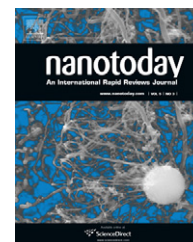


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## REVIEW

# Functionalisation of nanoparticles for biomedical applications

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**Summary** Nanoparticles with cores composed of inorganic materials such as noble, magnetic metals, their alloys and oxides, and semiconductors have been most studied and have vast potential for application in many different areas of biomedicine, from diagnostics to treatment of diseases. The effects of nanoparticles must be predictable and controllable, and deliver the desired result with minimum cytotoxicity. These criteria can be met by careful tailoring of the ligand shell, allowing stabilisation, specific targeting and recognition of biochemical species. For these reasons, this review is focused on the synthesis and biofunctionalisation of inorganic metal, semiconductor and magnetic nanoparticles for biomedical applications.

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## Introduction

Nanoparticles (NPs) are attracting considerable interest as viable biomedical materials and research into them is growing due to their unique physical and chemical properties. These NPs can be composed of a variety of materials including noble metals (e.g. Au [2], Ag [3,4], Pt [5], Pd [6]), semiconductors (e.g. CdSe, CdS, ZnS [2,7], TiO<sub>2</sub> [8], PbS [9], InP [9], Si [10]), magnetic compounds (e.g. Fe<sub>3</sub>O<sub>4</sub> [11], Co [12], CoFe<sub>2</sub>O<sub>4</sub> [13], FePt [6], CoPt [14]) and their

combinations (core–shell NPs and other composite nanostructures). Biomedical applications of NPs include drug carriers, labelling and tracking agents [2,7], vectors for gene therapy, hyperthermia treatments and magnetic resonance imaging (MRI) contrast agents [5,15]. In order for the NPs to be useful in biomedicine, they must satisfy certain criteria. For *in vitro* applications such as fluorescent staining of proteins and TEM imaging, NPs must outperform the conventional agents while having minimal cytotoxicity. *In vivo*, NPs have to avoid non-specific interactions with plasma proteins (opsonisation) and either evade or allow uptake by the reticuloendothelial system (RES) depending on the application, to reach their intended target efficiently. They must also maintain colloidal stability under physiological conditions, preferably including a wide range of pH. NPs carrying a payload, such as drug molecules or DNA for gene therapy must

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avoid premature release, yet specifically deliver the load to the desired site. Chemical modification of the NP surface is necessary for specific interactions with biomolecules of interest.

## Synthesis of nanoparticles

### Synthesis in water

Nano-structured materials including Au [16,17], Ag [4], Co [3], Ni<sub>x</sub> [18] Fe<sub>3</sub>O<sub>4</sub>, Fe<sub>2</sub>O<sub>3</sub>, α-FeO(OH) [19], SiO<sub>2</sub> [20] and CdTe [21,22] have been synthesised in aqueous solution. These methods provide water-dispersible NPs, a necessity for the application in biological systems; however, control over particle size distribution is still limited for many semiconductor and magnetic NP systems. NP size affects the properties so a narrow size distribution is essential [23]. This can be reduced post-synthesis by using a hydrophobic ligand such as oleic acid to transfer the NPs to an apolar solvent, from which particles of the desired size may be obtained by size-selective precipitation. However, this is a tedious, low yielding multistep procedure and the particles are then no longer in the desired medium, water [24].

Gold nanoparticles can be prepared by reduction of Au (III) salts using reducing agents such as citric acid, sodium citrate, sodium ascorbate or amines [25]. Iron (hydr)oxides are synthesised by the alkali coprecipitation process, with the composition and morphology of the resultant NPs depending on the precise reaction conditions [19,26]. Alkali precipitation is also suited for the preparation of more complex multi-metallic ferrites [27]. Borohydride reduction is another approach suited for the preparation of NPs composed of Co, Fe, Au and CdTe [3,25,28–30]. Ag NPs can be synthesised following a green synthesis protocol where starch was used as a stabiliser [4]. Synthesis of SiO<sub>2</sub> is well established *via* the Stober method [20]. Success has also been achieved in the synthesis of water soluble Co and CoPt structures through the use of multithiol ligands, thermoresponsive polymers and thioether end-functionalised polymers [3,31,32].

### Synthesis of nanoparticles in non-polar solvents

Syntheses in organic solvents have been published for a wide range of NPs composed of noble metals, transition metals, oxides and semiconducting materials [3,33–36]. The growth of NPs, the crystal structure and the cessation of the growth depend on the environment and are fundamentally regulated by the ligands. Thus, in the absence of ligand, particle surfaces are exposed, so they fused together and precipitate. These ligands tend to be either surfactant species such as fatty acids or alkane thiols, rendering the particles highly hydrophobic. In some cases the solvent itself acts as a ligand, semiconductor quantum dots (QDs) were capped with tri-*n*-octyl phosphine oxide (TOPO) [36].

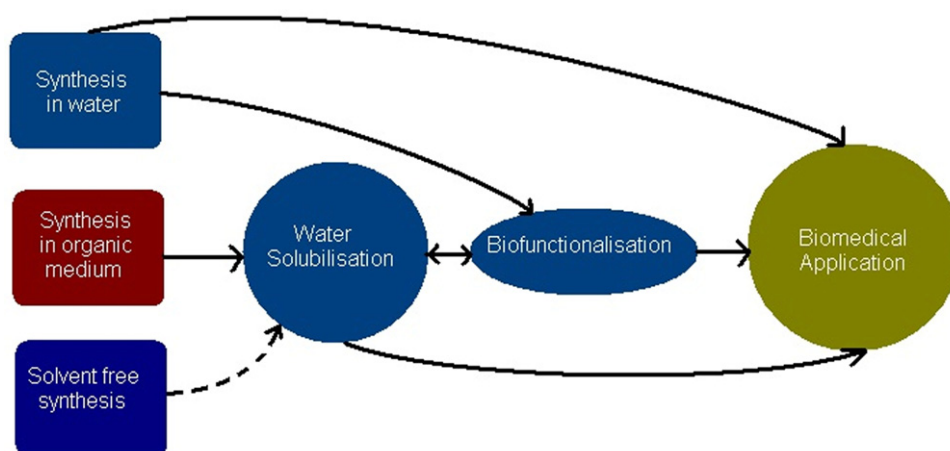
Water soluble NPs may be prepared in a one-step synthesis in organic solvents by judicious choice of stabilisation agents. Amphiphilic ligands such as peptides [12] and thermoresponsive polymers [31], have been used to produce Co NPs.

### Synthesis of nanoparticles by other methods

Other methods for NP synthesis have been reported including gel templating and solvent-free methods such as chemical vapour deposition (CVD) [37], electrical explosion [38] and mechanical milling [39].

In gel templating, metal salts are reduced in a porous gel matrix. The choice of gel is crucial to the mechanism; agarose gel has an inherent reducing effect [40]. Closely related to gel templating is the synthesis of subnanometre Au NPs in the dendrimer polyamidoamine. Again, this method leads to very small Au NPs, termed “gold quantum dots” due to the fluorescence exhibited by both the dendrimer [41] and the Au core in this size regime [41–43].

CVD, mechanical milling and other solvent-free methods are especially useful for the synthesis of other interesting materials such as NdFeB and carbon nanotubes for which there are no established “wet” synthesis routes [44]. To date, NPs prepared by CVD have been used almost exclusively for heterogeneous catalysis, magnetic data storage and nanoelectronic devices rather than biomedicine.



**Figure 1** Synthetic pathways to biomedically applicable nanoparticles, the dotted line indicates that materials synthesised in solid state are not water soluble or may have no biomedical application.

Electrical explosion and mechanical milling are currently unsuited to production of NPs for biomedicine due to their relatively large particle size and poor control over size distribution (Fig. 1).

## Water solubilisation of nanoparticles

Water solubilisation may be carried out either as the final stage of the functionalisation process of NPs, or as an intermediate stage. It should be noted that the terms "solubilisation" and "solution" when applied to NPs does not refer to the solvation of the inorganic cores but rather the physically and chemically stable colloidal suspensions where NPs do not aggregate, dissociate, or chemically react to the solvent or any dissolved gas with time. Water solubilisation refers to the conjugation of colloidal unstable NPs with hydrophilic ligands to give stable NPs in aqueous solution.

### Ionic stabilisation

Functional groups on the surface of NPs allow conjugation with ionic ligands. With the molecule bound to the nanoparticle, the charge resides on the outside of the particle giving way to coulombic repulsion and thus dispersion of the NPs.

