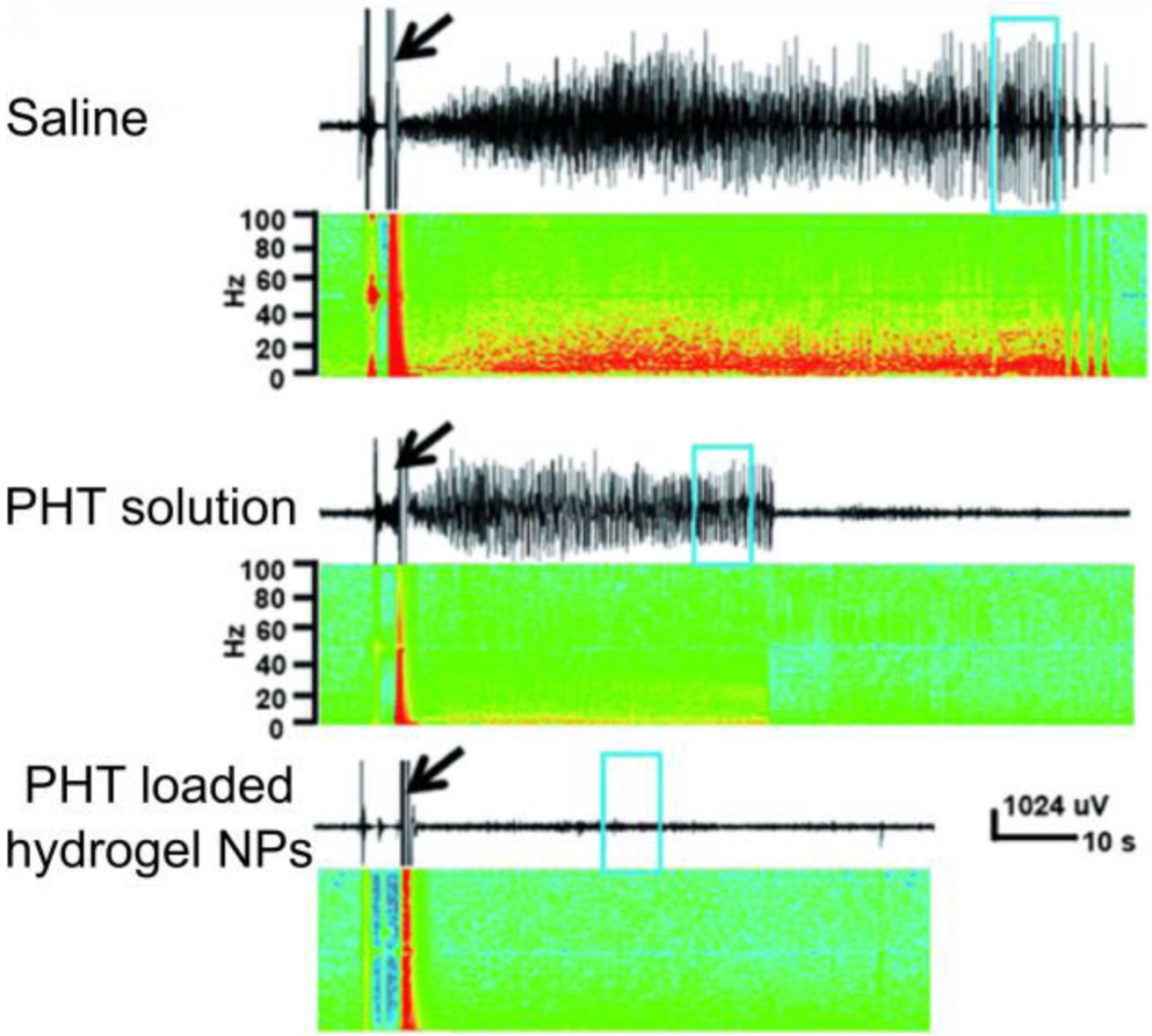

Figure 12.

Electroencephalographs from epileptic mice, injected with the anti-epileptic drug PHT. When supplied in solution, little benefit is observed. However, delivery within angiopep-2 functionalised hydrogel NPs enables penetration of the BBB and a therapeutic output. Adapted from Ying *et al* with permission from John Wiley and Sons.226

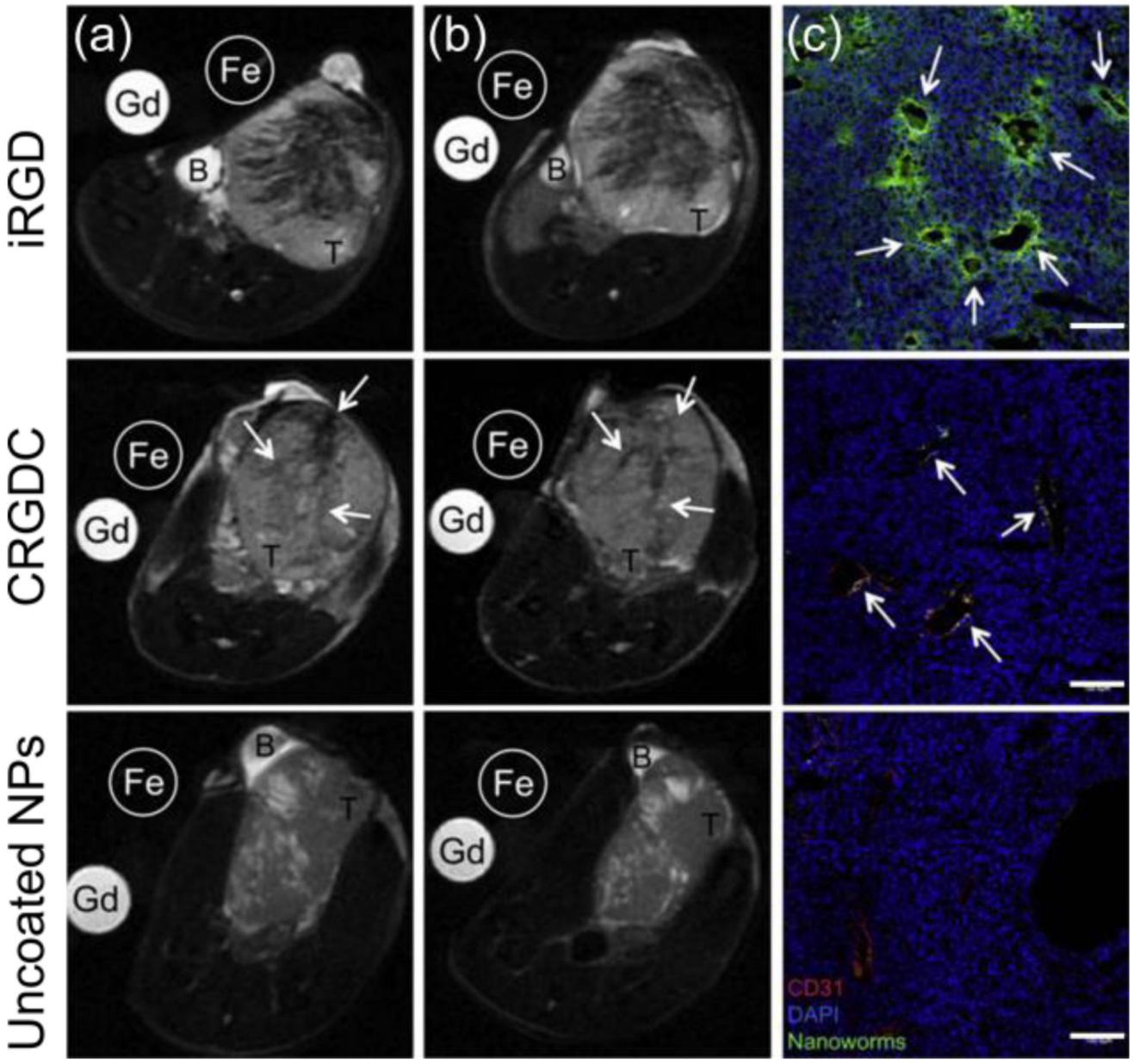

Figure 13.

