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**Chapter 5. MAIN CHALLENGES ABOUT SURFACE
BIOFUNCTIONALIZATION FOR THE *IN VIVO* TARGETING OF
MAGNETIC NANOPARTICLES**

Laurent Adumeau, Marie-Hélène Delville and Stéphane Mornet*

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ABSTRACT

Magnetic nanoparticles have attracted attention in nanomedicine owing to their potentialities offered in bioimaging, drug release, magnetic hyperthermia and diagnosis devices. The conjugation of selected affinity ligands on their surface enables the specific targeting of overexpressed receptors in diseased tissues or cells and can increase for instance the therapeutic index of drugs used in chemotherapy or the diagnosis value of images in magnetic resonance imaging. The bioconjugation appears as the ultimate step in the design of targeted-MNPs and is therefore critical since it can determinate their final *in vivo* biodistribution. This chapter summarizes the main bioconjugation procedures, followed by the description of the interface between MNP and the biological environment. In a last part, the critical parameters affecting the targeting efficiency and the MNP biodistribution is presented as well as pre-assessment methods.

5.1. Introduction

Magnetic nanoparticles (MNPs) have found applications in various fields of biomedicine and biotechnology. Their magnetic properties makes them useful as efficient contrast agents for nuclear magnetic resonance imaging (MRI),^{16,35,49} in hyperthermia therapy¹⁶ and also in magnetically-guided nanoparticles.⁵⁰ Moreover, MNPs could be functionalized with other components, such as ligands, metal oxides, enzymes, antibodies etc. As a result, these biofunctionalized MNPs have shown many applications in various areas, such as molecular MRI, drug and gene delivery, hyperthermia, proteomics and peptidomics analysis in recent years. With the advantages of a large surface area and a possible manipulation by an external magnetic force, MNPs have also been considered as extractive substrates for efficient enzymatic reaction. Among the different varieties of iron oxide nanoparticles (NPs), the nontoxic (at low clinical dose for humans between 0.56 to 3 mg Fe/kg of patient body weight¹⁵), biodegradable and biocompatible γ -Fe₂O₃ and Fe₃O₄ revealed to be the most commonly used for applications in bio-related fields.^{13-14,38,42,51-52}

The design of biofunctionalized MNPs for *in vivo* targeting applications is complex because it

must take into account all the specifications required by the biological character of the application. For the MNP design of a given architecture, the chemist must integrate in a conjugation strategy of biomolecules (called thereafter ligand), the choice of the ligand-receptor system and the specifications of the administration route that will contribute to a better targeting efficiency. This chapter dedicates to chemists who want to embark on this adventure. The first part consists of (i) the main available bioconjugation routes depending on a selected MNP synthetic method and (ii) the surface modifications steps required before conjugation to make the MNP biocompatible. The second part is dedicated to the description of the interactions between the NP surface and its biological environment and a common way to manage them. Finally, in the last part, the description of the current challenges is introduced in terms of benefits and drawbacks identified in conjugation of MNPs designed for the *in vivo* active targeting.

5.2. Basic principles of surface bioconjugation of MNPs

5.2.1. Different surface types of MNPs as a function of the synthetic methods

The design and preparation of functionalized MNPs for their applications in bio-related fields such as drug delivery for example require a strong control of the physicochemical properties of materials in terms of payload drugs and drug carriers, of their behaviours in biological and physiological environments, as well as the targeted functions that will address the relevant medical problems. In order to have an efficient drug delivery, the drug carriers based on these MNPs should be able to, (i) exhibit as high as possible loading of drug molecules, (ii) protect the drug bioactivity and enhance its biocompatibility, (iii) specifically target the anticipated delivery keeping the uptake by the normal organs and/or tissue as low as possible. The preparation methods of the magnetic cores with uniform size, controlled shape, and desirable compositions,^{5,42,43,52-54} are also covered in Chapter 1 “Controlling the size and the shape of uniform magnetic iron oxide nanoparticles for biomedical applications” and Chapter 3 “Carbon coated transition magnetic metal nanoparticles for

clinical applications” of this book. In brief, the most classical ones: co-precipitation,^{30,55} thermal decomposition,^{5-6,11,14-15,24,30-31,38,41-43,46,49,54,56-57} hydrothermal synthesis,^{43,54,58} microemulsion,^{57,59} and polyol synthesis,^{7,11,14-15,20-21,31,41-42,49,58,60-61} sonochemistry,^{5,14,42-43,47,59} microwave-assisted synthesis,^{42,47,62-63} reduction–precipitation.^{22,32,64}

The available surface of the MNPs will then strongly depend on the given synthetic approach, even if it is generally assumed of hydroxyl groups covering the coordinating sphere of the NPs. These MNPs are therefore amphoteric and may develop surface charges in interaction with their environment depending on the pH of the solution, that is higher or lower than the MNPs point of zero charge. Some of these synthetic methods⁵² require the use of surfactants and ligands during the reaction, species which then remain on the particle surface. Table 1 summarizes the surface states of MNPs with regards to their synthetic pathways.

<i>Surface features</i> <i>Synthesis methods</i> <i>(references)</i>	Dispersion medium	Surface state of resulting particles	Surface-capping agents
Coprecipitation (30,54,59)	Water	Uncoated NPs, M-OH groups, no molecules, potentially positively or negatively charged	None, added during or after reaction
Hydrothermal/ Solvothermal (22,43,53,54,56,57)	Alcohols/ water	Coating needed, added during reaction	None or coated with polyethylene glycol (54)
Sonochemistry (5,14,42,43,57,59)	Water	Uncoated NPs, M-OH groups, no molecules, potentially positively or negatively charged	Needed, added during or after reaction, possible direct grafting of biomolecules on the surface
Polyol (7,11,14,15,20-22,31,41,42,49,52)	Alcohols (DEG, EG, ...)	Coating needed, added during or after reaction	Chemisorption of (poly)alcohols
Thermal decomposition (5,6,11,14,15,24,30,31,38,41-43,46,49,53-56,62)	Organic solvent of high boiling T (i.e. octadecene)	Coating needed, added during reaction	Coated with various surfactants carbonyls, fatty acids such as oleic acid, hexadecanediol, oleylamine, hexadecylamine
Microemulsion (31,43,54,56-59)	Non-polar organic solvent (alkanes)	Coating needed, added during reaction	Coated with various ionic and/or non-ionic surfactants i.e. cetyltrimethylammonium bromide, sodium dodecylbenzenesulfonate, or AOT, Brij®, NP5,...
Microwave-assisted synthesis (42,47-62)	Organic solvent (i.e. Benzyl alcohol)	Coating needed, added during reaction	Coated with fatty acids (oleic acid)
Reduction–Precipitation (22,32,63)	Water	Uncoated NPs, OH groups, no molecules, potentially positively or negatively charged	None, added during or after reaction

Table 1. Comparison of MNP surface features according to their synthetic methods.

5.2.2. Subsequent relevant pre-functionalization steps

Controlling the interactions of nanomaterials with the biological environment is a fundamental challenge. In particular, the NP surface must be covered by a suitable biocompatible coating that must exhibit different functions such as: (i) to protect the iron oxide NPs and prevent their degradation when exposed to different environments such as blood or lysosomes, (ii) to prevent the aggregation of the NPs by controlling the attractive van der Waals forces and last but not least (iii) to provide the right functional group(s) in the right amount.^{2-8,14-15,20-21,24-25,31,37-43,45-47,59,65-68} The resulting inorganic/organic objects then exhibit core/shell architectures, with a shell which may be formed of organic molecules, polymers or inorganic shells and be covalently or adsorbed to the NPs and which may be functionalized by adding various functional groups via classical (bio)-organic

Reaction type	Functionalized NP	Reactant	Final NP
Amide bond formation			
Amide bond formation			
Epoxide opening			
Addition of amine to cyanates			
Michael addition			
Amide bond formation			
Imine bond formation			
Imine bond formation			
Azide-alkyne cycloaddition			
Ring closing or opening metathesis			
Diels-Alder reaction			

routes (See Table 2).^{69,70}

Table 2 List of the different conjugation processes of MNPs from implying functional groups either

on the ligand (schematically represented by a piece of puzzle) or on the MNPs.