Once the charge screening is large enough, the extent of the coulombic repulsion can be diminished by the addition of a salt, leading to precipitation of NPs or "salting out" [45]. As the physiological salt concentration is around 100 mM [46] this is generally sufficient to cause precipitation of NPs lacking additional stabilisation. For this reason, unfortunately, ionically stabilised NPs are generally unsuitable for biomedical application. The stability of NPs coated with species such as citric acid/citrate [16], orthophosphoric acid/phosphate [47] and other species that may easily gain or lose protons is highly sensitive to pH as protonation/deprotonation affects the surface charge ( $\zeta$ -potential). If the magnitude of the  $\zeta$ -potential is reduced below the point at which coulombic repulsion is effective, NPs will aggregate. This occurs at pH values around the  $pK_a$  of the surface functional group, making acidic anionic ligands (phosphate, citrate) suitable for stabilisation in basic to mild acidic conditions [48,49], whereas cationic ligands such as alkylammoniums offer stabilisation from acidic to mildly alkaline conditions [33].

While ionic stabilisation alone is generally insufficient to prevent aggregation of NPs, ionic interactions with charged species in biological media can have a significant effect on the overall stability of the ligand shell and the NPs' function. Positively charged NPs tend to be removed rapidly from the blood, ending up predominantly in the liver and spleen whereas negative NPs have a longer circulation time and are mainly taken up by the lymph nodes [50]. Charge neutrality may be achieved by using either uncharged or zwitterionic ligands with no overall charge. Neutral ligands must be bulky in order to compensate for the lack of coulombic repulsion, leading to a larger hydrodynamic radius and a generally longer circulation time in the blood. Zwitterionic ligands, on the other hand, have been reported to yield NPs with smaller hydrodynamic radii and much lower degrees of opsonisation [51]. Coulombic repulsion is also useful as it can protect ligands on the NPs from exchanging with biomolecules [52];

the disadvantage of highly charged NPs is that they are more readily opsonised [53].

However, inhomogeneous surface chemistry, segregated surface charge distribution and detachment of ligands in various environments can compromise the reproducibility and long-term stability. Therefore, the stability of the system should be tested for a wide range of electrolyte concentrations, values of pH at various time points so that its validity can be verified.

### Steric stabilisation

An alternative to ionic stabilisation is to provide a physical barrier to prevent aggregation. Steric stabilisation can be achieved by coating NPs with a ligand shell or embedding them with an inorganic or polymeric matrix.

### Polymeric ligands

Polymers make excellent ligands as they surround the NPs with a substantial physical barrier preventing the core NPs from coming into contact. The consequence of this enhanced core separation is an increase in the hydrodynamic radius of the NPs [54]. This is desirable for *in vivo* applications requiring a long circulation time, but disadvantageous if rapid diffusion to the extravascular space is required; essentially, size is a very important factor in the biodistribution of NPs [51]. There are many suitable polymeric ligands for the provision of water solubility (Table 1), the most common of which are based on poly(ethylene glycol) (PEG) and carbohydrates such as starch [55], dextran [56] and chitosan [57].

PEG is especially suitable as a ligand for NPs requiring long circulation times in blood, as it reduces the degree of opsonisation [51] and provides excellent long-term stability in high salt concentrations and pH extremes [58–60]. Conjugation and alteration of the head groups of PEG derivatives not only allows selective attachment to NP surfaces, but also makes way for biofunctionalisation [60].

Like small-molecule stabilising agents [61], the concentration of polymeric stabilisers may be used to control the NP core morphology [3,62]. As a result of the polymerisation method and reaction conditions used, various NP structures are formed. Such structures include single microparticles incorporating multiple NP cores [63,64], individual core-shell systems [65] and cases where the polymer is larger than the NP, leading to a templating effect [42,66].

### Small-molecule ligands

The advantage of small molecules as ligands is that they offer a certain degree of physical barrier, similar to polymeric ligands, but give a smaller hydrodynamic radius. *In vivo* applications require a small hydrodynamic radius for efficient trans membrane permeation and excretion [51]. However, care must be taken not to make the molecular shell too thin, as this leads to an insufficient steric barrier, resulting in reduced NP stability [82] and aggregation [83]. Molecular species suitable for water solubilisation of NPs tend to incorporate functional groups allowing ionic stabilisation and further (bio)chemical modification once in water. A common small-molecule form of PEG is an  $\omega$ -thiol func-

**Table 1** Different polymers used to coat NPs.

Polymer	Ligand addition method	Core	Chemical functional groups
PAMAM <sup>a</sup> [42,67]	Ligand addition in methanol	Au	Amine, amide groups. Dendrimer provides a steric framework.
PGAMA <sup>b</sup> and PLAMA <sup>c</sup> [68]	Reduction of chloroauric acid in the presence of glycopolymer	Au	Multiple –OH groups offer stability, solubility and resistance to high salt concentrations.
PEG-phosphine	Ligand addition	Au	Multidentate –PO groups offer binding to Au, the hydrophobic tails offer solubility in organic solvents.
PMAA-DDT <sup>d</sup> [3,62]	Reduction of CoCl <sub>2</sub> and chloroauric acid in the presence of polymer	Co, Au	–COOH group offers stability, solubility and morphology control.
PVP <sup>e</sup> [69]	Ligand exchange via hydrophobic interactions	CdSe/ZnS QDs, Au, Fe <sub>2</sub> O <sub>3</sub>	Amide within pyrrolidone structure gives water soluble properties. PVP acts as a stabilising agent for coupling to amino functionalised colloids.
PAA-octylamine [70]	Carbodiimide coupling in the presence of QDs	CdSe, CdS, ZnS QDs	–OH surface coating minimises non-specific cellular binding.
PAA modified <sup>f</sup> [71]	Carbodiimide coupling to create the multidentate ligand, followed by ligand exchange	CdTe QDs	–SH, NH <sub>2</sub> groups interact with QDs to give a small hydrodynamic radius and no degradation of optical properties.
PMAO <sup>g</sup> -block-PEG [72]	Ligand addition by hydrophobic interactions	CdSe QDs	–COOH groups offer biofunctionalisation possibilities, ester groups on hydrophilic side chains offer water solubility.
PBA <sup>h</sup> -block-PEA <sup>i</sup> -block-PMAA [73]	Ligand addition by hydrophobic interactions	CdSe, –ZnS QDs	Alkyl chains give hydrophobic interactions, while the –COOH groups give hydrophilicity and further functionalisation.
Pyr-PDMAEMA <sup>j</sup> [74]	Ligand exchange with TOPO	CdSe QDs	Pyrene is a fluorescent tracer
PMPC-PGMA <sup>k</sup> [75]	Coprecipitation of ferric and ferrous salts in the presence of the block polymer	Fe <sub>3</sub> O <sub>4</sub>	PO <sub>4</sub> <sup>–</sup> and NMe <sub>4</sub> <sup>+</sup> mimic phospholipid head groups of cell membranes, while the glycerol group holds a 1,2-diol group which creates a five membered chelate between glycerol residue and the NP.
ptBA <sup>l</sup> [1]	Ligand exchange followed by Cu <sup>I</sup> catalysed 'click chemistry' and polymerisation	Fe <sub>2</sub> O <sub>3</sub>	PO <sub>4</sub> <sup>3–</sup> and COO <sup>–</sup> .
PNIPAM <sup>m</sup> [63,76]	Silica coating and functionalisation followed by precipitation polymerisation	Fe <sub>3</sub> O <sub>4</sub> ,	–CONH– allows hydrogen bond networking.

Table 1 (Continued)

Polymer	Ligand addition method	Core	Chemical functional groups
PLLA <sup>a</sup> and PCL <sup>o</sup> [77]	In situ polymerisation with Sn <sup>II</sup> initiator	Fe <sub>3</sub> O <sub>4</sub>	–COOC– and chiral nature are biocompatible.
PMEMA <sup>p</sup> [65]	Surface activation and atom transfer radical polymerisation	Fe <sub>3</sub> O <sub>4</sub>	–COC– and –COOC–.
PVA <sup>q</sup> [78,79]	Alkali coprecipitation of ferric and ferrous chlorides followed by direct ligand addition	γ-Fe <sub>2</sub> O <sub>3</sub> and Fe <sub>3</sub> O <sub>4</sub>	–OH groups make hydrogels and a physical barrier.
PAA <sup>r</sup> [80]	Coprecipitation in the presence of polymer	γ-Fe <sub>2</sub> O <sub>3</sub> and Fe <sub>3</sub> O <sub>4</sub>	–COOH gives pH adjustable, solubility and stability in water at pH >5.
PNIPco-t-Bam <sup>s</sup> [31,81]	Thermal decomposition in the presence of polymer	Fe <sub>2</sub> O <sub>3</sub> and Co	Amphiphilic, allowing single step syntheses, –CONH– groups offer hydrogen bond networking.