Mice bearing tumours were injected with iron oxide nanoworms functionalised with iRGD, or the analogue CRGDC which retains targeting ability but with no CendR motif for penetration. a) MRI imaging after 3 hrs; b) MRI at 7 hrs; c) Fluorescence imaging at 7 hrs. Penetration away from the tumour vasculature into the tissue is only observed for iRGD functionalised particles. Scale bars 100 μm. Adapted from Sugahara *et al* with permission from Elsevier.254

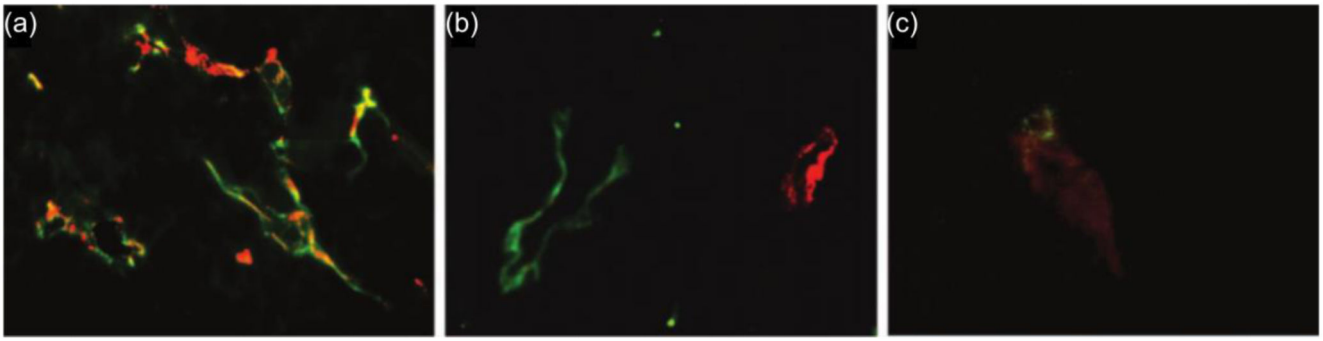

Figure 14.

Fluorescence imaging of breast cancer xenografts in mice, following intravenous injection of peptide labelled QDs. a) F3 labelled QDs (red) colocalise with tumour vasculature (green); b) Lyp-1 (red) labelled particles have a distinct, lymphatic distribution from the vasculature (green); c) F3 (red) and Lyp-1 (green) labelled QDs label different portions of the tumour. Original magnifications are x 400 (a) and x 600 (b and c). Adapted from Åkerman *et al*, copyright 2002 National Academy of Sciences.284

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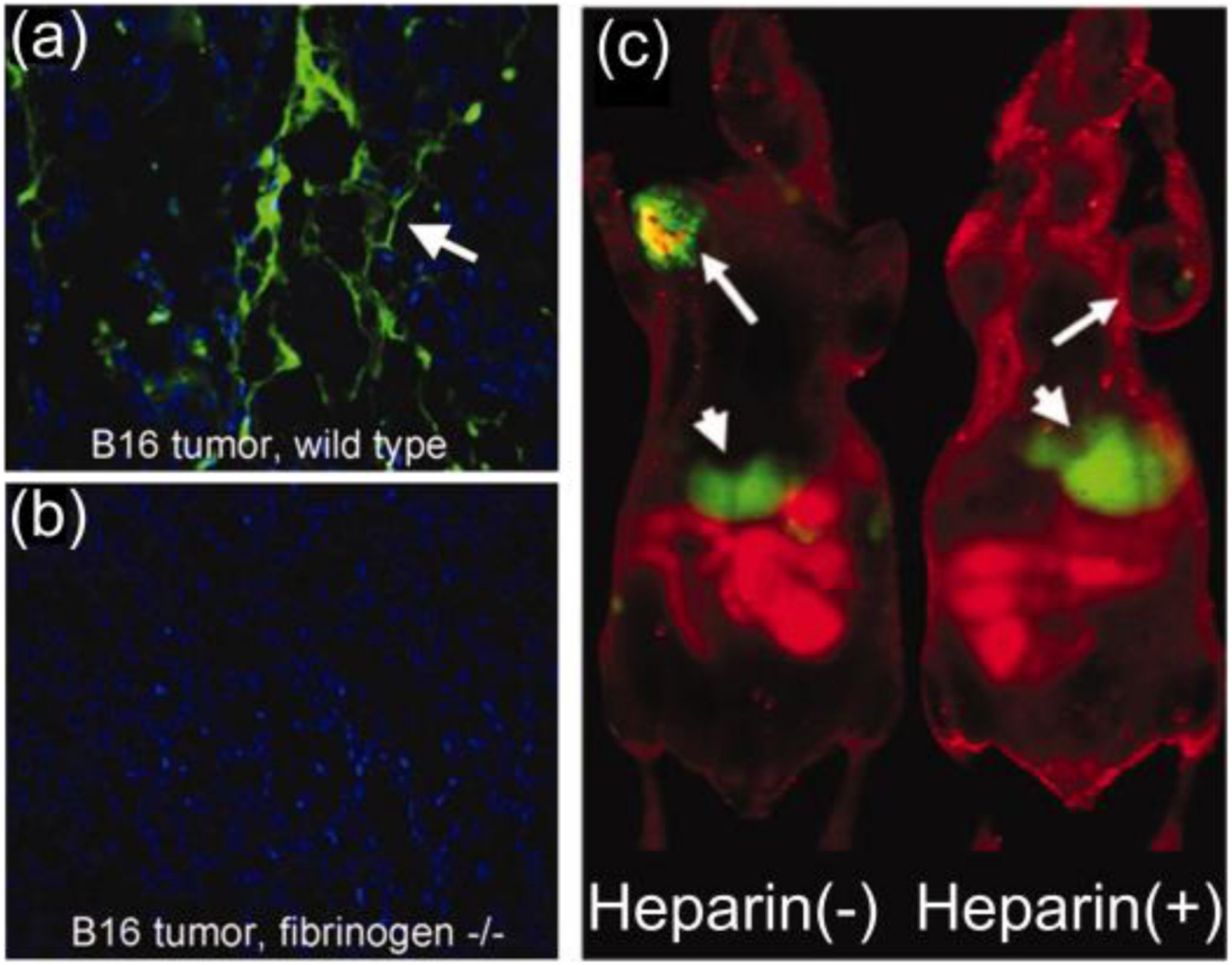

Figure 15.