Most of the time, a pre-functionalization step of the surface is compulsory to provide the attachment of the targeted biomolecules. They are summarized in Figure 1, which gathers the different possibilities of functionalization depending on whether the MNP surface is bare and hydrophilic or pre-coated with the ligand used for their synthesis.

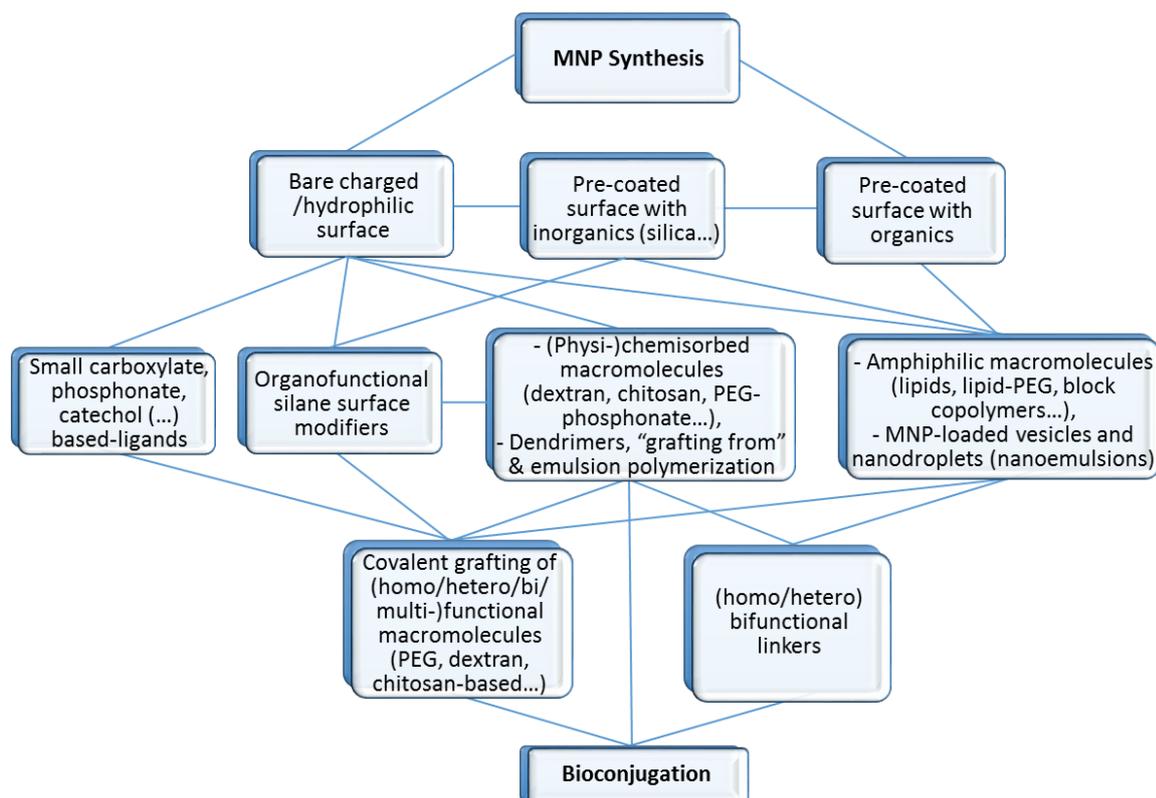


Figure 1. Chart of different main routes for the development of bioconjugated MNP-based formulation in the context of *in vivo* applications. For a better readability, only the boxes showing the main routes used are connected together. Other connections can be added by applying additional treatment for compatibility such as ligand exchange, phase transfer or chemical surface leaching procedures.

Therefore, the design and preparation of biofunctionalized MNPs require multiple approaches integrating multiple criteria according to the given targeted application *in vivo* (drug delivery, MRI, hyperthermia, theranostic approaches). They depends on (i) the physicochemical properties of the nanomaterial itself (degradation, metabolism, potential toxicity and immunogenicity, drug payload

and release capacity), (ii) its colloidal stability in physiological media; (iii) its behaviour and responses in the biological or physiological environment (circulation time in blood, ability to cross through physiological barriers, ...), (iv) and the intended functions that address relevant medical problems.

5.2.2.1. Different types of anchoring groups

The preparation of the surface for a further bio targeting depends as mentioned above on its state surface. The bond generated between the surface of iron atoms and the anchoring groups are not so obvious, it is between pure ionic nature or pure covalent one, then be called ionocovalent bond, the degree of covalence being strongly dependent on the chemical nature or the anchoring atom (O, N, S, P).^{11,15,42,53,71,72}

Organofunctional silanes coupling agents

A large variety of surface functional groups such as amine, carboxyl, hydroxyl, pyridine, amide, aldehyde, epoxy, thiol, and others has been described so far (Table 2).^{14,73} One of the easiest way to promote the functionalization of MNPs with such organic molecules is to proceed to the surface silanization and the subsequent strengthening of the polysiloxane network. This reaction provides a new surface, which presents satisfying responsivity, low cytotoxicity, high stability under acidic conditions, inertness to redox reactions; it then becomes easy to perform surface chemical modification. Functional alkoxysilane agents include mercapto-propyltriethoxysilane, 3-aminopropyltriethoxyloxysilane, and p-aminophenyltrimethoxysilane as the most frequently used to control the surface of the MNP and provide colloidal stability as well as potential interactions with biological molecules such as antibodies (Ab), nucleic acids, enzymes, or proteins.^{5-6,74-79}

Carboxylates and Catechol derivatives

Other frequently used small stabilizers are based on monomers bearing multiple carboxylate functionalities for the colloidal stabilization of MNPs and at least one carboxylic acid group

exposed to the medium. These free carboxylic groups provide negative charges on the surface of particles, making them hydrophilic.⁷⁹

When the MNPs are synthesized in organic solvents, their biocompatibility can be obtained through a ligand exchange of the hydrophobic surfactants with hydrophilic ligands which consist of two functional groups; one group strongly binding to the NP surface and the other is hydrophilic so that the NPs can be dispersed in an aqueous solution or be further functionalized. . Modification of the iron oxide NP surface with dopamine anchoring group dis a nice example of such a ligand exchange with a strong binding of dopamine with the surface Fe(III) via the diol bidentate bonding.^{80,81} Other examples of biocompatible ligands¹¹ with anchoring groups can also be found in the literature such as water-soluble zwitterionic dopamine sulfonate⁸¹, 3,4-dihydroxyhydrocinnamic acid⁸², 2,3-dimercaptosuccinic acid (DMSA)^{51,82,83}. The ligand shell can further be coupled with other targeted functional molecules via thiol-based linker chemistry.