<sup>a</sup> Poly(amidoamine).

<sup>b</sup> Poly(D-glucosamido ethylmethacrylate).

<sup>c</sup> Poly(2-lactobionamido ethylmethacrylate).

<sup>d</sup> Alkyl thioether end-functionalised poly(methacrylic acid).

<sup>e</sup> Poly(vinyl pyrrolidone).

<sup>f</sup> PAA modified with cysteamine and ethylene diamine.

<sup>g</sup> Poly(maleic anhydride-alt-1-octadecene).

<sup>h</sup> Poly(butylacrylate).

<sup>i</sup> Poly(ethylacrylate).

<sup>j</sup> Pyrene-poly(dimethylaminoethyl methacrylate).

<sup>k</sup> Poly[2-(methacryloyloxy)ethyl phosphorylcholine]-*block*-(glycerol monomethacrylate).

<sup>l</sup> α-Acetylene-poly(*tert*-butyl acrylate).

<sup>m</sup> Poly(N-isopropylacrylamide).

<sup>n</sup> Poly(L,L-lactic acid).

<sup>o</sup> Poly(ε-caprolactone).

<sup>p</sup> Poly(2-methoxyethyl methacrylate).

<sup>q</sup> Poly(vinyl alcohol).

<sup>r</sup> Poly(acrylic acid).

<sup>s</sup> Poly(N-isopropyl-co-*t*-butylacrylamide).

tionalised alkane ether of tetra(ethylene glycol) which is commonly used for water solubilising and stabilisation of Au NPs [84]. The exposed end of the ethylene glycol chain can also be modified to provide chemical functionality or ionic stabilisation [52,85].

### Phase transfer (PT)

Almost all biochemical reactions are conducted in an aqueous environment. However, due to the synthetic methods generally used, NPs are capped with hydrophobic ligands, meaning they are unstable in aqueous suspension. In order to overcome this barrier, a variety of PT methods have been developed to transfer NPs from organic to aqueous solution. PT agents include tetraalkylammonium salts such as tetraoctylammonium bromide (TOAB) for transfer of Au NPs; 4-(dimethylamino)pyridine (DMAP) for transfer of Au and Pd NPs [34], hexadecyltrimethylammonium bromide (CTAB) for magnetic NPs (MNPs), [58,86] and other amphiphilic species such as 2,3-dimercaptosuccinic acid (DMSA) [87], α-cyclodextrin [88] and copolymers [89] for transfer of oleic acid capped NPs. These PT agents act as labile ligands, being

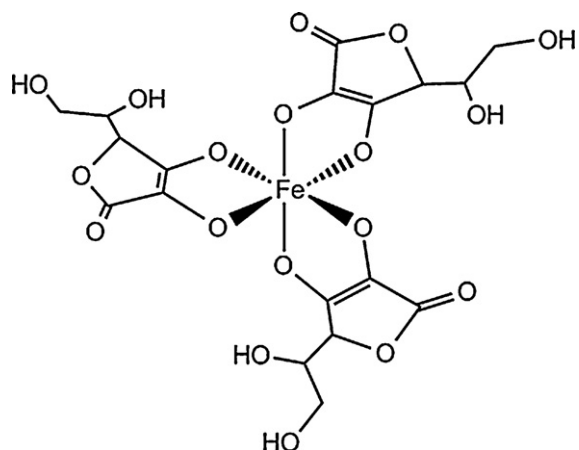
easily replaced with the desired biofunctional ligand after transfer is complete.

An alternative to using labile PT agents is to carry out a ligand exchange/ligand addition step in the organic phase, using an amphiphilic ligand capable of forming a strong NP-ligand bond (Fig. 2). A common example of this is mercaptoundecanoic acid and other mono- and dimercapto alkane carboxylic acids [42,83,90]. The advantage of this system is that the carboxylic acid terminal group provides not only water solubility but also a site for further chemical functionalisation.

A different approach takes a partially solvent-free ligand exchange and PT process in which phosphine oxide-terminated PEG is mixed with OA-capped NPs in THF to give NPs which can then be dispersed in water [91].

### Ligand exchange

Ligand exchange with increased binding strength is common for the formation of self-assembled monolayers of alkane thiols and their derivatives on the surface of Au NPs coated with ionic stabilisation agents such as citrate or tetraocty-



**Figure 2** Ascorbic acid binding to iron in an octahedral manner.

lammonium bromide (TOAB). The sulfur-gold bond is one of the most frequently used bonds in the functionalisation of NPs, having a bond strength of approximately  $210 \text{ kJ mol}^{-1}$  [92].

The alternative to exchanging weakly bound ligands for strongly binding ligands is to replace a strongly bound ligand with a ligand present in high concentration during the exchange procedure. This drives the equilibrium towards the thermodynamically less stable NPs coated with weakly binding ligands; thus satisfying Le Chatellier's principle (Eq. (1)), where  $n$  and  $m$  are the number of molecules of  $X$  and  $Y$  respectively.

$$K = \frac{[\text{NP-X}_n][\text{Y}]^m}{[\text{NP-X}_m][\text{X}]^n} \quad (1)$$

This approach can be used to replace strongly bound oleic acid with ethanol [1] and to introduce peptide sequences to PEGylated QDs [93]. However, due to the relatively high prevalence of potential ligands *in vivo*, including thiols, carboxylic acids, peptides, sugars and phosphates; ligands on NPs intended for *in vivo* use must have a high enough affinity for the NP that they will not undergo significant exchange with these species [94].

### Oxygen based ligands

**Neutral ligands.** This class of ligands includes alcohols, polyols, polyethers, carbonyls and carbohydrates. In general, there is little coordination chemistry between NPs and simple alcohols as they are insufficiently electron-donating to serve as good ligands. Moreover, the size-selective precipitation process relies on addition of ethanol, methanol, acetone and other polar solvents, to alter the polarity such that NPs are no longer stable in solution, precipitating at different polarities dependent on their size. However, serotonin has been shown to bind to CdSe nanocrystals *via* the hydroxyl groups and acetone has been demonstrated to be a stable ligand under  $\text{BH}_4^-$  and HCl conditions [25].

Polyols and ene-diols, bearing multiple alcohol groups, are capable of binding to NPs composed of transition metal oxides [95]. Dopamine and ascorbic acid [96,97] co-ordinate Fe atoms in iron oxide NPs in a favourable octahedral manner

(Fig. 2). Meanwhile in the polyol process for reduction of ionic NP precursors in organic solution hexadecane-1,2-diol is also used as a multico-ordinate ligand [35].

Polyols can be used as a selective binding agent in block polymer ligands; the double hydrophilic block copolymer, poly[2-(methacryloyloxy)ethyl phosphorylcholine]-*block*-(glycerol monomethacrylate) binds to  $\text{Fe}_3\text{O}_4$  NPs *via* the glycerol monomethacrylate block [75]. Meanwhile the zwitterionic phosphorylcholine groups do not compete with the glycerol for NP binding, but instead provide water solubility through the charged groups.

More common cases of neutral oxygen donor ligands are PEG and carbohydrates such as glucose, sucrose, and starch. However, as PEG itself is simply a polyether, it must generally be modified to bear a more reactive terminal functional group to serve as an anchor to the NP.

Glucose and sucrose have also been used in the reduction and post-synthesis stabilisation of aqueous salts of Ag and Au [98]. Starch may likewise be used for the stabilisation of noble metal and iron oxide NPs [29].

**Anionic ligands.** This class of ligands is composed predominantly of carboxylic acids and their derivatives. Two of the most well-known of these ligands are the hydrophilic citrate anion used to cap Au NPs and the hydrophobic oleic acid (OA), used in the synthesis of Fe-containing NPs in organic solvents. Oleic acid is a particularly ubiquitous carboxylic acid as it also finds use in binding and stabilising almost all core NPs. Other examples of carboxylic acid stabilisers include poly(acrylic acid), poly(methacrylic acid) and derivatives [3,80,99].

Hydroxamic acids have a high affinity for many metal oxide surfaces and can be used to introduce dendrons to the surface of NPs, providing a high degree of protection against acid etching [100].