CREKA coated superparamagnetic iron oxide NPs were intravenously injected into cancer xenograft containing mice where they targeted clotted plasma proteins. a) and b) Fluorescent imaging of melanoma xenograft in fibrinogen deficient mice, no labeling is observed; c) NIR imaging of mice treated with or without heparin. In the presence of heparin, clotting is inhibited and therefore no tumour labeling is observed. Original magnifications x 200 (a and b). Adapted from Simberg *et al*, copyright 2007 National Academy of Sciences.285

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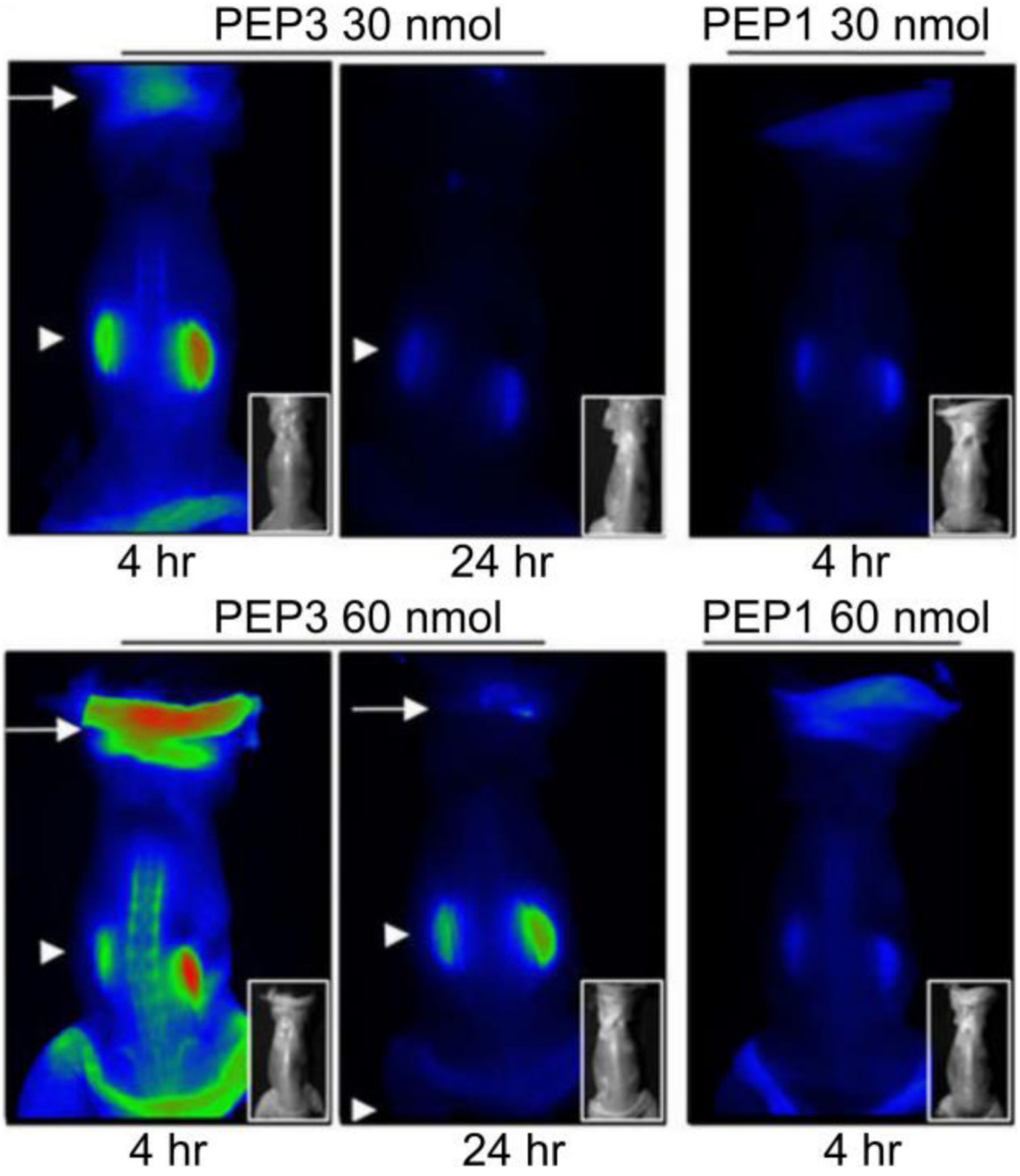

Figure 16.

NIR fluorescent imaging of mice intraveneously injected with fluorescently labelled peptides targeting brown adipose tissue (PEP3), or a control untargeted peptide (PEP1). Reproduced from Azhdarinia *et al* with permission from Nature Publishing Group.301

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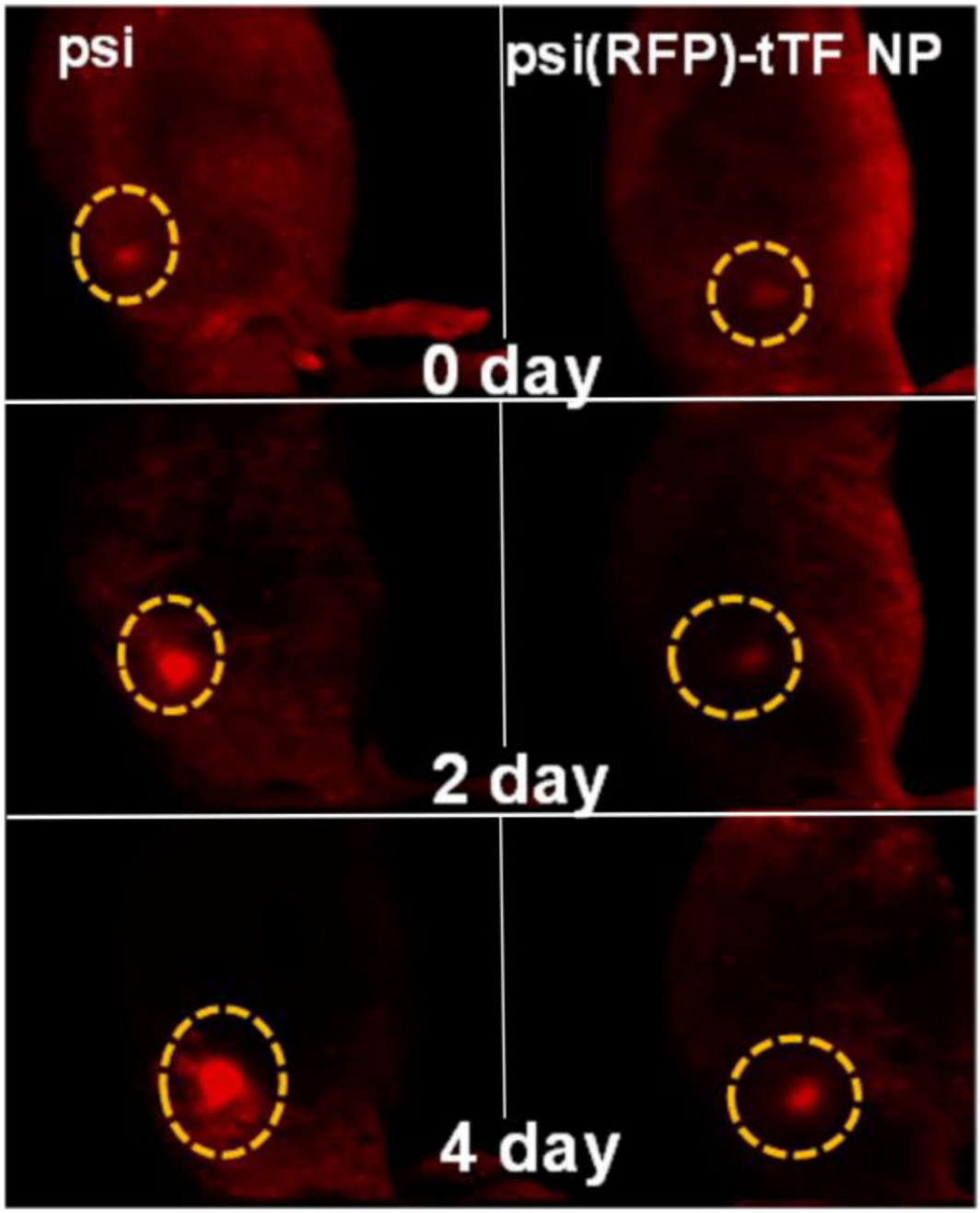