Phosphorous derivatives

However, phosphate- and phosphonate- based ligands reveal to be more efficient due a much stronger Fe-O-P bond on the oxide surface. These ligands are promising provided they also exhibit other functional groups as in the case of poly(vinylalcohol phosphate) macromolecules to make the MNPs hydrophilic and biocompatible.⁸⁴

5.2.2.2. Polymers

Coating MNPs with polymer provides an alternative option for the previously mentioned small anchoring agents, leading to particles with the properties of the macromolecular systems grafted to the particle surface. These polymer-functionalized MNPs have been receiving much attention, since the polymer coatings not only provide excellent colloidal stability but also increase repulsive forces and balance the magnetic and the van der Waals attractive forces acting on the MNPs. The main advantages of polymer macromolecules are that they (i) have multiple functional groups on the polymer backbone, which introduce multiple anchoring points to the NP surface, (ii) improve the

colloidal stability, and (iii) give rise to further reactions with the remaining groups. Both natural^{5-6,13-14,20,35-36,39-40, 44,47,57,61,64,67,79} (dextran, chitosan, starch, cellulose, gelatin, alginate) and synthetic^{6,38-39,52,75,79} polymers polyethylene glycol (PEG), polyvinyl alcohol (PVA), polylactic acid (PLA) polymethylmethacrylate (PMMA), polyacrylic acid (PAA) for the most important and many others⁷⁹) have been used.

Most common coating materials for MNPs include polystyrene,^{85,86} polysaccharides,^{13-14,20,37-39,42,46,47,86} polyacids,⁸⁷⁻⁸⁹ polyols,^{47,86,90-92} polypeptides,^{13,24,31,35,38,42} and polyamines.^{86,93-96} Some of these polymers are even able to replace oleate ligands with minor alteration of the hydrodynamic sizes of the now water-dispersible particles.⁷¹

Hydrophobic effects

Monodisperse MNPs are often obtained in organic solvents (Table 1) with a hydrophobic capping layer due to the long alkyl chains of the ligands. Therefore, there is a real need to obtain NPs which are water-dispersible, biocompatible, readily surface-functionalized for biomedical applications.¹¹ A smart approach based on the use of amphiphilic molecule encapsulation consists of taking advantage of the hydrophobic effects between the surface ligands and the hydrophobic segments of chosen amphiphilic molecules such as poly(maleic anhydride) derivatives,⁹⁷ PEG-derivatized phosphine oxide or PEG-phospholipid. It is also possible to play with hydrophobic effects coupled with coordinating interaction of Tween derivatives.⁹⁸ A PEG-phospholipid copolymer, has also been used to modify hydrophobic magnetic NPs through a ligand addition strategy.⁹⁹ By simply mixing the as-synthesized magnetic NPs with an amphiphilic ligand in a proper solvent, effective ligand addition could be achieved.⁹⁹⁻¹⁰⁰

Amphiphilic polymers

Many amphiphilic polymers have also been developed, such as poly(maleic anhydride-alt-1-octadecene), poly-(maleic anhydride alt-1-tetradecene), poly(styrene-block-acrylic acid) copolymers, and polyethylene glycol-block-poly lactide.^{35,38,42,95,101} An interesting polymer

candidate for coating of MNPs such as polyethylene glycol-g-poly(ethylene imine), was shown as a good alternative to pure poly(ethylene imine) in terms of colloidal stability and cytotoxicity due to the introduction of the PEG shielding moieties.⁹⁵ The carboxylic and amino functional groups present in these polymer backbones are potential sites for further targeted bioconjugation.¹⁰²

5.2.2.3. *Inorganic coating*

Besides the polymeric materials described above, inorganic materials⁴⁶ such as silica,^{5-7,13-15,20,35,38-43,52, 65,67,76,79,80,102-107} carbon,^{6,41,52} and graphene-based materials have also been used as coating materials. Silica has been so far the most used coating due to its characteristics (easy regulation of the coating process, processability combined with chemical inertness, controlled porosity, and optical transparency).⁵³ The further use of organofunctional coupling agents is once again compulsory (*as mentioned above in section 5.2.2.1*) and generates the formation of an outer layer exhibiting various functional groups such as $-\text{NH}_2$, $-\text{Br}$, $-\text{CN}$, $-\text{OH}$, $-\text{CO}_2\text{H}$, $-\text{SH}$, and epoxy which themselves may undergo transformation to other groups using standard organic methods or methods more dedicated to biofunctionalization.⁷⁰ These silica shells may also exhibit interesting properties for biomedical application such as incorporation of fluorescent dyes into the SiO_2 matrix and controlled mesoporosity useful for the drug loading.⁵³

The coating of hydrophobic NPs with inorganic materials was also performed to generate other metal oxides at the surface of MNPs. For example, a sol-gel reaction of tantalum(V) ethoxide in a microemulsion containing Fe_3O_4 NPs provided multifunctional $\text{Fe}_3\text{O}_4@ \text{TaOx}$ core-shell NPs with a good biocompatibility and a prolonged circulation time. When intravenously injected, these particles could enhance the contrast of X-ray computer tomography (CT).¹⁰⁸ A TiO_2 coating was also performed on $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ and the resulting particles could be modified with dopamine, which attaches onto the surface of the titania substrate. The further immobilization of succinic anhydride onto the surface of the $\text{Fe}_3\text{O}_4@ \text{TiO}_2$ NPs via dopamine was then followed by the immobilization of immunoglobulin G (IgG) via amide bonding. The resulting magnetic NPs not only had the capacity

to target different pathogenic bacteria, but could also effectively inhibit their growth under a low-power UV lamp irradiation within a short period.¹⁰⁹

5.2.3. Common synthetic strategies for bioconjugation

The functional groups grafted on NPs for the subsequent conjugation reactions, are also found on and complementary to the naturally occurring biomolecules (proteins, DNA, carbohydrates, lipids, amino acids etc.). They are limited to the following: carboxylic acids R-CO₂H, amines R-NH₂, hydroxyls R-OH, ketones R-COR', thiols R-SH, aldehydes R-CHO and to groups involved in hydrogen bonds RH interactions^{48,71} which also limits the number and type of reactions that can be used to form a linkage between the MNP and the target biomolecule. The most common chemical reactions involving these groups include *N*-hydroxysuccinimidyl (NHS) ester modification of amines along with carbodiimide-mediated (EDC) condensation of carboxyl groups with amines, maleimide conjugation to thiols, and diazonium modification of the phenolic side chain on tyrosine. Table 2 illustrates the main conjugation reactions that have already been applied to NPs for their functionalization. It is noteworthy to mention that chemistries can be applied with reactive groups on either the NP or the biological molecule of interest or both.

Many synthetic biomolecules such as peptides and nucleic acids, revealed to be very useful because they can be modified by any functional group (amines, thiols, carboxyls, biotin, azides, alkynes) and introduced as needed on the NPs. Another commonly used biochemical technique involves the biotin-avidin strong interactions. It has been used for bioconjugation of many NP materials using the grafting of either biotin or avidin on the NP surface.

5.3. The nano-bio interface

5.3.1. Specification analysis for *in vivo* applications of bioconjugated MNPs

MNPs synthesized according to the procedures given in Table 1 are not usable directly for *in vivo* applications. A strategy of surface modification, formulation and bioconjugation integrating as

much as possible the overall specifications have to be defined depending on the type of use.

After the suspension of NPs in a biological medium, proteins and other biomolecules spontaneously adsorb onto their surface. This leads to the formation of a biomolecular corona. This corona alters the physicochemical interfacial properties of the nanomaterials and provides a new biological identity to the NPs.¹¹¹ This biological identity governs the interactions of the NPs with the biological environment and determines the biological functionality of the synthetic objects.