### Nitrogen-based ligands

**Neutral ligands.** As well as acting as an organic-aqueous PT agent, zwitterionic DMAP can be used to cap Au NPs [34]. Unlike most nitrogen-based PT agents (see below) DMAP is proposed to bind to the NPs *via* the nitrogen atom bearing the negative charge in the zwitterionic form of the molecule.

The bidentate 5-methyl-6-carboxy-2,2'-bipyridine is capable of binding to  $\text{La}_{0.95}\text{Eu}_{0.05}\text{F}_3$  NPs, replacing the 2-aminoethane phosphate ligands used in the synthesis procedure [101]. The ligand assumes an energetically favourable tridentate binding mode, with both nitrogen atoms and the carboxylate group binding to the NP surface thus explaining why, despite the presence of polar groups in the ligand, such NPs are insoluble in water.

**Cationic ligands.** Tetraalkylammonium salts have a variety of applications in the synthesis and stabilisation of NPs. For example, TOAB is used as a PT agent and ionic stabiliser during the two-phase synthesis of Au NPs, which are colloidally stable for up to two weeks without further functionalisation [33]. Similarly, tetramethylammonium hydroxide provides ionic stabilisation of aqueous  $\text{Fe}_3\text{O}_4$  NPs prepared by alkali coprecipitation, protecting them from aggregation for up to a week. However, these ionically stabilised NPs are very susceptible to salting out in the presence of electrolyte concentrations well below physiological conditions.

Alkylammonium salts are also used as templates in the formation of mesoporous silica shells on NPs. The most common reagent for this purpose is cetyltrimethylammonium bromide (CTAB), which may additionally be used as a PT agent [86,102].

### Phosphorous-based ligands

One of the most important phosphorous-based ligands is TOPO. It is used primarily during the synthesis of semiconductor QDs, either as the solvent or to provide colloidal stability in the chosen organic solvent. PEG-based ligands bearing phosphine oxide groups provide stabilisation and solubilisation for NPs composed of transition metal oxides, semiconductors and noble metals [91,95].

Organophosphates and phosphonates are suitable for binding to a range of surfaces similar to those accessible by phosphine oxide ligands [1,64,103,104]. However, due to the additional bulkiness of the phosphate group compared to the carboxylate group, the reported coverage of phosphates is around an order of magnitude lower than for carboxylic acids [1].

The orthophosphate anion,  $\text{PO}_4^{3-}$ , has a relatively low affinity for NP surfaces at pH values greater than 5, but can adsorb well onto iron (hydr)oxides at low pH [47]. This is particularly important as almost all ligand-stabilised NPs for which stability data are available were studied in solutions of phosphate buffered saline (PBS).

Phosphines (e.g. triphenyl phosphine) may serve as labile ligands for Au NPs; providing steric stabilisation yet being exchanged readily for ligands with higher affinity for the metal surface [24,105,106].

### Sulfur-based ligands

*Neutral ligands.* Alkane thiols make good soft Lewis bases which makes them complementary to the soft acid properties of NPs composed of Au, Ag, Pt, Pd and their alloys [25,107,108]. With Pd NPs care must be taken with the corresponding ligand shell, as they are less stable than Au NPs, and they will decompose upon exposure to air [109].

There is evidence to suggest that even with the use of thiol terminated ligands, disulfide-like bonds form on the NP surface [109]. However, NPs with monolayers prepared from either species are indistinguishable [82].

It is also reported that disulfide ligands provide Au NPs with enhanced colloidal stability compared to monothiols [110,111], suggesting that the choice of thiol or disulfide could be used to tune the stability of the ligand shell without significantly affecting the packing of the ligand molecules.

It has been shown that mono-, dithiol and amine terminated peptides provide superior stability to peptides with no thiol groups [3,48]. Similarly the incorporation of a thioether group to the stabilising agent poly(methacrylic acid) (PMAA) allowed cobalt NPs to be stored for up to eight weeks as opposed to only 11 days with pure PMAA. This indicates the importance of thiol groups in stabilising cobalt NPs.

In view of the fact that QDs containing S, Se and Te are unstable in the presence of a monodentate thiol, polythiol ligands have been successfully employed as capping agents [112,113].

It has been demonstrated that NPs can be coated with a self-assembled and highly ordered monolayer composed of a binary mixture of ligands with a hydrophobic content as high as 66% [114]. Boal and Rotello [115] exploited this ligand self-organisation to create a self-assembled two-component recognition site for binding flavin *via* hydrogen bonding and  $\pi$ -stacking.

As well as introducing additional chemical functionality, thiol-modified biofunctional species may be added directly to the NP surface in this manner, for example, DNA bearing a 3' or 5' thiol modification can be conjugated to Au NPs [105].

Other sulfur-containing functional groups such as thiocarbamates and xanthates can be used as ligands for a variety of transition metal-based NPs, but they have lower affinity for the NP surface than thiols [82,116]. On the other hand, xanthates can provide high protection for Au NPs against etching by cyanide.

*Anionic ligands.* Sulfonates are capable of binding to iron oxide [104] but their binding affinity to Au surfaces is lower than that of thiols [114]. Micelles formed from the anionic surfactant bis(2-ethylhexyl) sodium sulfosuccinate (AOT) can be used as a surfactant in the synthesis of Ag NPs [117,118]. AOT is a labile ligand, which could be ligand exchanged to introduce chemo and biofunctional species.

### Other factors affecting ligand shell stability

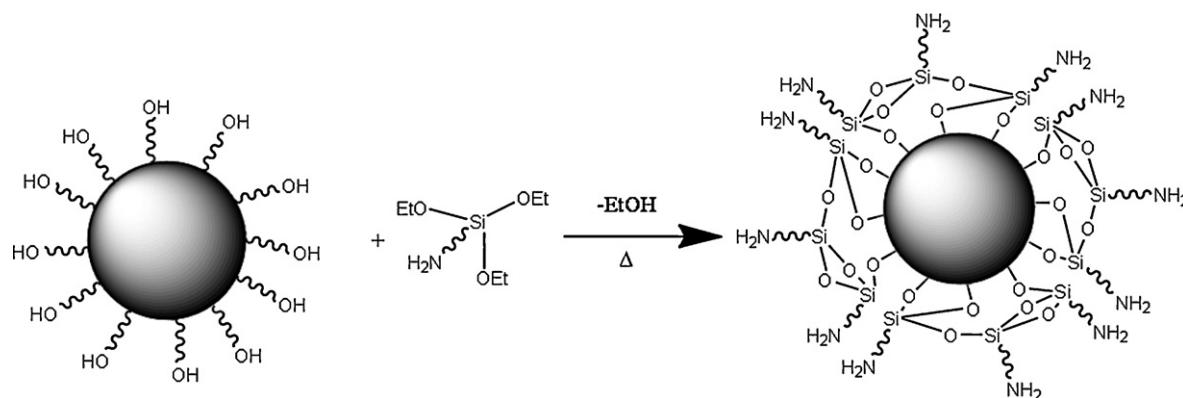
The stability of ligand shells is governed by many of the same factors that stabilise self-assembled monolayers. Stability is dominated by the strength of the ligand-surface bond, but the ability of the ligand tails to pack in an ordered fashion is also significant. The structure and hence the stability of a ligand shell can be altered by the position of a single methyl group [119], the presence or absence of a single unsaturated bond [120] or other packing factors such as chain length [121] and  $\pi$ - $\pi$  stacking [108]. The concentration of free ligands available to form a monolayer also influences the ligand orientation and hence degree of stabilisation afforded [122]. Light induced reactions at the nanocrystals/ligand interface can also often lead to desorption of the ligands [123].

### Ligand addition

Ligand addition involves a modification of the external surface of the NP-ligand shell without removal of any pre-existing ligands. The four approaches to ligand addition are outlined as follows: (1) addition of ligands to NPs initially prepared with no capping agent; (2) indirect ligand addition; growth of a layer of inorganic material such as amorphous or mesoporous  $\text{SiO}_2$ , Au, iron oxide, carbon, onto the NP surface with subsequent adsorption of a ligand species directly onto this surface by ionic or other non-specific interactions; (3) exploitation of the "hydrophobic attraction force" to intercalate hydrophobic species into the hydrocarbon shell of NPs capped by ligands such as OA and (4) formation of a covalent bond between the existing ligand and the incoming ligand.

### Core-shell structures

As expected, ligand-NP affinity is highly dependent on the NP surface and the ligand head group. In many cases the NP core is chosen for its desirable physical properties such



**Figure 3** Simplified scheme of silanisation reaction of APTES on NP surface, MPTES differs only by having  $-SH$  end groups as opposed to  $NH_2$ .

as electronic, magnetic or optical behaviour, but presents a surface incompatible with the chemical functionality of the desired ligand. In order to overcome this, the surface can be coated with a thin shell of material for which the ligand has a high affinity [124].