Figure 17.

Fluorescence imaging of mice bearing red fluorescent protein (RFP) expressing tumours. Treatment with free siRNA failed to impact tumour growth. However, Tf labelling of poly-siRNA (psi) particles enabled efficient tumour delivery and growth suppression. Adapted with permission from Yhee *et al.*318 Copyright 2013 American Chemical Society.

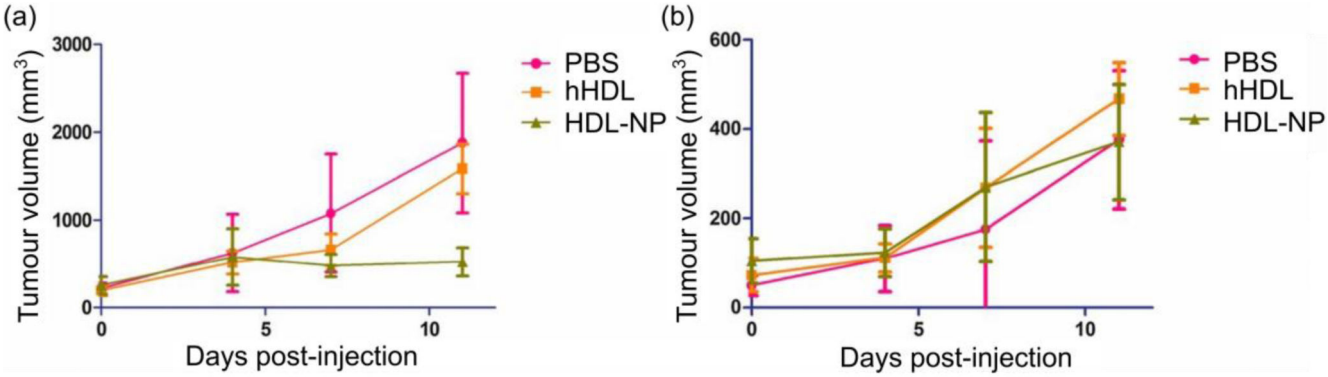

Figure 18.

AuNP templated HDLP particles were injected into xenograft tumour bearing mice: a) Particles selectively target and are uptaken by B-cell lymphoma cells. Disruption of cholesterol flux as a result limits tumour growth; b) In contrast, particles are not targeted to T-cell lymphoma cells and have no effect on tumour growth. Reproduced from Yang *et al*, copyright 2013 National Academy of Sciences.329

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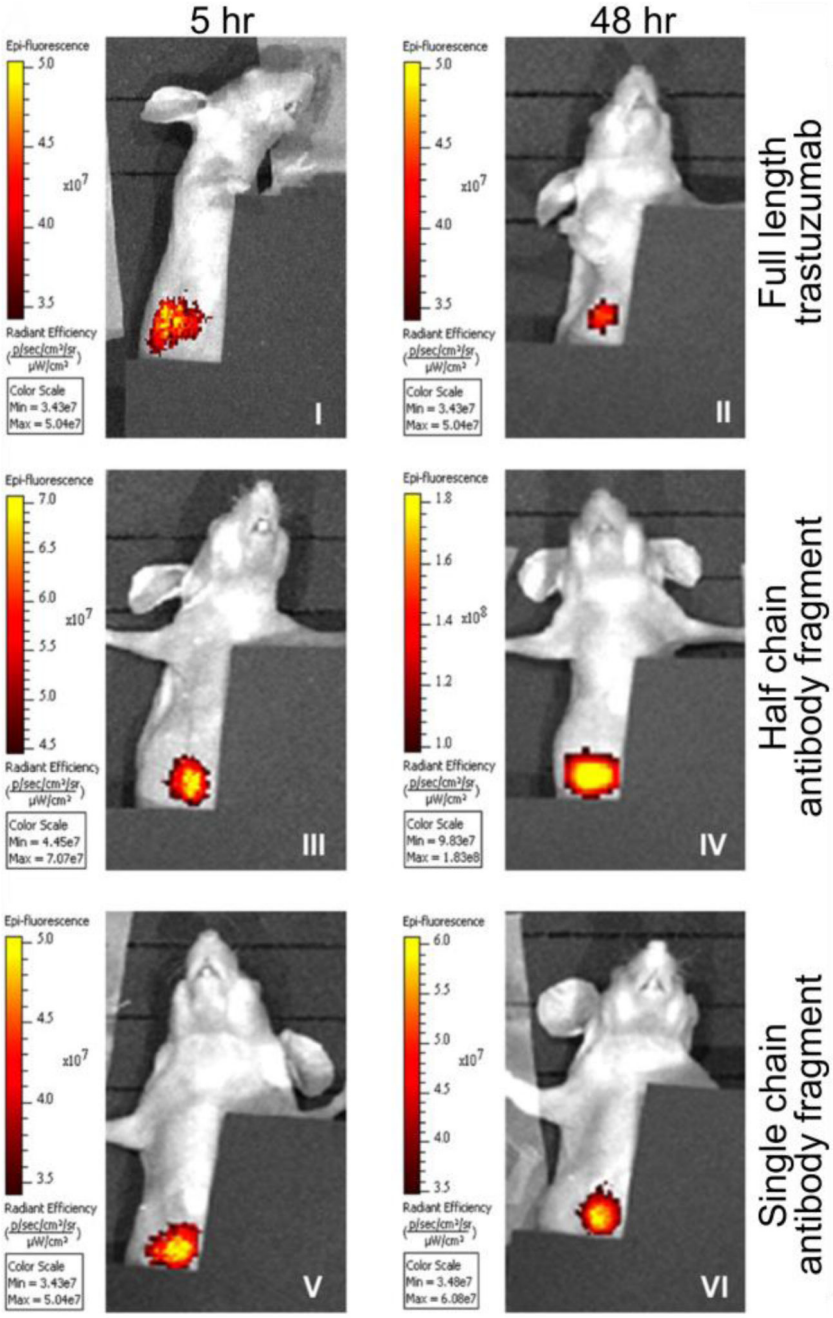

Figure 19.