The biomolecular corona formation is driven by the presence of fundamental forces, i.e., electrostatic interactions, van der Waals interactions, hydrophobic effects.¹¹¹ Thus, its composition depends on the surfaces properties of the nano-objects and on the composition of the medium. The number of parameters influencing the biomolecular corona and its interactions with the biological environment are numerous so that it is rather difficult to predict accurately the interactions (Figure 2). Bioinformatics-inspired approach are developed to help in this prediction.¹¹²

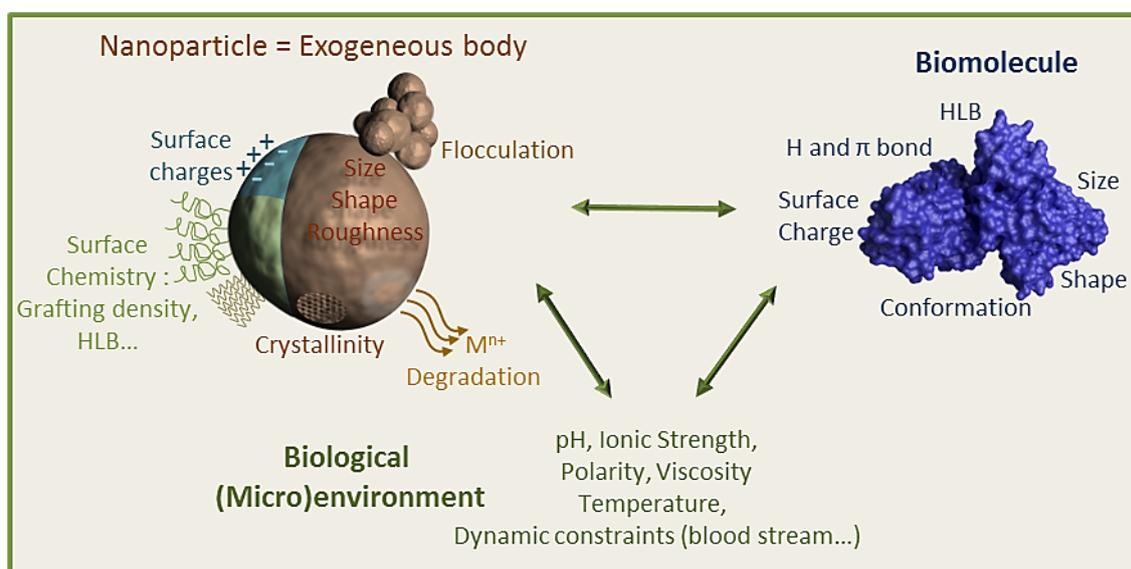


Figure 2. Illustration of the different physicochemical factors affecting NPs-biological environment interactions (HLB, hydrophilic-lipophilic balance).

The biomolecular corona forms almost instantaneously, but the biomolecules adsorption is a dynamic process depending on their affinity for the nanomaterial. During the first stage, the surface is covered with abundant and mobile biomolecules. Then, these biomolecules can be replaced by

less abundant and less mobile ones, with a higher affinity for the surface. As a result, the quantitative composition of the corona is not directly correlated to the composition of the medium. The determined dissociation constant (K_d) of complexes between biomolecules and nanomaterials varies from approximately 10^{-4} M to 10^{-9} M,¹¹³ similar to the range of physiological complexes. For example, the K_d of the complex human serum albumin-FePt NPs functionalized with carboxylic acids is of the order of 10^{-6} M.¹¹⁴ In a dynamic environment, e.g., *in vivo*, the exposure of the NP to a new environment with a different composition, by crossing biological barriers, may only lead to partial displacement of the original corona. Therefore, the resulting corona would contain the history of all the environments the NP went through. As a result, the targeting behaviour of bioconjugated NPs optimized *in vitro* is not predictive of the *in vivo* performance.¹¹⁵ Accordingly, the administration route of MNPs may also drastically affect their biological identity and so, their biodistribution.

For *in vivo* application, non-controlled adsorption on the NP surface is an important issue. In the blood, administered NPs are exogenous materials liable to be eliminated by the immune defence system, which represents one of the major barriers for systematically administered nanomedicines. Opsonins are plasma biomolecules, in particular immunoglobulin and proteins of the complement, marking antigens and promoting their phagocytosis. Adsorption of opsonins onto the surface of NPs, a process termed as opsonisation, enhances the recognition and uptake of these NPs by the macrophages belonging to the reticuloendothelial system (RES), present in the liver (Kupffer cells), in the spleen and in the bone marrow. This opsonisation leads to the rapid clearance of the nanomaterials from the vascular compartment. Such an effect can be advantageous when these organs are the intended target sites, while in the other case, it may limit the NP delivery to the targeted zone.¹¹⁶

Proteins are macromolecules consisting of one or more long chains of amino acids residues. They fold into functional 3-dimensional structures thanks to weak interactions. During adsorption,

proteins may undergo conformational changes. While a preformed albumin corona onto a polymeric NP can extend the blood circulation time by decreasing the complement activation,¹¹⁷ conformational change undergone by albumin when adsorbed to the silica NPs surface increases the NP uptake by human macrophages.¹¹⁸ Albumin unfolding reveals a cryptic epitope capable of recognition by class A scavenger receptors, a major receptor family associated with the mononuclear phagocyte system. Protein corona can also interfere with signalling processes. For example, unfolding of adsorbed fibrinogen onto negatively charged poly(acrylic acid) conjugated gold NPs leads to the release of inflammatory cytokines by promoting the interactions with the integrin receptor Mac-1.¹¹⁹ Lynch and co-workers assumed that iron oxide NPs may increase the rate of amyloid β protein fibrillation, depending on the charge of the dextran polymer coating.¹²⁰ Protein fibrillation is implicated in amyloidogenic diseases like Parkinson's disease or Alzheimer's disease.

For *in vitro* applications, these interactions may also be harmful and MNPs can be disrupted. Moreover, it can be easily understood that non-controlled interactions may drastically reduce the targeting efficiency, e.g., cell labelling, magnetic extraction and purification.

5.3.2. Shielding approaches

A solution to limit the uncontrolled interactions between NPs and biomolecules consists in modifying their surfaces with charge-neutral, highly hydrophilic polymers to render protein adsorption thermodynamically unfavourable. Macromolecules can be covalently bonded to the surface or just physically adsorbed, but physically adsorbed macromolecules tend to desorb over time in a biological environment. Different polymers have been used to "passivate" surfaces, such as dextran or poly(vinyl alcohol) but one of the most used polymer is PEG, which has the chemical formula $\text{HO}-(\text{CH}_2-\text{CH}_2-\text{O})_n-\text{H}$, and its derivatives. This approach is so commonplace that it is known as "PEGylation".

The surface density of PEG chains and its molecular weight are the most important parameters determining the interactions of a nanomaterial with proteins. At low density, when macromolecules are too far to interact with each other, the chains adopt a “mushroom” conformation, and do not create an effective barrier against protein adsorption. At high grafting density, macromolecules strongly interact with each other and adopt a “brush” conformation, creating a large thermodynamic barrier to adsorption. The surface density is crucial to limit biomolecule diffusions to the NP surface, and the shell thickness is important to decrease the intensity of long distance interactions, i.e., van der Waals interactions and electrostatic interactions.