While this strategy is suitable for creating NPs with composite attributes, it must be noted that physical properties of the core such as saturation magnetisation,  $M_s$ , can deteriorate with increasing shell thickness. As little as four extra methylene groups in a ligand tail can have a large impact on the measured magnetic properties [125]. Meanwhile, ZnS a shell can also improve the properties of semiconducting materials such as CdSe [126].

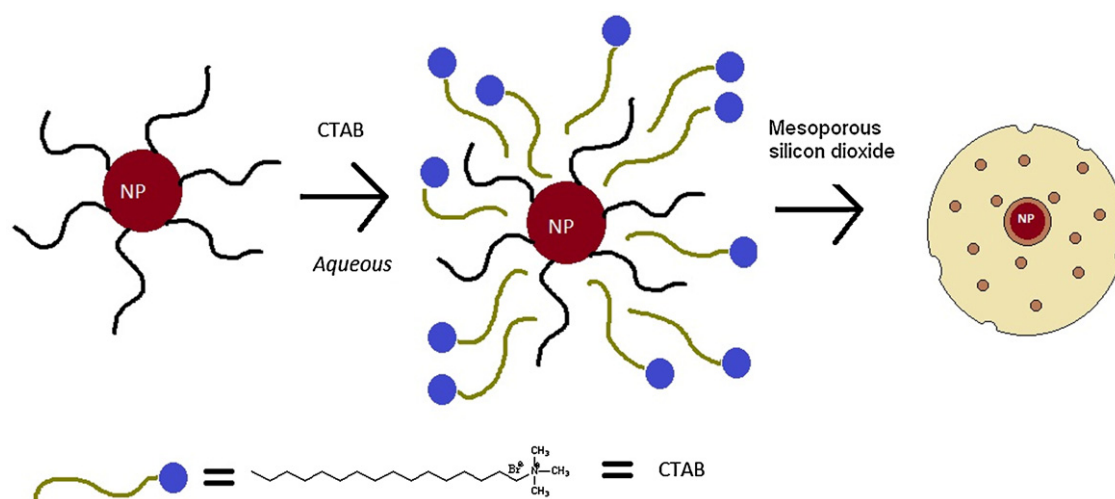
The NP shell can provide protection for the core, added modes of functionality or introduce different chemical groups for further functionalisation, in the following sections we have outlined a variety of shells that have been investigated.

**Amorphous silica.** Two main silicon-based ligands are frequently used to change the surface chemistry of NPs, silica ( $SiO_2$ ) and trialkoxysilylpropane bearing a functional group on the 3 position of the propyl chain. Common examples of the latter are (3-aminopropyl)triethoxysilane (APTES) (Fig. 3), the trimethoxy analogue APTMS and (3-

mercaptopropyl) triethoxysilane (MPTES). These silanes can be used to introduce surface functionality for further chemo and biofunctionalisation.

Using a modified version of the Stöber process, a layer of amorphous silica may be grown on the surface of NPs [20]. Modern methods involve the base-catalysed hydrolysis of a tetraalkyl orthosilicate, commonly tetraethyl orthosilicate (TEOS) in a 4:1 mix of ethanol:water; the thickness of the silica shell can be controlled by altering this ratio [27]. The growth of amorphous silica depends very strongly on the pH of the reaction mixture. At low pH, NPs become encased in a silica matrix, at mildly alkaline pH (7.5) core-shell structures are formed with individual NP cores coated by a thin layer of silica. At high pH, a mixture is formed containing the original NPs and silica NPs with no coating observed. An alternative process proposed by Graf et al. [69] involves coating NPs in PVP. The PVP-coated NPs can then be adsorbed onto the surface of aminated silica NPs and a further layer of  $SiO_2$  is then deposited by hydrolysis of TEOS.

By using a silanisation agent such as APTES instead of TEOS, a very thin layer of  $SiO_2$  may be deposited on the surface of the NPs. This layer of  $SiO_2$  is terminated with the functionalised propylene groups, effectively allowing the



**Figure 4** Use of CTAB in the conversion of hydrophobic NPs into mesoporous 'cargo transfer agents'.

introduction of any surface functional group *via* the same ligand-core chemistry. This functionality can be introduced either before or after the silanisation is carried out [58].

**Mesoporous silica.** Alternatively, mesoporous SiO<sub>2</sub> may be grown on the surface of NPs, generally after the deposition of a thin layer of amorphous SiO<sub>2</sub> as described above. The presence of a templating agent such as cetyltrimethylammonium bromide (CTAB) leads to the formation of mesopores around 2–4 nm (Fig. 4). This allows for the loading of drugs, e.g. doxorubicin, ibuprofen, and other molecular species [58,102]. Not only can such pores be used to deliver cargo, but they can also be used to scavenge biological species such as microcystins [127].

Shells of mSiO<sub>2</sub> can be deposited on NP surfaces composed of metal oxides [58], semiconductors [102], amorphous SiO<sub>2</sub> [127], and Au [128]. The emission spectrum of the QDs encapsulated with mSiO<sub>2</sub> by Kim et al. [102] is red shifted relative to the free QDs, suggesting that the QDs are held in close proximity within the silica shell. It should be noted that such encapsulation using amorphous SiO<sub>2</sub> is pH dependant [128].

**Gold and other noble metals.** Procedures for the preparation of Au NP structures include seed mediated growth and sacrificial reduction. In seed mediated growth, Au ions are reduced in the presence of the NP cores requiring alteration [86,129,130]. However, where TEM evidence is presented nucleated nano-structures are formed. This occurs because the surface of the core NP is suited to nucleation of the noble metal (i.e. Au and Ag). Due to the high interfacial energy, the noble metal grows outwards from the "core" forming a twinned structure rather than forming an encapsulating layer [86,131].

Sacrificial reduction, as the name suggests, involves the sacrifice of the outer layer of the NP core, which acts as a reducing agent to Au<sup>3+</sup> ions in solution. Oxidised core material is lost to the solution which leads to a decrease in size of the magnetic core and lower coercivity and saturation magnetisation values. However, thanks to loss of some of the initial core material the resulting NP size is roughly the same after coating as before.

Amine terminated SiO<sub>2</sub> NPs can be used as frameworks. Fe<sub>3</sub>O<sub>4</sub> NPs were grown on amine terminated SiO<sub>2</sub> NPs followed by the deposition of Au seeds (1–3 nm) and a 15 nm Au shell [86,132]. Liz-Marzan and his team have also contributed a wealth of knowledge concerning the coating of NPs with SiO<sub>2</sub>, a review of some synthetic methods for coating Au and Fe<sub>3</sub>O<sub>4</sub> and the topological effects on material function has been published and this work has since been advanced [132].

**Semiconductors.** Transfer of QD semiconductors to aqueous phase has been covered elsewhere [133,134]. By coating a narrow band-gap semiconductor such as CdSe with a large band-gap layer such as ZnS, electron/hole pairs generated by absorption of photons are confined to the core by the potential difference between the electronic bands of the core and shell thus preventing photon emission. By depositing a shell of ZnS on CdSe QDs, the quantum yield can be increased from about 10% to 50–60% [126,135,136]. By adopting a large initial Se:Cd ratio of the precursors, it is also possible to gain QYs of up to 80% [137]. Coating  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> with a CdSe/ZnS shell has also been performed and offers the advantage of dual functionality [138]. The ZnS also offers

the advantage of a physiologically stable barrier which prevents hydrolysis of the core and dissolution of poisonous Cd into the bloodstream.

**Iron oxide and other magnetic materials.** Due to the increased ease of biofunctionalisation of iron oxide, it is commonly used to coat other magnetic materials such as FePt [139,140], Co and SmCo<sub>5,2</sub> [96]. These shells are deposited by methods similar to the preparation of Fe<sub>3</sub>O<sub>4</sub> NPs by the thermal decomposition of Fe(CO)<sub>5</sub> followed by oxidation, or reduction of iron salts such as Fe(acac)<sub>2</sub>. The advantage of this technique is that non-biocompatible materials with magnetic properties superior to magnetite may be encased in iron oxide while retaining enhanced magnetic behaviour.