Fluorescence imaging of xenograft bearing mice systemically injected with Ab labelled iron oxide NPs against HER2. Targeting is enabled by the use of full length Ab, or half-chain and single-chain Ab fragments with little difference in efficiency. Adapted with permission from Fiandra *et al.*357 Copyright 2013 American Chemical Society.

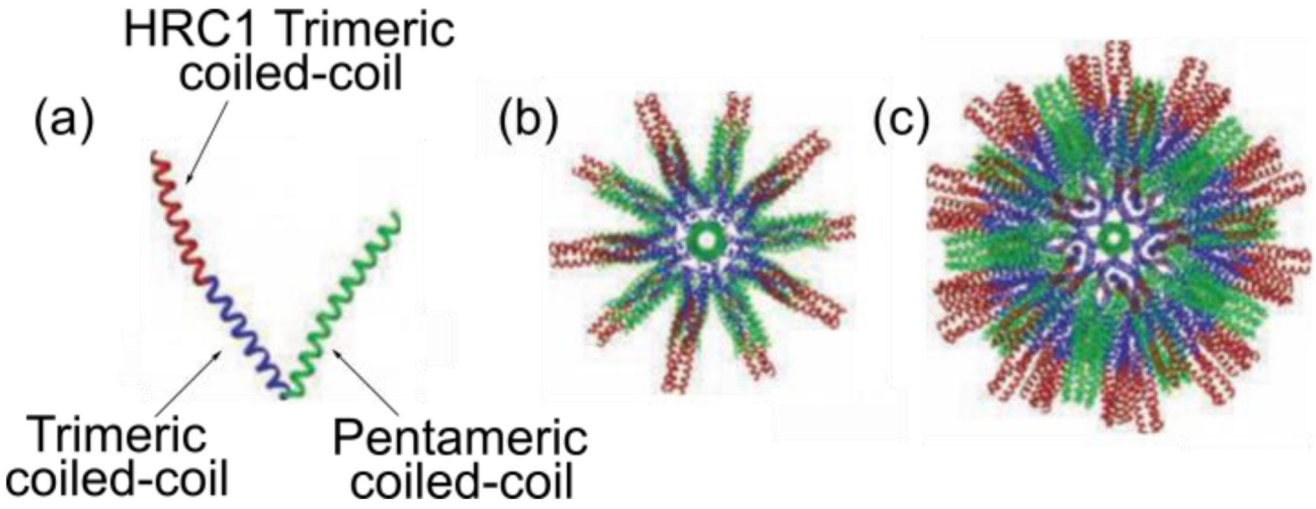

Figure 20.

a) Linear peptide, composed of a pentameric coiled-coil repeat from COMP and a *de novo* designed trimeric coiled-coil repeat, bearing a terminal SARS antigen; Peptides can self-assemble into an antigen displaying NP in a 60 (b) or 180 (c) peptide chain icosahedron. Adapted from Pimentel *et al* with permission from John Wiley and Sons.474

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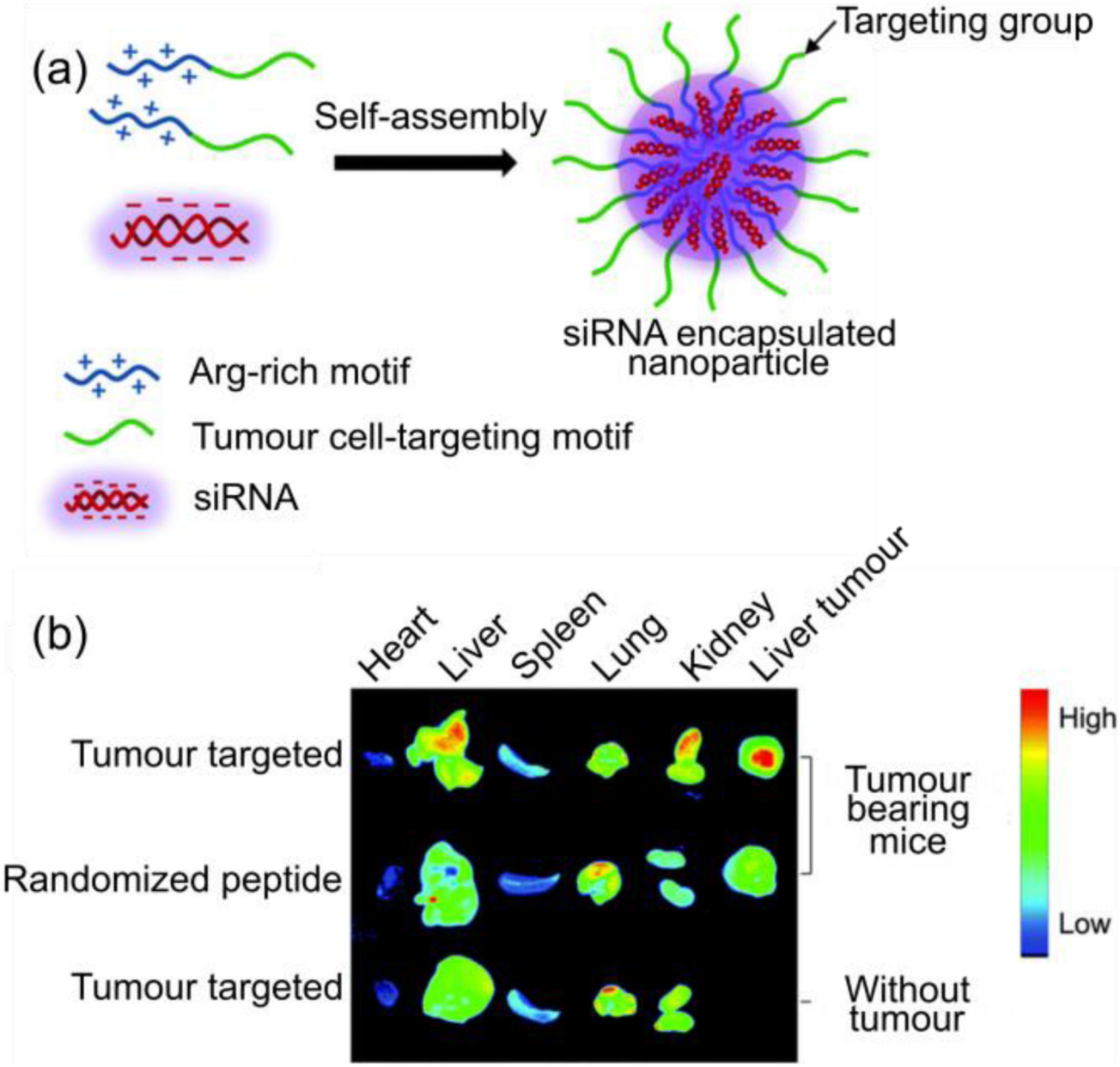