The effect of a PEG shell on the protein corona formation has been evaluated. Walkey *et al.* presented that at constant gold NP size, an increase in PEG grafting density resulted in decreased serum protein adsorption, and for the highest PEG density tested (from 0.8 to 1.2 macromolecules/nm² depending on the NP size) 94-99% of serum protein adsorption were eliminated relative to non-grafted NPs.¹²¹ The study presents also the importance of the NP surface curvature. It has been found an inverse correlation between particle size and protein adsorption. For a fixed PEG density, the volume occupied by macromolecules grafted on small NPs is bigger than the one of the same macromolecules grafted on a bigger NP. The increase of this volume implies that the conformational freedom of grafted PEG macromolecules increases, weakening the thermodynamic barrier to protein adsorption on smaller NPs. Efficiency of NP uptake by macrophages correlated with variations in serum protein adsorption. At ~0.5 PEG/nm², macrophage uptake was reduced by a factor of 20 to 169 relative to non-grafted NPs.¹²¹ In general, PEGylated NPs in brush conformational regimes display enhanced NP lifetime in the blood stream, but it is not a sufficient condition. In particular, higher grafting densities must compensate the intermolecular spacing generated by high curvature radius.¹²² It can be defined a threshold value R_f/D (with the R_f , Flory radius and D the average distance between neighbouring PEG chains ($D = 2(\pi\sigma)^{-\frac{1}{2}}$)) for which it can be observed an effective stealth behaviour with prolonged blood circulation of NPs. This threshold was assessed at 2.8 for PEGylated 100 nm polystyrene NPs¹²³ whereas it was 4 for

19 nm fluorescent silica NPs.¹²² In this last case, R/D of about 4.2 was sufficiently high to increase the blood circulation time up to 24 h and to label xenograft tumours showing enhanced permeation and retention (EPR) effect.

Although PEGylation is effective for lowering biomolecules adsorption at the surface of NPs, even at high densities, the PEG corona can never truly eliminate it because of direct interactions between biomolecules and PEG macromolecules.¹²⁴ Furthermore, PEGylated NPs may suffer from an “accelerated blood clearance” (ABC) phenomenon corresponding to the enhanced clearance of second and/or subsequent doses of PEGylated nanomaterials. This phenomenon is coupled with the presence of anti-PEG IgM. However, the immunogenicity of PEG seems to be function of the nano-object composition.¹²⁵

The limitations of PEG have led to the development of a range of alternative antifouling polymers, such as poly(2-oxazoline)s, peptides and peptoids, and zwitterionic polymers.¹²⁶ Zwitterionic polymers are electrically neutral materials, but each monomer is composed of both positively charged functions and negatively charged functions. The high hydration of these polymers due to the presence of electric charges may be responsible of their antifouling properties.¹²⁶ One of these promising polymers is poly(carboxybetaine methacrylate) (pCBMA). Recently, the study of Yang *et al.* on gold NPs coated with PEG or pCBMA shows that pCBMA coating allowed a longer circulation plasma half-life for the NPs compared to those coated by PEG macromolecules (55.8 h instead of 8.7 h) with no visible ABC phenomenon.¹²⁷ Synthetic peptides composed of neutral hydrophilic amino acids may be able to avoid the ABC phenomenon¹²⁸ and may overcome the biodegradability issues of PEG and other alternatives.

5.4. Current challenges in MNP bioconjugation for the *in vivo* targeting

The biological function of the ligand is dictated by specific interactions, e.g., molecular recognition of a specific ligand with a target or interactions between the enzyme and its substrate. Non-specific interactions can occur directly at the surface of the NP and interfere with the

conjugation affecting the orientation or the conformational structure of the ligand. For example, nucleic acids, negatively charged, adsorb onto the surface of positively charged NPs.¹²⁹

For an effective biofunctionalization by conjugation, different criteria have to be taken into account. First, mobility and orientation of the conjugated biomolecule may influence its activity. For example, access to the binding site of a protein, e.g., active site of enzymes, paratope, may be blocked by a bad orientation of the biomolecule. Second, the activity of the biomolecules has to be maintained after conjugation. Biomolecule function is widely dependent of its structure. As mentioned before, adsorption of biomolecules on the NPs may lead to conformational changes and to the loss of their activity. Moreover, adsorption of biomolecules on NPs may be stronger than their affinity for their biological partners, which decreases their bioavailability. For example, transcription by T7 RNA polymerase of DNA adsorbed to cationic gold NPs is completely inhibited *in vitro*.¹²⁹ The surface of NPs has to be carefully prepared for the bioconjugation. In the context of the development of theranostic strategies, MNPs are incorporated into a polymer shell, a mesoporous silica matrix, or into lipid vesicles constituting the nanocarriers (Figure 1).^{12,13,33,116,130,131} In this case, the surface chemistry of the nanocarrier must be considered according to the requirements of the theranostic application notably in regards of the physico-chemical and chemical stability of the carrier or also of the drug loading and release.

5.4.1. Categories of targeting ligands

Numerous (bio)-molecules possessing molecular recognition properties for tissue-specific targeting can be grafted onto MNPs and then allow molecular imaging as well as therapeutic applications. Targeting ligands can be nucleic acids, monoclonal antibodies (MAbs) or their fragments (Figure 3), proteins, peptides, or smaller molecules such as sugars or vitamins. Here, the most common classes are presented with some benefits and limitations.

5.4.1.1 Nucleic acid based ligands

Nucleic acid polymers are well known for their properties of complementary binding. Complementary DNA or RNA strands interact with each other with the formation of hydrogen bonds between pair bases. Adenine interacts with thymine *via* two hydrogen bonds and cytosine interacts with guanine *via* three hydrogen bonds. This property has been extensively used for the development of sensitive biosensors.¹³² For example, magnetic NPs biofunctionalized with single-stranded DNA have been used for the sensitive detection of bacteria. The detection is based on the aggregation of magnetic NPs hybridized to the bacterial DNA target.¹³³

Aptamers are single-stranded nucleic acids that can fold into specific 3D structures and selectively bind to specific antigen types overexpressed in cancer cells such as prostate-specific membrane antigen (PSMA).¹³⁴ Most aptamers are obtained through a combinatorial selection process called “systematic evolution of ligands by exponential enrichment” (SELEX).¹³⁵ Aptamers can be in principle selected for any given target. Due to their unique conformational structures, aptamers display high affinity and specificity for their target. They are more stable than proteins and can achieve a higher density for immobilization due to their smaller size. They also have low immunogenicity or toxicity. This advantage makes aptamers excellent sensing elements.¹³⁶ The main concern regarding their use is related to their degradation by the nucleases present in high amounts in biological environments.

5.4.1.2 Peptides

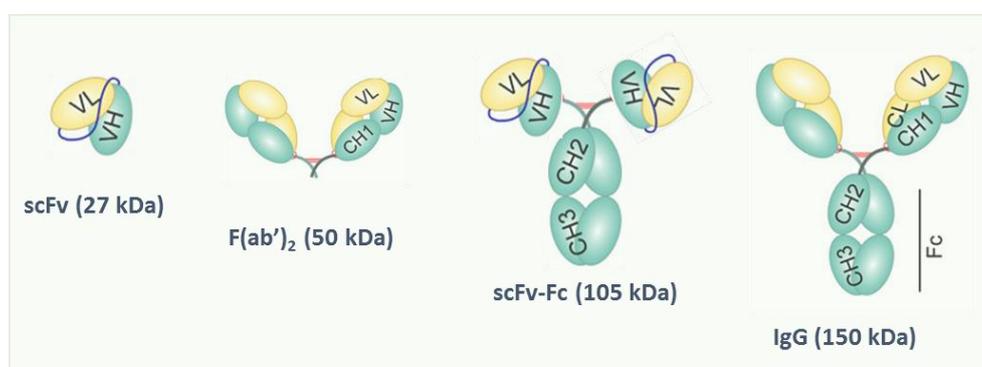
Peptides are linear or cyclic sequences composed of two to a few dozen of amino acids residues. Their small size combined with screening techniques to isolate ligand-substrate combinations has contributed to increase the use of peptides for targeting approaches in the past decade¹³⁷⁻¹³⁹. Natural peptides may have the propensity to bind to cell membrane. For example, the tripeptide Arg-Gly-Asp (RGD sequence) is well known to strongly bind $\alpha_v\beta_3$ integrin receptors that are overexpressed on a variety of angiogenic tumour endothelial cells.¹⁴⁰ This property was used to develop RGD-conjugated USPIOs for MRI of tumour angiogenesis targeting¹⁴¹ and for cancer