**Carbon.** Bulk carbon is a bioinert material which makes it a suitable material for NP shells. However, there are some suggestions that nanoparticulate amorphous carbon may display higher cytotoxicity than the bulk [141]. There are also well-published concerns about the health risks of carbon nanotubes and fullerenes, discussion of which is beyond the scope of this review. Carbon-coating of NPs can be carried out by addition of a carbon-bearing ligand followed by heating until the ligand is carbonised [55]. This technique has been proposed to be suitable for drug delivery, but no evidence has yet been published to support this idea.

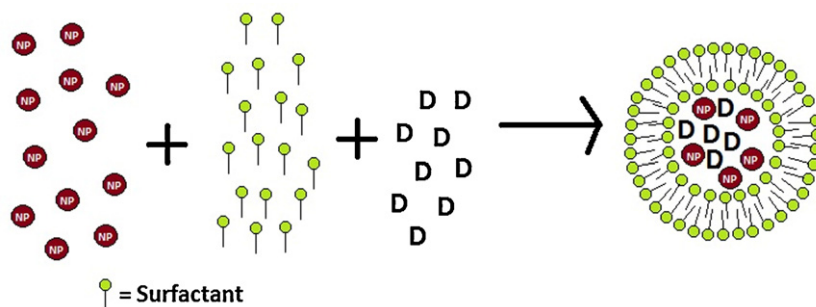
### Hydrophobic interactions

The phospholipid membrane bilayer structure in living systems is well known and there is evidence for similar structures forming during the capping of NPs with ligands bearing long hydrocarbon chains [103]. In these situations a complex system of forces and interactions attributed to the "hydrophobic effect" causes the hydrocarbon chains to interlace, forming entropically favoured structures such as micelles and bilayers [45].

These interactions can be exploited to attach other species bearing unreactive hydrocarbon chains without prior modification of the chain. Poly(maleic anhydride-*alt*-1-tetradecene) bears hydrophobic C<sub>14</sub> chains on every other repeat unit, allowing it to form an external shell around NPs capped with hydrophobic surfactants. The polymer can then be cross-linked with a hydrophilic agent allowing the NP to be further modified under aqueous conditions [142]. This approach is applicable to all core types as the only requirement is the hydrophobicity of the ligand tail group [70,142].

Phospholipids, e.g. phosphatidylcholine and phosphatidylethanolamine are zwitterionic, bearing both phosphate and ammonium groups. Such ligands can form micelles with a 5 nm hydrophobic interior capable of encapsulating TOPO-capped NPs leaving the hydrophilic head group exposed for further functionalisation after PT to water [60,143,144]. This class of nanostructure is known as solid lipid nanoparticles (SLNs).

An alternative to SLNs is to encapsulate multiple NPs in the hydrophobic interior of larger liposomes [145]. This approach has the advantage that liposomes are biocompatible and may fuse with the cell wall, allowing for relatively facile transfection. Additionally, the liposome may be loaded with a cargo species without having to bind the drug to the NPs directly (Fig. 5). This means that the cargo



**Figure 5** Use of liposomes as magnetic drug delivery moieties, D represents a chosen drug.

can be magnetically targeted, but requires no chemical modification.

### Effects of the ligand shell on the physical and biomedical properties

#### Toxicity

Ligands are essential to the reduction of cytotoxicity of many NPs, as uncoated NPs are generally cytotoxic themselves [146–148]. It is likely that significant quantities of NPs will be taken up by phagocytes and macrophages, so they must be protected from the harsh chemical environments present through the employment of a chemically inert shell [149]. However, ligands do affect the properties of NPs and these changes should be noted. A study investigated the genotoxicity of amine terminated FePt NPs in the Ames test and *in vitro* chromosomal aberration test, but further studies are required to establish whether a positive aberration result is irrelevant [150]. Details of interaction of NPs with the immune system have been published in a recent mini-review [151]. The Stellacci group have also presented a critical review of current understanding of how synthetic and natural chemical moieties on NPs interact in the body and the challenges of systematic studies [152]. For information detailing *in vitro* studies investigating the cytotoxicity of metal and semiconductor nanoparticles please see the review by Drezek et al. [153].

#### Magnetic properties

As ligands bind to NPs by donation of electrons it is inevitable that there will be some effect on the electronic state of the NP, at least near the surface. Tanaka and Maenosono [154] studied a variety of thiols, amines and carboxylic acids and report that the saturation magnetisation of the NPs is significantly lower than that of the bulk phase and the uncapped particles, with carboxylic acids showing the greatest decrease for the FePt alloy NPs studied.

However, there is some confusion over the extent of this effect due to variations in reporting magnetic data for NPs. When the ligand layer is substantial, there will clearly be a large difference between magnetisation values reported per unit mass of NPs [27,38,64,75,96,155] and that of core magnetic material. There is also evidence that non-magnetic shells can alter the physical and chemical nature (i.e. oxidation states) of atoms in the core of the NPs [129,156].

#### Optical properties

The addition of a ligand layer to semiconductor QDs can be used to passivate the surface, thereby enhancing the quantum yield [135]. Surface passivation can be carried out using species such as hexadecylamine [126], mercaptosuccinic acid [21] or PVP [157]. Conversely, conjugation of Au NPs to QDs leads to quenching of luminescence by around 70%. Cleavage of the QD-Au tether and the subsequent increase in luminescence could be used as a probe for real-time monitoring of biochemical events such as proteolysis [93].

Alternatively, the ligand shell may act as an antenna, harvesting light and delivering it to the NP core. This sensitization can lead to a large increase in the absorbance or luminescence spectra of the NPs [101,108,158].

### Biofunctionalisation

#### Common biofunctional species and their uses

##### Nucleic acids

The specific nature of DNA complementary binding is exploited in colorimetric assays for gene detection [159]. This strong, specific, non-covalent binding can also be employed to attach DNA-tagged NPs [60,105,160] to the surface of other NPs. However, it is worth noting that DNA is also capable of non-specific binding interactions with NPs, leading to less selective complementary binding interactions [161]. Non-specific binding of QDs to DNA has been shown to be entropically driven, this behaviour is frequently observed between proteins and DNA; such QDs have been labelled 'inorganic proteins' [162]. Functionalisation of QDs with hydroxyl groups reduces solubility issues encountered when attempting to detect chromosome abnormalities or mutations when using fluorescence *in situ* hybridisation (FISH) procedures [163].

##### Proteins

Like nucleic acids, proteins are known for their specific binding interactions. However, nucleic acids are limited to interactions with other nucleic acids whereas proteins can interact with a wide range of substrates and synthetic analogues [146,164,165]. Another advantage of proteins is their enzymatic activity, which may be harnessed in catalytic applications [166]. In bionanotechnology, specific functions of proteins such as antibody–antigen detection and receptor–substrate recognition such as the biotin–avidin

interaction are very useful. They can either be used to immobilise species on the NP surface (i.e. as an intermediate in the functionalisation process) [167] or constitute the desired functionality of the NP itself (e.g. for immunoassays or targeted NP delivery) [87,146,165]. Proteins can be incorporated into NPs during the coprecipitation step, in a reported one-step synthesis and biofunctionalisation [168]; however, there is little evidence that such treatment leads to retention of the protein function. More sophisticated bioconjugation methods are desirable to make sure that the protein is bound to the NP surface in such an orientation that the biological activity is retained. Such methods are discussed later in this review. An added bonus of proteins such as albumin [56] and transferrin [83] is that they may also be used in order to reduce cytotoxicity and facilitate cellular uptake of NPs. Another consideration when binding proteins to NPs is that these molecules are very large (generally tens to hundreds of kDa, or a few nm across), so this can add a relatively thick ligand shell [87,169]. In order to keep the hydrodynamic radius small enough for *in vivo* application it is desirable to conjugate only the active fragment of the protein to the NPs [170,171].

### Peptides

Natural and synthetic peptide ligands both have potential for the stabilisation and biofunctionalisation of NPs. Such biofunctional peptide sequences include "membrane translocation signals" like the HIV-Tat peptide sequence [172,173], which is capable of transporting nanoscale materials (proteins and NPs) across cellular membranes. Additional peptide sequences such as "nuclear locating signals" could be used for further intracellular targeting [84]. While peptides such as CALNN may provide stabilisation in aqueous solutions [48], there is evidence of CALNN-capped NPs aggregating when endocytosed [84]. This could be overcome by combining peptides with other steric stabilisation agents such as PEG.

### Phospholipids

Phospholipids are inherently suited to encapsulating both hydrophobic and hydrophilic payloads, allowing them to serve as the outer envelope of nanocomposite structures with potential for drug delivery and hyperthermia applications [174]. Encapsulation of silica NPs has been shown to reduce non-specific interactions thanks to the biocompatibility of the phospholipid head group. As well as biocompatibility, the head group offers the possibility for biofunctionalisation, e.g. biotinylation, allowing for further bioconjugation steps exploiting biotin-avidin interactions [175].