Figure 21.

a) Self-assembly of tumour-homing arginine-rich peptide hybrids with siRNA creates targeted siRNA encapsulated NPs; b) Particle delivery enables accumulation and siRNA delivery to tumour tissue. Adapted from Wang *et al* with permission from The Royal Society of Chemistry.495

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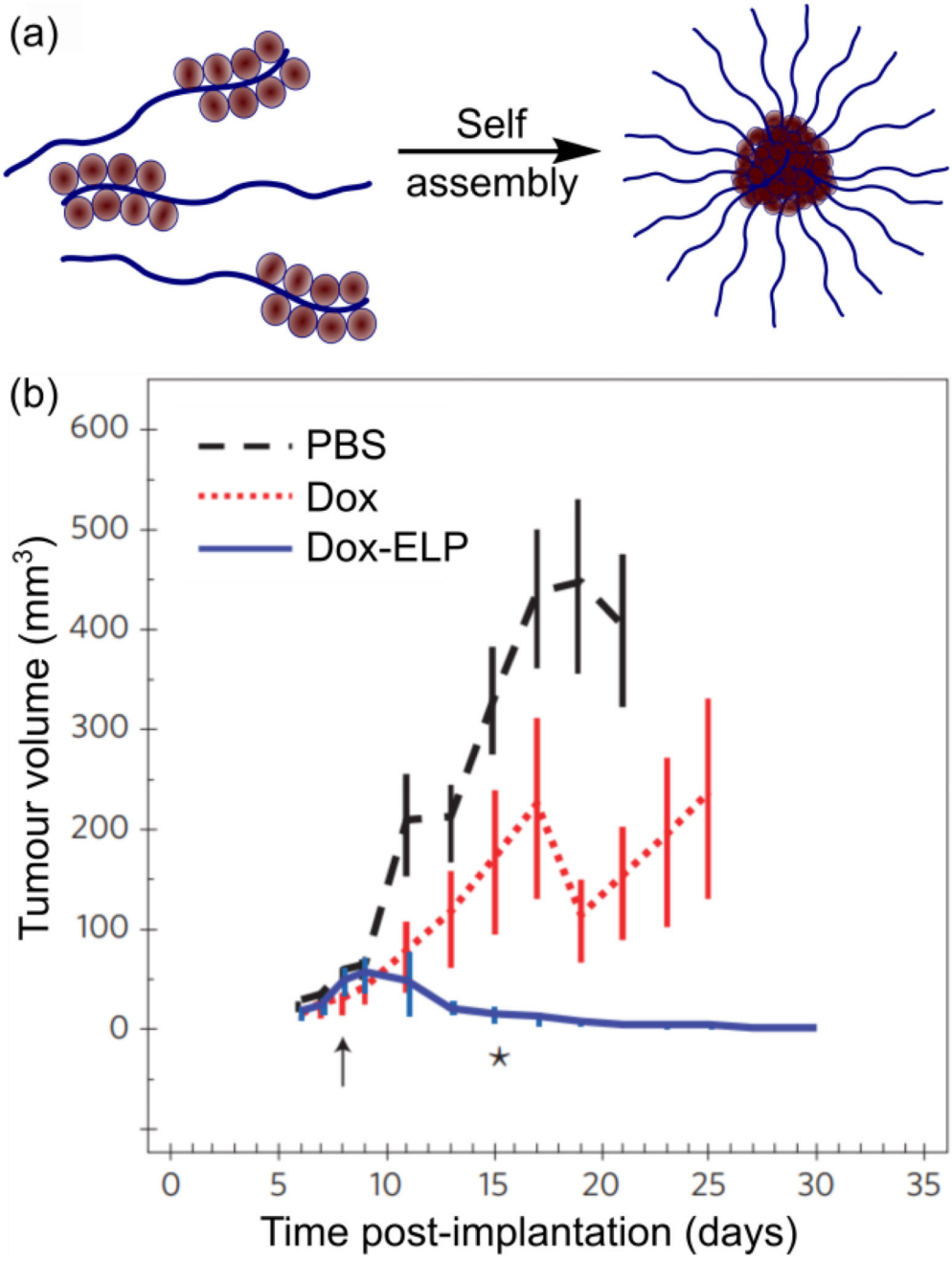

Figure 22.

a) Conjugation of mulitple doxorubicin groups at the terminus of a hydrophilic ELP generates an amphiphilic linear peptide that can self-assemble into stable micelles with a drug rich hydrophobic core; b) Tumour growth in mice is greatly reduced when doxorubicin (Dox) is conjugated to ELP in the form of micelles via an acid cleavable hydrazone bond, when compared to treatment with free doxorubicin. Adapted from Mackay *et al* with permission from Nature Publishing Group.504

