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Interfacing Engineered Nanoparticles with Biological Systems: Anticipating Adverse Nano–Bio Interactions

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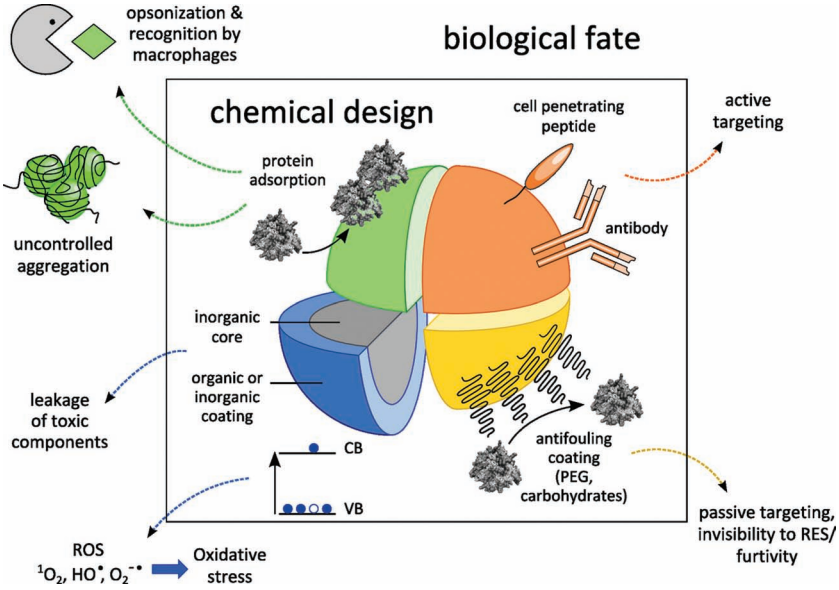

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The innovative use of engineered nanomaterials in medicine, be it in therapy or diagnosis, is growing dramatically. This is motivated by the current extraordinary control over the synthesis of complex nanomaterials with a variety of biological functions (e.g. contrast agents, drug-delivery systems, transducers, amplifiers, etc.). Engineered nanomaterials are found in the bio-context with a variety of applications in fields such as sensing, imaging, therapy or diagnosis. As the degree of control to fabricate customized novel and/or enhanced nanomaterials evolves, often new applications, devices with enhanced performance or unprecedented sensing limits can be achieved. Of course, interfacing any novel material with biological systems has to be critically analyzed as many undesirable adverse effects can be triggered (e.g. toxicity, allergy, genotoxicity, etc.) and/or the performance of the nanomaterial can be compromised due to the unexpected phenomena in physiological environments (e.g. corrosion, aggregation, unspecific absorption of biomolecules, etc.). Despite the need for standard protocols for assessing the toxicity and bio-performance of each new functional nanomaterial, these are still scarce or currently under development. Nonetheless, nanotoxicology and relating adverse effects to the physico-chemical properties of nanomaterials are emerging areas of the utmost importance which have to be continuously revisited as any new material emerges. This review highlights recent progress concerning the interaction of nanomaterials with biological systems and following adverse effects.

1. Introduction

Since the term nanomaterial includes many different materials, i.e. inorganic nanocrystals, organic nanoparticles, films, inorganic/organic hybrid materials, etc., for the sake of clarity, this review focuses on colloidal nanoparticles (NPs) containing inorganic materials (typically these are referred to as nanocrystals or inorganic NPs), although many of the critical aspects discussed here also apply to other nanoparticulate materials, e.g. purely organic nanoparticles.

Functional inorganic colloids have been used in modern biology and medicine for long time. Already in 1971, an original antibody–colloidal gold complex was proposed for cell surface antigen localization for electron microscopy.[1] In the last decade, nanoscience has grown to what appears to be one of the richest research areas in terms of spin-off effects on our society.[2] Importantly, engineered nanomaterials have been proven to deliver important technological features in a wide variety of medical applications (e.g. imaging,[3] therapy,[4] sensing,[5] diagnosis[6]). Maybe since the pioneering works where semiconductor nanocrystals were proposed as cellular probes,[7] and DNA to arrange NPs,[8] there has been an increasing and widely extended interest for 'mixing' inorganic NPs with living organisms and biomolecules. This is motivated by three main reasons: (i) NP size lies in the "right" range where most bio-interactions occur, (ii) when bulk materials are reduced to the nanoscale (increasing the surface to volume ratio dramatically), they present useful properties such as bright fluorescence, plasmonic effects or superparamagnetism[9] and, remarkably, (iii) these 'nano' properties can be accessed synthetically by controlling the design of the NPs (e.g. size, shape, structure), enabling tailoring for specific purposes.