therapy.¹³⁸ The structure-activity relationship of linear and cyclic RGD peptides was compared. The cyclic peptide showed more than 10-fold higher efficiency for tumour targeting compared with its linear counterpart.¹³⁹ Cell-penetrating peptides are known to facilitate cellular uptake of large macromolecules, and can be used for the intracellular delivery of NPs.¹⁴² These properties combined with the EPR effect have been used in vasculature targeting strategy to favour the NPs penetration through blood vessels.¹⁴³ Other peptides such as the $A\beta_{1-42}$ peptide have been conjugated to MNPs to detect the amyloid deposition in Alzheimer's disease.¹⁴⁴

Thanks to the phage display technology, new synthetic peptides with a random sequence are selected to bind the desired target.¹⁴⁵ Compared to proteins, peptides can be easily prepared, and their small size enables their bioconjugation with high grafting density on the NPs. However, they are sensitive to the degradation by proteases.

5.4.1.3 Proteins

Proteins are also polymers composed of amino acids residues but they are longer than peptides. These macromolecules fold to adopt a 3D structure thanks to weak interactions between amino acids residues. Among them, MAbs are one of the most important classes of proteins known for their highly specific binding interaction. The antigen-binding site (paratope) represent a small part of the Ab, the Fc fragment at the base of the Y shape is much less variable and is responsible for the recognition of the protein by the MPS and immune system. MAbs such as IgG are large proteins with a molecular weight of 150 kDa with a hydrodynamic diameter of 10-20 nm that increase the size of the NPs. Smaller Ab formats such as scFv, Fab'2 have been produced by recombinant protein expression to reduce the MAbs to their essential part (Figure 3). This limits both the



increase of the hydrodynamic radius of the final hybrid NPs, their inherent immunogenicity and a rather poor diffusion through physiological barriers.¹⁴⁶

Figure 3. Examples of antibody fragments (scFv, single-chain variable fragment; Fc, constant fragment) and canonical full length IgG (right).

Other proteins present a high affinity for specific receptors overexpressed on a variety of tumour cells.¹⁴⁷ Among them, transferrin, a glycoprotein of 80 kDa, has been extensively studied as a targeting ligand due to the presence of up-regulated endogenous transferrin receptors 1 (TfR1) on the surface of cancer cells.¹⁴⁸ TfR1 were also targeted by a more exotic MNPs design involving encapsulation of iron oxide NPs within human ferritin protein shells called magnetoferritin.¹⁴⁹ Some proteins are also used for bioconjugation because of their specific bio-recognition interactions useful to control the orientation of a protein ligand in order to promote the binding site exposition. Once bound to the NPs surface, protein G was used to immobilize in a well-oriented manner anti-HRP IgG via interactions with the Fc region of the Ab.¹⁵⁰ Another representative example of affinity interactions is the well-known streptavidin-biotin binding which displays the highest affinity constant ($K_d = 4.10^{-14}$ M).¹⁵¹ Biotinylated Herceptins (HER2/neu) were conjugated to commercially available streptavidin-conjugated superparamagnetic iron oxide NPs.¹⁵² HER2/neu receptors are highly expressed in 25% of breast cancers. This bioconjugated MRI contrast agent was used to correlate the different levels of HER2/neu expression on the breast cell membrane. Like streptavidin-biotin system, the barnase/ barstar based recognition system also possesses a strong binding affinity, K_d , of the order of 10^{-14} M. One of them can be conjugated to a NP while the other can be fused to a protein of interest by genetic engineering, which allows the region-selective grafting of the protein of interest on the NP.¹⁵³ However, the barnase is a bacterial protein lethal to the cell when expressed without its inhibitor barstar. Even if such strategies are useful to accurately control the orientation of protein ligands, these supramolecular structures are accompanied by a significant increase of the hydrodynamic diameter of NPs and may be at the origin of non-specific

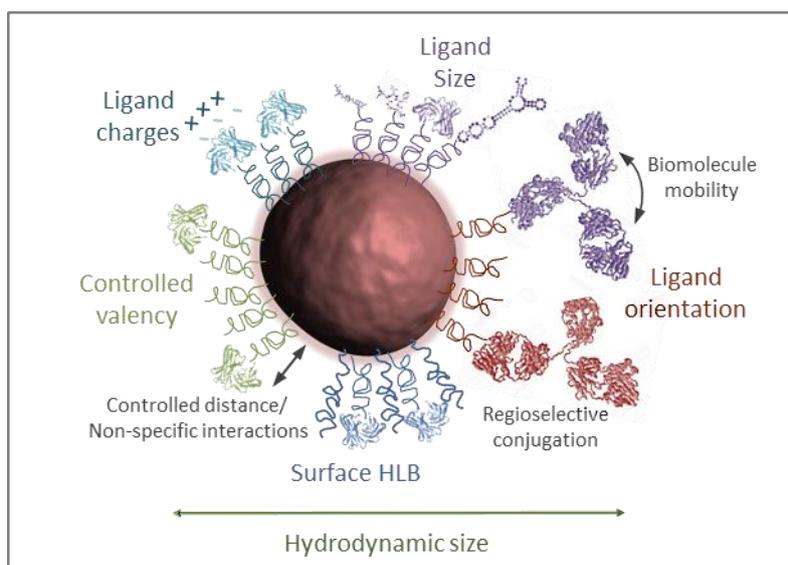
interactions that can result in an increased uptake from the RES (*see also section 4.4.2*). Enzymes are other proteins of interest with their catalytic activity under mild conditions. Immobilization of enzymes on magnetic NPs allows an efficient recovery of the enzyme complex thereby preventing the enzyme contamination of the final product.¹⁵⁴

5.4.1.4. Small molecules

Numerous small biomolecules of interest can be conjugated to NPs. One of the most widely studied small molecules, as targeting moiety is folate. Folate is a water-soluble vitamin B6 essential for cell division and growth. In cancers, folate receptors are overexpressed in a wide variety of tumour cells including ovarian, brain, breast, colon, renal and lung cancers.^{155,156} The folate ligand has a quite high binding affinity for its receptor ($K_d = 10^{-9}$ M) and thus enables the binding of the conjugated NP.¹³⁰ This ligand have been extensively conjugated on MNPs. For instance, MNPs were decorated by PEG and folate to deliver doxorubicin, an anti-cancer drug, to tumour cells.¹⁵⁷ Carbohydrate moieties such as galactose¹⁵⁸, glucose¹⁵⁹ and mannose¹⁶⁰, which are recognized by ubiquitous cellular membrane glycoproteins from lectins family, have also been widely used as targeting ligands¹⁶¹. For instance, galactose moieties were conjugated to amino-functionalized (ASPIONs) and lactose-derivatized galactose-terminal ASPION.¹⁶² for the specific targeting of asialoglycoprotein receptors (ASGP-R) overexpressed only on hepatocytes at a high density of 500,000 receptors per cell.¹⁶³ These smaller molecular ligands offer the possibility of increasing the affinity of MNPs toward cancer cells through multivalent attachment. In addition, they exhibit a better chemical stability than proteins, peptides or even aptamers or nucleic acids.