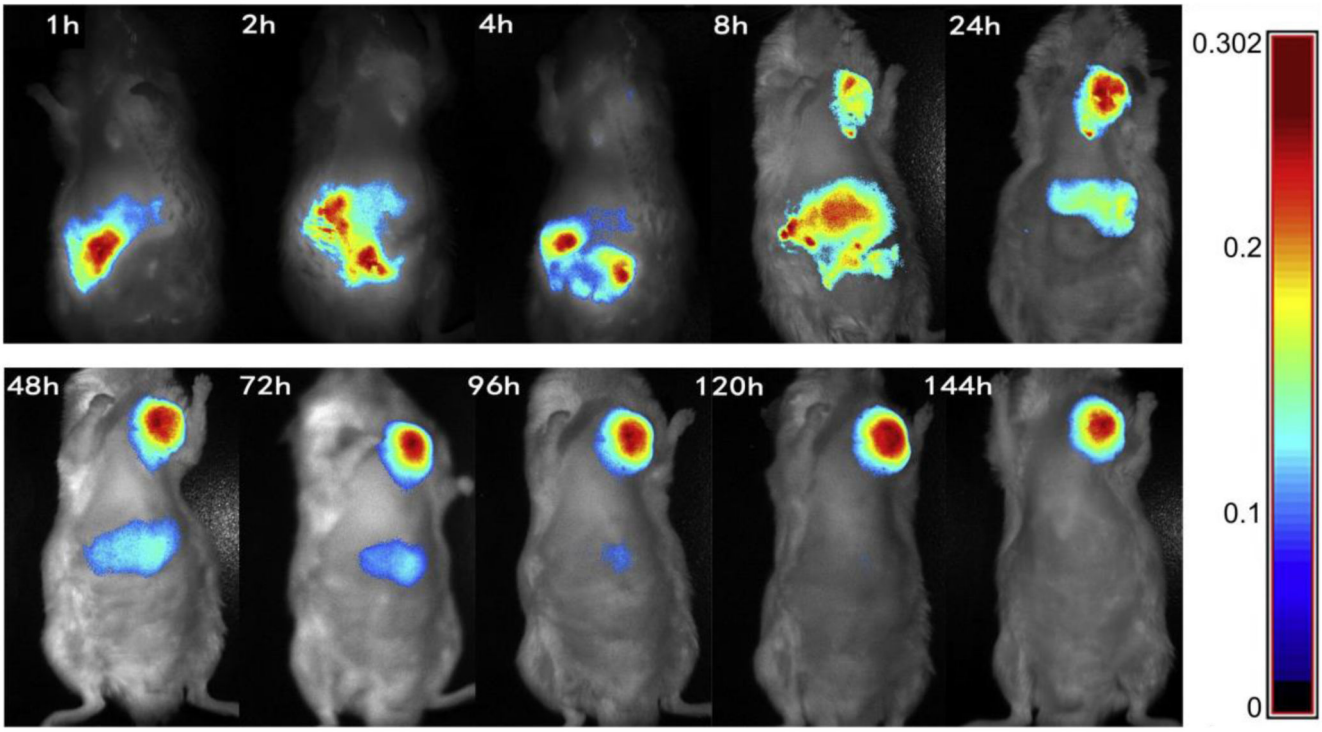

Figure 23.
NIR fluorescence imaging of tumour xenograft bearing mice, injected with cisplatin loaded casein NPs. Gradual accumulation within the tumour is enabled by the EPR effect and long circulation time of casein particles. Reproduced from Zhen *et al* with permission from Elsevier.520

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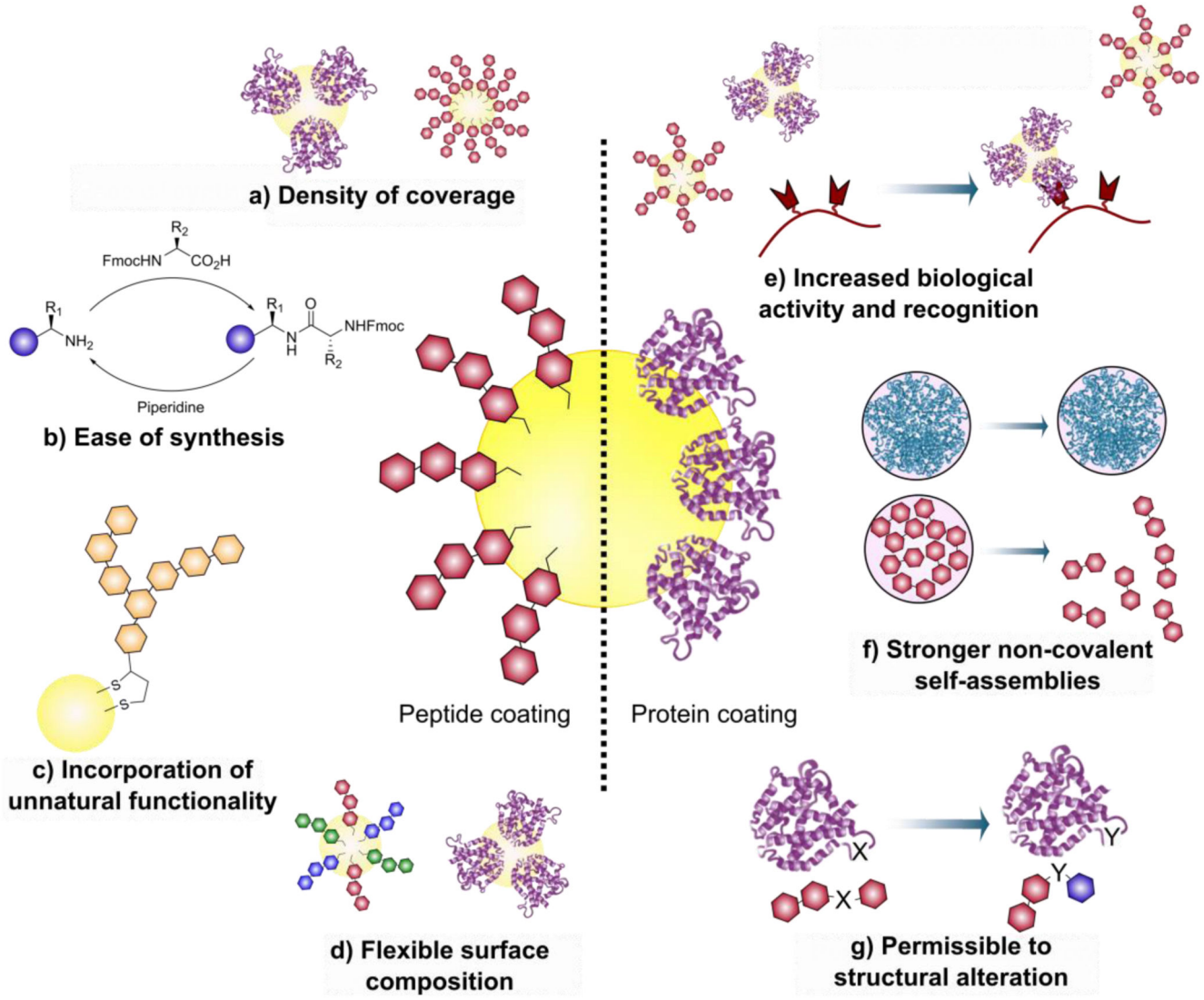

Figure 24.

Making the choice between a peptide- or protein-NP conjugate is an important decision during the design of biomedical nanotechnologies. Both have significant advantages over the other, as well as drawbacks which may limit their applicability in certain scenarios. It is therefore imperative to consider the interaction of the polypeptide component with the NP surface, core, and cargo, as well as the end application, interplay with natural systems, and route of administration: a) The small size of peptides allows a high surface density of coating to be achieved; b) Solid-phase peptide synthesis enables the straightforward production of large volumes of short peptides, as well as c) Enabling the facile introduction of unnatural amino acids, backbones, and architectures; d) Multiple peptides can be introduced within a single NP construct, enabling multi-functional coatings to be accessed; e) Proteins typically display enhanced bioactivity and binding affinity when compared to peptide substrates, though many examples of potent peptide substrates also exist; f) The high molecular weight of proteins leads to strong non-covalent interactions which can drive the

formation of stable self-assemblies; g) Single amino acid alterations to protein sequences lead to significantly less structural disruption when compared to the analogous peptide substrate.