### Carbohydrates

Carbohydrates such as dextran have long been used in biocompatible iron oxide NPs for MRI enhancement thanks to their low cytotoxicity. There is some evidence to suggest that dextran-coated NPs may still induce cytotoxic effects – although the severity is reduced compared to the uncoated NPs [56]. However, dextran-coated iron oxide is generally claimed to be especially non-toxic as the whole system degrades *in vivo*, being safely metabolised and eliminated [176]. Another advantage of dextran is that it may

be aminated using ammonia, allowing facile conjugation with complementary chemical groups [172]. Chitosan can be used as a biocompatible coating for a range of NP core materials and, like aminated dextran, it presents amine functionality for subsequent attachment of biofunctional ligands [57,177]. Starch vermicelli has been used as a templating agent in the formation of Au and Ag NPs which can be heated to obtain potentially biomedically useful carbon-coated nanostructures [55,178].

Including starch in the alkali coprecipitation process leads to the formation of small NPs aggregated into clusters of 200–300 nm in size, such clusters have potential in the preparation of iron oxide-carbon nanocomposite materials [55].

Smaller carbohydrates such as lactose, glucose, mannose [179–181] can be thiolated for attachment to Au NPs by ligand exchange. The particles prepared this way may be used as sensitive colorimetric probes for a variety of metal ions. Mannose and lactose have also been used for the reduction of Au and Ag salts and stabilisation of the resultant NPs [98].

Closely related to carbohydrates, oligosaccharide aldonic acid shells can be used to alter cellular uptake of NPs, with maltotrionic acid leading to reduced cellular uptake whereas lactobionic acid increases uptake [182]. Again, these NPs exhibit low cytotoxicity at NP concentrations of 200  $\mu\text{g mL}^{-1}$ .

## Coupling strategies for biofunctionalisation

### Carbodiimide coupling

While it is possible to form amide bonds directly between a terminal carboxylic acid group on the surface of a NP and a free amine on a biological species like proteins [83], reaction efficiency can be enhanced through the use of additives.

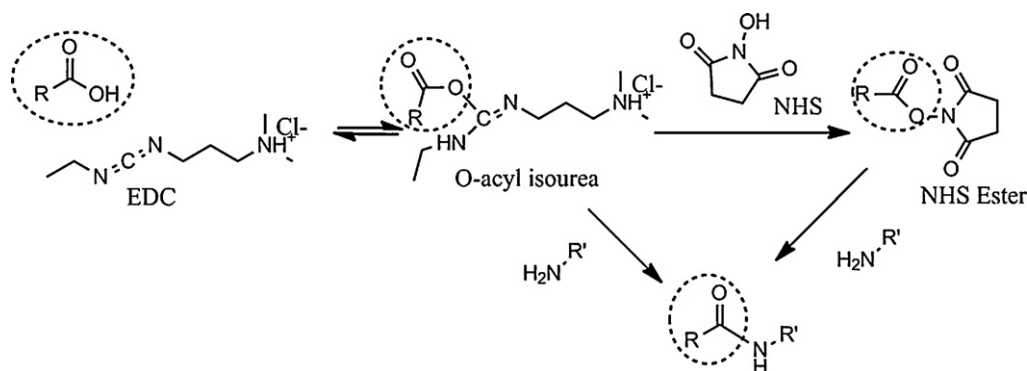
Carbodiimide coupling is used to covalently link carboxylic acids to amines *via* formation of a "zero length" amide bond [73]. The key advantage of this procedure is that it involves no lengthy linker species, allowing the hydrodynamic radius of the NP to be minimised. The most common carbodiimide coupling strategy used 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC or EDAC) (Fig. 6) as the coupling agent.

This strategy has been applied to coupling of enzymes to NPs, with retention of up to 50–80% of enzymatic activity, depending on the enzyme [169]. The efficiency of the coupling reaction can be increased by stabilising the *O*-acylisourea intermediate by formation of the succinimide ester. This is achieved by addition of NHS or sulfo-NHS. Rather usefully bis-*N*-hydroxysuccinimide can be used without a carbodiimide activation agent allowing the conjugation of two amines thanks to the two NHS ester groups present [165].

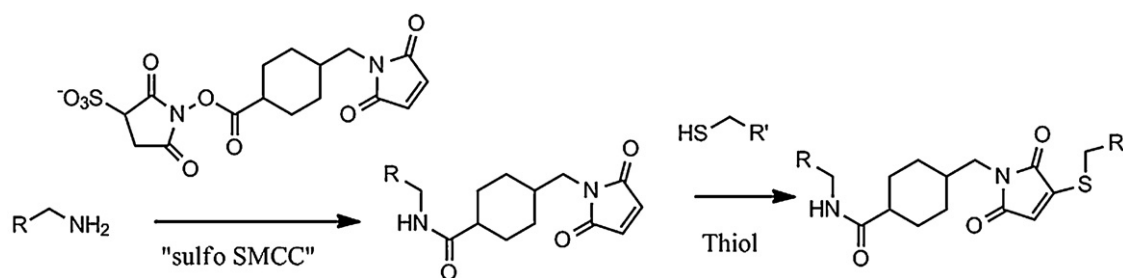
EDC-activated species may be coupled to existing ligands [183] or, if the synthesised NP bears hydroxyl groups on the surface, the activated species may be directly coupled to the NP surface *via* a net dehydration reaction, leading to an ester linkage as opposed to an amide [146,167,184].

### Maleimide coupling

A maleimide may be used to conjugate primary amines to thiols as illustrated in Fig. 7 [185]. The most commonly used



**Figure 6** Schematic of carbodiimide coupling of an acid to an amine using EDC as the coupling agent. An *N*-hydroxy succinimide (NHS) ester intermediate may be formed to increase reaction efficiency.



**Figure 7** Maleimide coupling of an amine and a thiol using sulfo-SMCC as the linker.

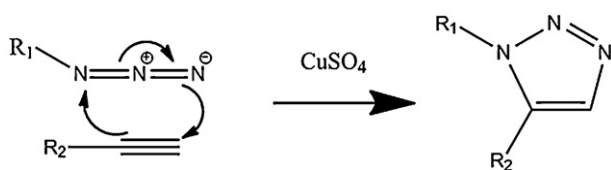
maleimide-derived coupling reagent is sulfo-succinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC).

Maleimide coupling has been used to conjugate biomolecules such as DNA [60], herceptin [87] and proteins [164,186] to NPs. Lee et al. [187] used maleimide coupling to link iron oxide and dye-doped silica NPs, creating MRI- and fluorescence-active imaging agents.

#### Click chemistry

Another common route for bioconjugation, the Cu(I)-catalysed alkyne-azide cycloaddition reaction also known as “CuAAC” or “click chemistry” involves the coupling of an alkyne to an azide giving a 1,2,3-triazole ring, which serves as a strong covalent bond between the NP and the biofunctional moiety (Fig. 8). This process has been demonstrated to be highly versatile, suitable for conjugation of a variety of species including small molecules.

This procedure provides versatility through the fact that either the alkyne or the azide could be expressed on the biofunctional moiety, with the complementary functional group expressed on the NP surface. Combined with the variety of



**Figure 8** Schematic of Cu-mediated alkyne-azide cycloaddition (“click reaction”). The reaction proceeds *via* a Cu(I) intermediate (CuSO<sub>4</sub>) [1].

ligand head groups available for NP-ligand bond formation, this procedure has a lot of potential as a coupling approach for bioconjugation [1]. Furthermore, the one-step click process has been shown to give the possibility of introducing multiple functionalities onto the NP [188]. However, the procedure does have the disadvantage that preparation of the azide or alkyne-functionalised bioactive species is often lengthy and low yielding [189]. So, while the final cycloaddition step itself proceeds rapidly and with high yield, the overall functionalisation procedure can end up being long and inefficient.

#### Disulfide bridges

Disulfide bridges have been used for the reversible chemical coupling of NPs. Tian et al. [190] showed that the oxidation and reduction of disulfide bridges between silica NPs and Fe<sub>3</sub>O<sub>4</sub> NPs was facilitated by glutathione disulfide (oxidative bond formation) and dithiothreitol (DTT) (reductive bond cleavage). While current work appears to be focussed on the formation of hybrid nanostructures, this approach could also be adapted for drug delivery because cleaving agents such as glutathione (GSH) [191], are present at appreciable concentrations *in vivo*.