5.4.2. Main critical parameters involved in active targeting approaches

The bioconjugation of MNPs changes the physicochemical properties of both targeting molecules and NPs. The size, shape, hydrophilic-lipophilic balance and composition are all physicochemical parameters that can affect the blood circulation time and the interactions with their targets (Figure 4). The immobilized ligands that lose their rotational and translational freedom must



be also well oriented in order to promote their binding site exposition. Finally, the density of ligand molecules must be optimized to compensate the rotational motion of MNPs and achieve improved avidity while preserving the selectivity with respect to the targeted zone (*see section 5.4.2.4*). The use of too high densities of ligands can also modify the blood circulation and biodistribution kinetics of the biofunctionalized MNPs.

Figure 4. Schematic representation of the influence of physicochemical properties of the ligand affecting both the pharmacokinetics and the biodistribution of systemic administered bioconjugated MNPs.

5.4.2.1. Effect of the bioconjugation on the physicochemical parameters (size, surface charge and HLB) of the NP surface

The size of NPs influences their pharmacokinetics and biodistribution. Thus, it must be taken into consideration in the design of bioconjugated NPs. It is also commonly accepted that the charge of NPs affects the systemic circulation times by altering the opsonisation profiles and their recognition by the RES.^{164,165} Positively charged particles have been known to form aggregates in the presence of negatively charged serum proteins once intravenously administered.¹⁶⁶ The aggregates are large and often induce transient embolism in the lung capillaries. Negative surface

charges can either increase, decrease or have no impact on the NPs blood clearance. Parameters like surface charge density in physiological medium and their distribution inside the nanostructure (i.e. core and polymer corona) are at the origin of conflicting findings. Some ligands such as folic acid or proteins can display amphiphilic character, which can also be at the origin of non-specific interactions of the MNP surface with cells. This effect may be counterbalanced for instance by grafting of long hydrophilic PEG chains.

5.4.2.3. *Ligand orientation*

In the case of ligands of great structural complexity notably when the epitope is localized in a well-specified region such as MAbs and their fragments or aptamers, a regioselective conjugation guaranteeing the correct orientation must be controlled. The conjugation procedure can be complicated when the ligand does not have an anchoring function sufficiently far from the molecular recognition site. The many reactive functional groups present in protein (mainly amines, carboxylates, cysteine residues and carbohydrate moieties) complicate their oriented immobilization on MNP surface and can lead to cross-linking between NPs. In the case of MAbs, a wrong orientation could further expose the Fc fragment to the outside of the surface, which can result in an increased clearance from the blood. Regioselective strategies have been developed considering the repartition of amino groups in MAbs. At least two types of amine functions are exposed to the medium, the terminal amine that has a pK around 7–8, and the ϵ -amine moiety of lysine residues that have a pK close to 10.¹⁶⁷ At pH values less than 8.0, the Ab amino terminal groups are the most reactive. At pH values higher than 8.0, the ϵ -amino groups of Lys residues are more reactive and as the majority of the lysine residues are located in the Fc portion, the modification should occur preferentially in the Fc portion.¹⁶⁷⁻¹⁶⁹ This property can be exploited by using sulfonated NHS as good leaving groups in order to promote electrostatically the Ab orientation before the formation of peptide bonds¹⁷⁰ or with the same idea, by leaving carboxylate functions bearing negative charges.¹⁷¹ A post-functionalization of the Ab by iminothiolane (Traut's reagent) is also performed

before coupling on NPs bearing maleimide functional groups.^{172,173} Another strategy lies in the presence of a carbohydrate moiety in Fc region to conjugate the Ab in a proper position on aminated NPs by reductive amination.^{167,174} Anti-P selectin half-Ab have been also experimented by reduction of disulphide bonds of the hinge region before conjugation on a MRI contrast agent (Versatile USPIOs) for atherosclerosis diseases¹⁷⁵, but this strategy requires long biochemical studies and the conditions found to enrich the half-Ab fraction can change from one Ab to another. The conjugation of such ligands in principle requires the use of a chemical spacer to move the protein away from the NP thus preventing non-specific interactions (electrostatic, VdW, HLB) with the surface. Such interactions can interfere with the orientation and the conformation of the ligand. Long hetero- or homobifunctional PEG macromolecules with different lengths are often used in this aim.¹⁷⁶

5.4.2.4. *Effect of the multivalence on the affinity/avidity and specificity*

One of the most important notions for the molecular recognition is the affinity of the NP for its target. In theory, a high affinity increases the efficiency of the targeting because interactions are stronger. It may allow a better *in vivo* delivery to the biological target by compensating the non-specific interactions described above. The affinity of the conjugated NP is dictated by the affinity of the grafted biomolecules, but the strength of interactions between NP and its target depends also on its valence.

Multivalent interaction mechanisms are complexes and some of them still need to be finely understood. However, the effect of multivalent interactions can be easily understood with simple notions: Affinity of a couple of biomolecules is defined by the equilibrium dissociation constant K_d , corresponding to the ratio k_{off}/k_{on} where k_{off} and k_{on} are the kinetic dissociation and association constants, respectively. When low affinity ligands are used, it is often necessary to increase the valence of MNPs. The Brownian rotational diffusion of the NPs must be also compensated in order to increase the kinetics of binding of the immobilized ligand to its receptor. Multivalent interactions

reduce the apparent rate of dissociation due to the clustering and concentration of membrane receptors. Thus, the NPs can leave from the cell membrane only if all the ligand-receptor interactions dissociate at the same time. This clustering is accompanied by the wrapping of the cell membrane leading to the NP internalisation.¹⁷⁷ The dissociation rate decrease results in the increase of the apparent affinity of the NP for its target, compared to a monovalent NP. The term of “avidity” is used instead of affinity for multivalent interactions. Avidity of the NP can be increased by up to several orders of magnitude¹⁷⁸ but it can also decrease the specificity of the targeting. It can be explained by steric hindrance between neighbouring molecules, wrong ligand orientation or competition between ligands for a given receptor. An excess of ligands can also promote non-specific binding with endothelial or other cells, increase immunogenicity and increase the macrophage uptake of the RES.^{179,180}

NP avidity depends also on the surface receptor density.¹⁸¹ In fact, if the mean distance between the receptors is too large to allow multi-binding of the multivalent NP, avidity effect can be discussed. Nevertheless, the control of the valence of the NP is a key parameter for the optimization of the selectivity of the targeting,¹⁸² even if most of the time, the valence corresponds to the average number of biomolecules per NP while the distribution follows the Poisson statistics¹⁸³ leading to heterogeneous NP avidities.¹⁸⁴ Another issue impacting the avidity of multivalent NPs, in correlation with the receptor density, is the decrease of mobility or freedom degree of the immobilized ligands. In this case, the use of longer spacer arms based on heterobifunctional PEG appears once again as a solution but it has also been demonstrated that they can also prevent the ligands from reaching their receptors.^{176,185} A high ligand density is not always the most effective way to improve the targeting since it can result in a decrease of selectivity. For instance, it has been demonstrated that polymer particles coated with lower density of radiolabelled MAbs ICAM-1 are more advantageous for PET detection of pulmonary inflammation.¹⁸² In the present case, this behaviour can be explained by the receptor mobility at the cell surface leading to clustering phenomena during the ligand binding.¹⁸⁶ This suggests that the ligand spacing should be

considered during the surface bioconjugation step. This trend has also been observed with others nanoparticles conjugated with others targeting agents. PLGA nanoparticles were conjugated with cLABEL peptides at different grafting levels¹⁸⁷ to target ICAM-1 receptors present onto A549 cancer cells. Different grafting conditions were achieved by playing with the ratio of carboxylic and hydroxyl terminations of Pluronic[®] surfactant constituting the surface of these NPs. The conditions corresponding to the highest ligand densities led to the lowest cellular uptake showing that too high amounts of peptides disturb the receptors clustering.