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Table 1

Most commonly used NPs in biomedical applications.4

Particle type	Advantages	Disadvantages
Gold NPs	• Ease of surface modification • Active in range of imaging modalities • Size dependent properties • Can be utilised for photodynamic therapy	• Non-biodegradable • Potential for low colloidal stability
Magnetic NPs	• Active for high-contrast MRI imaging • Can be guided magnetically • Can be utilised for photodynamic therapy	• Cytotoxic and poor biocompatibility • Non-biodegradable
Semi-conducting NPs and QDs	• Size tunable, high quantum yield emission • Resistant to bleaching and degradation • Broad wavelength excitation • Narrow emission allows multiplexing	• Fluorescence sensitive to surface functionalisation • Toxicity of metal components • Non-biodegradable
Mesoporous silica NPs	• Controllable porosity • Biodegradable • High loading capacity for cargo delivery • Ease of surface modification	• Potential toxicity of degradation products
Liposomes	• Low immunogenicity and high biocompatibility • Ease of functionalization • Flexibility of formulation for tuning of structure • Can encapsulate hydrophilic and hydrophobic cargoes	• Low loading efficiency of valuable cargo • Poor stability and prone to leakage
Polymer NPs	• Versatile function and structure • Ease of modification and tunability • Can be made to be degradable or stimuli responsive	• Potential toxicity of both NP and degradation products

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Table 2
Peptides mediating biological and physical stability discussed in this review

Common name	Sequence (N-C)	M_w	Charge	pI	Origin	Role
	CLPFFD	741	-1.1	3.4	β-amyloid peptide derived	Biological stability
CD47 peptide	GNYTCEVTELTREGETIIELK	2399	-3.1	4.3	CD47 derived	Escape phagocytosis
	CALNN	534	-0.1	5.2	Synthetic	Physical stability
	CCVVVT	623	-0.1	5.1	Synthetic	Physical stability
Phytochelatin	(γE)C(γE)C(γE)CG	772	-3.1	3.4	Natural metal chelator	Metal NP stabilisation
GCK15	GCGGCGGKGGCGGCG	1129	+0.7	7.1	Synthetic	Metal NP stabilisation
Hexahistidine	HHHHHH	841	0.6	7.8	Synthetic	Metal affinity

pI estimated using the online tool at http://isoelectric.ovh.org/

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Table 3

Proteins mediating stability discussed in this review

Protein name	PDB #	M_w	pI	Role
CD47	2JJS	32000	6.0	Escape phagocytosis
BSA	3VO3	66500	4.8	Escape phagocytosis
Hydrophobin	2B97	14500	4.2	Modulate protein corona
Clusterin/ApoJ	P10909[1]	75000	3.8	Minimise cell uptake
Fibrinogen	3GHG	340000	5.5	BBB penetration

[1] UniProt; PDB - Protein data bank; pI estimated using http://isoelectric.ovh.org/

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Table 4

Peptides mediating penetration discussed in this review

Common name	Sequence (N-C)	M_w	Charge	pI	Origin	Role
TAT	GRKKRRQRRRPQ	1622	+8	12.5	HIV protein derived	CPP
R8	RRRRRRRR	1267	+8	12.7	Synthetic	CPP
Penetratin	RQIKIWFQNRRMKWKK	2247	+7	12.1	Drosophila Antennapedia homeodomain	CPP
HA2 peptide	GDIMGEWGNEIFGAIAGFLG	2054	-3	3.2	Influenza virus HA-2 derived	Endosomal escape
GALA	WEAALAEALAEALAEHLAEALAEALEALAA	3023	-6.9	3.7	Synthetic	Endosomal escape
Pas	FFLIPKG	821	+1	9.1	Cathepsin B substrate	Endosomal escape
	THRPPMWSPVWP	1491	+1.1	10.5	Phage display	BBB penetration
Angiopep-2	TFFYGGSRGKRNNFKTEEY	2301	+2	9.3	Kunitz domain	BBB penetration
Glutathione	(γE)CG	307	-1.1	3.6	Natural anti-oxidant	BBB penetration
CDX	FKESWREARGTRIERG	1978	+2	10.3	Snake toxin candoxin derived	BBB penetration
Chlorotoxin	MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR	4004	+2.6	7.3	Snake venom peptide	BBB penetration
MiniAP-4	c(DLATEPAL[Dap])	911	-2	3.3	Bee toxin apamin derived	BBB penetration
g7	GFTGFLS(Glucose)	889	0	N/A	Simil-opiod glycopeptide	BBB penetration
RV29	YTIWMPENPRPGTPCDIFTNSRGKRASNG	3266	+1.9	9.1	Rabies glycoprotein derived	BBB penetration
iRGD	CRGDKRGPDEC	1235	-0.1	5.9	Phage display	Tumour penetration
IL-13p	TAMRAVDKLLLHLKKLFREGQFNRNFESIIICRDRT	4334	+4	9.5	IL-13Rα2 binding domain	Tumour penetration
	CGEMGWVRC	1040	-0.1	5.8	Phage display	Tumour penetration
Lyp-1	c(CGNKRTRGC)	993	+3	9.1	Phage display	Tumour penetration

c - Cyclic; Dap - Diaminopimelic acid; pI estimated using the online tool at http://isoelectric.ovh.org/

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Table 5
Proteins mediating penetration discussed in this review

Protein name	PDB #	M_w	pI	Role
Transferrin	1D3K	76000	5.5	BBB penetration
OX26	N/A	N/A	N/A	BBB penetration
Lactoferrin	1LFG	77302	8.7	BBB penetration

PDB - Protein data bank; pI estimated at http://isoelectric.ovh.org/

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Table 6
Targeting peptides discussed in this review

Name	Sequence (N-C)	M_w	Charge	pI	Origin	Role
GFE	CGFECVRQCPERC	1530	-0.3	5.8	Phage display	Membrane dipeptidase targeting
F3	KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK	3433	+8	9.9	High mobility group protein 2 derived	Blood vessel and tumour targeting
Lyp-1	CGNKRTRGC	993	+2.9	9.1	Phage display	Tumour lymphatic vessel targeting
	CREKA	605	+0.9	8.1	Phage display	Clotted plasma/tumour targeting
RGD	RGD	346	0	6.5	Integrin binding sequence	Tumour targeting
	c(CNGRC)	550	+1	11.2	Phage display	CD13/tumour targeting
Bld-1	CSNRDARRC	1080	+1.9	8.5	Phage display	Bladder cancer targeting
AHNP	YCDGFYACYMDV	1450	-2.1	3.2	Trastuzumab derived	Breast cancer targeting
SP204	KQFSALPFNFYT	1462	9.6	9.1	Phage display	Prostate cancer targeting
	CKGGRAKDC	937	8.2	9.2	Phage display	White fat targeting
	GGGGYDRVTIHPF	1375	7.4	7.8	Angiotensin II derived	Infarcted cardiac tissue targeting
	PLGLAGGWGERDGS	1371	4.2	3.9	MMP cleavable peptide	Infarcted cardiac tissue targeting
	CAQK	448	8.1	9.1	Phage display	Extravascular brain tissue targeting
	$D(KLAKLAK)_2$	1523	9.8	11.4	Synthetic	Mitochondria directing
Eriss	MRYMILGLLALAAVCSA	1796	8.3	8.6	ER insertion sequence	ER directing

c - Cyclic; pI estimated using the online tool at http://isoelectric.ovh.org/

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Table 7

Targeting proteins discussed in this review

Protein name	PDB #	M_w	pI	Role
Transferrin	1D3K	76000	5.5	Tumour targeting
EGF	1NQL	134000	5.3	Tumour targeting
VEGF	2VPF	27400	8.1	Tumour targeting
LFA-1	N/A	N/A	N/A	Tumour/inflammation targeting
Apolipoprotein AI	3R2P	31000	5.4	Tumour targeting
WGA	2UVO	21200	6.4	Alveoli targeting
UEA-1	1JXN	27000	4.5	Intestinal targeting