#### Histidine tagged proteins

The addition of six terminal histidine residues (“6 His tag”) on a protein allows the protein to act as a chelating agent, coordinating to a metal cation held to the NP surface by nitriloacetic acid groups. This type of binding interaction is utilised in the magnetic assisted purification of proteins extracted directly from cell lysate [96,107] and specific enzyme binding to the surface of Au NPs giving almost

complete retention of enzymatic activity [90]. This binding interaction is capable of detecting 6 His-tagged proteins at concentrations as low as 0.5 pM without non-specific binding to undesired proteins occurring [107].

### Ionic coupling

Charged NPs may be coupled either with oppositely charged biological and polymeric species [192], or indeed to different oppositely charged NPs [193]. Some obvious examples of biological application are the coupling of negatively charged DNA [30,194] or liposomes to positively charged NPs [195,196]. With careful tuning of the pH it is possible to couple a variety of proteins, which can be either cationic, anionic or neutral, to the surface of oppositely charged NPs. Immunoglobulin G (IgG), a cationic species, can be immobilised onto the surface of citrate stabilised Au NPs at pH 6 [167].

Radionuclides [172] and imaging agents such as Gd [197] may also take advantage of ionic coupling to chelating agents allowing another means of NP detection or making way for a therapeutic guided delivery of radioactive compounds.

### Specific bio-recognition interactions

In order to retain the specific recognition ability of antibodies, it is desirable to bind the antibody in a fixed position relative to the NP so that the antigen binding site in the Fab fragment remains exposed. If the antibody has multiple antigen binding sites in the Fab fragment it is sometimes possible to bind the antibody to the NP *via* an active site irrelevant to the biomedical application. This is demonstrated by Ho et al. [167], in which protein G is bound to the NP surface, immobilising IgG *via* interactions with the Fc region of the antibody but leaving the Fab region of the protein free for specific *in vitro* recognition of *Staphylococcus* bacteria.

Complementary binding of base pairs is exhibited in double stranded DNA and it is desirable to emulate this in bioconjugation. For example, nucleotide specific interactions were used for reversible assembly of DNA conjugated NPs by Mirkin et al. in 1996 [198]. Through attachment of single stranded DNA to NPs, self-organisation was also achieved dimers and trimers of NPs [105]. A protocol for the conjugation of specific DNA aptamers onto Au NPs was reported to give an optical response upon the binding of different analytes [199].

Avidin and analogues such as streptavidin and neutravidin are capable of forming up to four interactions with biotin, making it an ideal cross-linker. Such functionality can be exploited through the conjugation of biotinylated PEG or phospholipids to NP surfaces [68,175,200,201] which can then interact with avidin or avidin-functionalised species. Synthesis of biotinylated NPs specific to free avidin could make good avidin sensors [68] or the incorporation of avidin species into the NP allows the possibility of using the site for addition of further biotinylated species to form a "B-A-B" linkage. While this is a strong and specific non-covalent interaction suitable for the formation of 3-D nanostructures [200,202], a significant setback is that a B-A or B-A-B linkage is long, adding significantly to the hydrodynamic radius of NPs and reducing their suitability for some *in vivo* applications.

## Biomedical applications

The small nature of NPs allows them to cross cellular membranes and avoid detection by the reticuloendothelial system and their high surface area to volume ratio can allow increased loading of therapeutics; such properties makes NPs desirable for diagnostic and therapeutic applications which are briefly detailed, as follows [133,203,204].

NPs are employed for imaging in a variety of ways, both for medical purposes and further understanding of biochemical processes *in vitro* and *in vivo* [205]. General reviews covering the applications of a variety of materials for biomedical imaging including Au, QDs, and MNPs have already been published elsewhere [148,206].

After Faraday studied finely divided Au in 1857, little attention was paid to its photophysical properties until Turkevich et al. published a paper detailing the nucleation and growth processes of colloidal Au in 1951. This resulted in a wide expansion of research into Au NPs to further our understanding and establish possible biomedical applications. The plasmon absorption and scattering properties of Au NPs make them favourable materials for imaging and sensing [159,207–211]. The author is directed to a review published by Boisselier and Astruc for extensive coverage of Au NPs including use of surface enhanced Raman scattering (SERS) imaging which has been used for targeting and imaging of cells and cancer markers [66]. Au NPs also find application in therapy; light has been demonstrated as a good orthogonal stimulus to effect drug release from gold NPs acting as both cage and carrier [212]. Au NPs with engineered surfaces have been shown to aggregate in low pH environment resulting in a NIR shifted absorption band; this shift has been exploited to yield a new proof of concept for photothermal cancer therapy [213]. Recently, site selective assembly of MNPs on to Au rods yielded NPs with tunable optical and magnetic properties, these probes were shown to exhibit simultaneous detection, separation, and thermal ablation of multiple pathogens [214]. Through the attachment of an antibody and platinum complex *via* a thiol based linker onto the gold part of dumbbell shaped Au-Fe<sub>3</sub>O<sub>4</sub> nanoparticles, target specificity and strong therapeutic effects have been demonstrated in the treatment of Her2-positive breast cancer cells [215].

The semiconducting nature of QDs gives them excellent, tuneable, photophysical properties [216]. Labelling of cellular proteins, cell tracking, pathogen and toxin detection, *in vivo* animal imaging and fluorescence resonance energy transfer (FRET), are all techniques that have been developed to take advantage of these properties; please see previously published reviews and articles for further information regarding biological applications of QDs [7,112,134,217–224]. Diffusion dynamics of glycine receptors have been revealed by single quantum dot tracking, thus demonstrating the power of QDs in furthering our understanding of *in vivo* molecular dynamics [225]. Photodynamic therapy (PDT) uses the combination of a photosensitizing drug and light in the presence of oxygen to cause selective damage to target tissue. There is growing interest in the potential for QDs to act as FRET donors in conjunction with conventional photosensitizing agents for PDT. QDs possess

superior properties to organic photosensitizers including a broad absorption spectra; these attributes are all described elsewhere [226].

Magnetic NPs have long been used as contrast agents for magnetic imaging; the reader is directed to recent reviews which cover progress towards improved contrast agents derived from Fe, Co and Pt for MRI and MPI applications [5,53,66]. Research has been growing in cellular MRI (CMRI); this type of cell tracking shows potential in the evaluation of novel drug therapies [206]. Apoptosis of tumour cells was reportedly detected with MRI using a contrast agent in 2001 [227]. Resolutions of up to 1 mm have been achieved by taking direct signals from magnetic NPs; magnetic particle imaging (MPI) technology also has the advantage that it does not require bulky full body scanners [5].

Drug delivery systems provide an important tool for increasing the efficacy of pharmaceuticals through improved pharmacokinetics and biodistribution. MNPs have been investigated as platforms for transport of drugs and genes; delivery can be performed *via* passive, active or direct means [228]. Research into the functionalisation of MNPs through different coatings and structures with emphasis on the active targeting approach continues to yield results [53,229].

The low pH conditions inside breast cancer cells can reportedly cause acid etching of herceptin conjugated porous Fe<sub>3</sub>O<sub>4</sub> NPs to further open the pores to give a regulated release of cis platin into the cell; this result indicates a possible delivery formulation for target specific therapeutic applications [230]. Magnetic separation technology has been implemented into a continuous flow microfluidic device to separate individual cells and can be observed visually using a low power microscope [231].

Magnetic actuation for *in vitro* non-viral transfection and tissue engineering, *in vivo* drug and gene delivery, and recent clinical results for magnetic hyperthermia treatments by direct injection of amino silane coated iron oxide NPs are presented by Pankhurst et al. [5]. Efforts are continuing in the ultimate goal of functionalising MNPs for target specific hyperthermia treatments [3,4,232]. Analysis has been done on single intracellular NPs to establish a mechanism of action for intracellular heating; this work indicated the formation of short lived 'nanobubbles' which have potentially explosive behaviour in cells and could be used for future cancer therapy [233]. By taking a synergistic approach, incorporation of cytotoxic carboplatin into Fe@C-loaded chitosan magnetic NPs followed by thermal induction has shown efficient destruction of tumour cells [234].

## Conclusion

In order to successfully prepare and biofunctionalise nanoparticles for a given biomedical application, a wide range of physical, chemical, biological and physiological factors and conditions must be taken into account. However, by tuning the nature of the core, shell and ligands, these factors can be taken advantage of to provide the desired, biocompatibility and biofunctionality, making inorganic nanocrystals suitable for a very wide range of applications in diagnostics and therapy for numerous medical conditions.

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