5.4.3. Pre-assessment of the targeting efficiency

Most of the ligands display proper physicochemical features and their conjugation to the MNP surface has to be optimized in terms of surface density and orientation to improve as much as possible their avidity for a target molecule. These optimisations can be performed by physical measurement methods such as zeta potential measurements and dynamic light scattering for charge and hydrodynamic diameter assessments. Analytical techniques such as surface plasmon resonance (SPR)¹⁸⁸, quartz crystal microbalance (QCM)¹⁸⁹ are powerful to quantitatively monitor in real-time the interactions between various biomolecules, such as proteins and nucleic acids. These techniques are particularly useful to quantify small aliquots of bioconjugated NPs for desired and undesired molecular interactions. It allows to study the kinetics of binding and to evaluate their binding affinity/avidity towards targeted molecules immobilized on the sensor surface^{188,189}. Immunological methods such as western blot, immunohistology or enzyme-linked immunosorbent assay (ELISA) allow also to reach information about avidity, molecular recognition and targeting efficiency etc. The ligands quantification is an important issue, which have to be considered in the development of targeted NPs. At the step of bioconjugation and depending on the nature of the ligand (Ab, DNA, Aptamer...) and the complexity of the formulation, thermogravimetric analysis is not adapted and requires too much product to estimate the ligand surface density. The amount of ligands is most of the time deduced from gel electrophoresis (PAGE for proteins or agarose for nucleic acids), by UV-

visible spectroscopy in micro-volumes, by liquid chromatography mass spectroscopy (LC-MS), or by others spectroscopies such as Fourier-transform infrared spectroscopy (FTIR) and surface-enhanced Raman spectroscopy (SERS)¹⁸⁹⁻¹⁹¹ that generally only need small aliquots. Fluorescence and radioisotope labelling can also be useful to control the average amount of ligands for a given developed surface. Even if, as already mentioned above, *in vitro* studies are not predictive of the *in vivo* results, they seem to remain prerequisites for toxicological data or for the examination of intracellular pathways (i.e. internalization, intracellular release). Finally, the optimisations in terms of surface ligand density have to be performed case by case according to the architecture of the MNP to improve the targeting efficiency/RES clearance balance. For this purpose, screening methods can be helpful to identify the bioconjugated MNPs, which display the highest specificity for a given target or cell line. MNPs-based immunoassays-on-chip were developed to screen or detect circulating cancer cells.⁴⁶ These screening methods are often coupled with computational approaches. Following the example of combinatorial approaches developed in medicinal chemistry, computational methods involving data analysis methods and statistics are also currently emerging to develop models for the accurate prediction of biological activities or properties of the compounds based on their nanostructure.¹⁹² Predicting methods called QNAR for “quantitative nanostructure-activity relationships” integrating diverse NPs features (morphological, size, coating) experimentally characterized, combined with surface modifiers characterized by computed chemical descriptors of modify organic molecules were developed to establish statistically significant relationships between the measured biological effects of nanoparticles and the intrinsic properties of the NPs. For instance, a combinatorial library of 146 nanoparticles designed from cross-linked iron oxide with amine groups (CLIO-NH₂) conjugated with different synthetic molecules was established with the aim to study the effect of multivalency on the specific binding affinity for different cell lines.¹⁹³ Different classes of small molecules with amino, sulfhydryl, carboxyl, or anhydride functionalities were anchored onto MNPs and stored in multi-well plates for testing. These NPs showed different binding properties toward different targeting proteins using

fluorescence detection. This screening enables to select the NPs displaying the highest specificity for endothelial cells, pancreatic cancer cells and macrophages.

5.5. Conclusion and future outlook

This chapter described the main requirements that chemists must consider in bioconjugation of MNPs for a realistic *in vivo* targeting. In particular, the design of bioconjugated MNPs must take into account the changes in physicochemical properties of the particle surface, which is the location of complex interactions with the biological environment. Among all the parameters to be controlled, the density of ligands seems to be the most important one because it not only affects the hydrodynamic diameter but also the surface properties which themselves influence (i) the stealth required for long blood circulation times and (ii) the avidity and specificity of the targeting.

The multicomponent character of targeted MNP requires preclinical, pharmacokinetics and long-term toxicity studies before use in humans. Even if the impact of each individual component can be distinctively evaluated in preclinical models, the systematic assessment of their synergy in humans become more complexed if not impossible due to the high cost required and regulatory hurdles. It is therefore necessary to develop pre-assessment approaches, screening methods and combinatorial approaches that take into account all the physicochemical features required for a particular application in nanomedicine. This type of approach is already being developed for manufactured nanoparticles with the aim of anticipating the toxicological impact on health and the environment during their life cycle ("safe by design" approaches)¹⁹². As it is not possible to accurately predict the MNP *in vivo* fate, these approaches, which could be termed "targeting-by-design", would certainly assist in optimizing and selecting the best surface strategies to test for a given pathological situation. To go further, additional information can be found in recent reviews dealing with the use of bioconjugated NPs for bioimaging, drug delivery and theranostic depending on their administration routes and for different pathological situations.^{116,148,192,194,195}

Abbreviations

ABC	accelerated blood clearance
AOT	
ASPION	amino-functionalized SPIONs
CLIO-NH ₂	cross-linked iron oxide with amine groups
CT	computed tomography
DEG	diethylene glycol
DMSA	dimercaptosuccinic acid
DNA	deoxyribonucleic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EG	ethylene glycol
ELISA	enzyme-linked immunosorbent assay
EPR	enhanced permeation and retention
FTIR	Fourier-transform infrared spectroscopy
HER2/neu	human epidermal growth factor receptor-2/
HLB	hydrophilic-lipophilic balance
ICAM	intercellular adhesion molecule 1
IgG	immunoglobulin G
LC-MS	liquid chromatography mass spectroscopy
MAbs	monoclonal antibodies
Mac-1	macrophage-1 antigen
MNPs	magnetic nanoparticles
MPS	mononuclear phagocyte system
MRI	magnetic resonance imaging
NHS	hydroxysuccinimidyl
NPs	nanoparticles
NP5	Polyoxyethylene (5) nonylphenylether
PAA	poly(acrylic acid)
PAGE	polyacrylamide gel electrophoresis
pCBMA	poly(carboxybetaine methacrylate)
PEG	polyethylene glycol
PEI	poly(ethylene imine)
PET	positron emission tomography
PLA	poly(lactic acid)
PMMA	poly(methylmethacrylate)
PSMA	prostate-specific membrane antigen
PVA	poly(vinyl alcohol)
QCM	quartz crystal microbalance
QNAR	quantitative nanostructure-activity relationships
RES	reticuloendothelial system
RGD	Arg-Gly-Asp tripeptide
RNA	ribonucleic acid
ScFv	single-chain variable fragment
SELEX	systematic evolution of ligands by exponential enrichment
SERS	surface-enhanced Raman spectroscopy

SPIONs	superparamagnetic iron oxide nanoparticles
SPR	surface plasmon resonance
TfR1	transferrin receptor 1
USPIO	ultrasmall superparamagnetic iron oxide
VdW	Van der Waals

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