Recent progress in nanotechnology applied to medicine, also called nanomedicine, has spread the general optimism that this applied discipline can solve many medical issues (therapy, early diagnosis, sensing, limits of detection, etc.).[10] However, many challenges must be overcome before nanomedicine becomes available for all and an actual breakthrough for our society.[11] Among a variety of challenges, one could highlight the following: (i) technology transference to the healthcare system (hospitals, pharmaceutical industry, etc.); (ii) regulation of application of nanomaterials in healthcare, or (iii) guidelines for nanosafety, i.e. the safe manipulation of nanomaterials and prevention of accidental exposure to nanomaterials. Most importantly, anticipating the effects of the interaction of nanomaterials with biological systems such as their potential toxicity and bio-performance is crucial. Although there are many recent and ongoing investigations in this direction, nanotoxicology, nanopharmacology, or nano-biosensing are still very young areas of research.[11b] We would like to highlight that, ultimately, the targeted delivery of functional nanomaterials remains the key issue for achieving 'real' medical approaches.

In this review, we discuss important issues concerning the functionalization and characterization of inorganic NPs for bioapplications and, importantly, how the outermost layer on the NP design affects the suitability and performance of nanomaterials.

2. Hybrid Nature of 'Inorganic' Functional NPs for Bioapplications

This review focuses on inorganic NPs where the term 'inorganic' refers to the nature of the core component of the material. Actually, the term 'inorganic NPs' is misleading since the nanomaterials designed for a bioapplications need to be of hybrid nature. A NP comprises an inorganic core, a purposely designed primary coating made of organic or inorganic material and, typically, an organic outermost layer incidentally derived from the interaction with the surrounding medium. In the following, we take a closer look at the structure and composition of these Russian doll-like structures while focusing on the inorganic core and the chemically designed primary coating (**Figure 1**). The principles governing the formation of the outermost biomaterial layer are discussed in detail in Section 3.

2.1. The Inorganic Core

Importantly, evaluation of the inorganic material itself represents the first task prior to success in any bioapplication. There is nowadays tremendous chemical expertise in the synthesis of inorganic nanocrystals. Size, shape, and composition, the parameters that govern their physical properties, can be varied almost continuously while retaining monodispersity.[12] These parameters not only govern the physical properties of the nanocrystals (quantum yield, position of the plasmon band, saturation magnetization) but will also strongly affect their bio-performance through their interaction with physiological media. For instance, in the context of passive targeting in cancer treatments, larger NPs are preferred over their smaller counterparts as large ones (greater than 100 nm) accumulate in areas of leaky vasculature (as in solid tumors)

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to a higher degree than smaller NPs by the enhanced permeability and retention (EPR) effect.[13] For details regarding tumor-selective delivery of macromolecular drugs via the EPR effect, we refer to the recent review of Maeda.[14] On the other hand, small NPs with a hydrodynamic diameter less than 5 nm can be excreted very conveniently, which is normally preferred over unintended accumulation in organs for extended periods of time.[15] Similarly, the shape of the nanocrystal can control the rate of internalization by cells; for instance, gold nanostars are less readily internalized than gold nanospheres of equivalent dimension.[16] In addition, the composition of the core material strongly affects the toxicity response, as will be detailed later on. Some inorganic materials are more prone to corrosion than others and, thus, the 'dissolution' of NPs and metal ion shedding is different depending on the identity of the core.[17]

2.2. Organic Coatings for Stabilization

Bare inorganic NPs are colloidally unstable due to attractive interparticle van der Waals interactions, and tend to form large agglomerates. Therefore, any bioapplications based on colloidally stable NPs will require their stabilization with a coating which prevents these interactions from being dominant. Stabilization in water is usually achieved through electrostatic repulsion between like-charged NPs or through steric hindrance using hydrophilic moieties, such as polyethylene glycol (PEG) chains,[18] carbohydrates,[19] or zwitterionic appended arms.[20]

Generally, for bioapplications, any as-synthesized NP will require a coating, whether it is initially synthesized in organic media or in water. The chosen coating represents a crucial part of the design of the hybrid inorganic NP as it ultimately determines their colloidal and chemical stabilities through imparting resilience to high ionic strengths, various pH, or nonspecific protein adsorption, and shielding them against corrosion. Ideally, the coating should ensure that the NPs do not agglomerate, dissociate, or suffer any chemical reaction with the surrounding media. The coating can also open the possibility to further derivatize the nanomaterials with molecules of biological relevance by adding functional groups on their surface (Figure 1).

There are a number of strategies to derive water colloidally stable inorganic nanocrystals including ligand exchange with small, macro, or biomolecules, polymerization of a silica shell, wrapping in organic polymers, encapsulation (e.g. polymer coating, micelles, liposomes), or combinations of the aforementioned.[21]

The ligand exchange strategy is based on the displacement of the ligands surrounding as-synthesized nanocrystals by ligands with a higher affinity for the inorganic core. The choice of ligand type (i.e., thiols, amines, or alcohols) depends on the composition of the core. The selection of a ligand chain relies on the type of stabilization sought (electrostatic or steric repulsions).[18,20] Silanization consists of growing a glass shell around the NPs.[22] Polymer coating methods can involve intercalating amphiphilic polymers in the aliphatic shell of the NPs[23] or sequentially wrapping them in layers of polymers of alternating charges, a method known as the layer-by-layer (LbL) technique.[24] Finally, inorganic NPs can be derivatized into inorganic/organic hybrid materials by trapping inorganic NPs within other organic structures such as liposomes or lipospheres.[21,25]

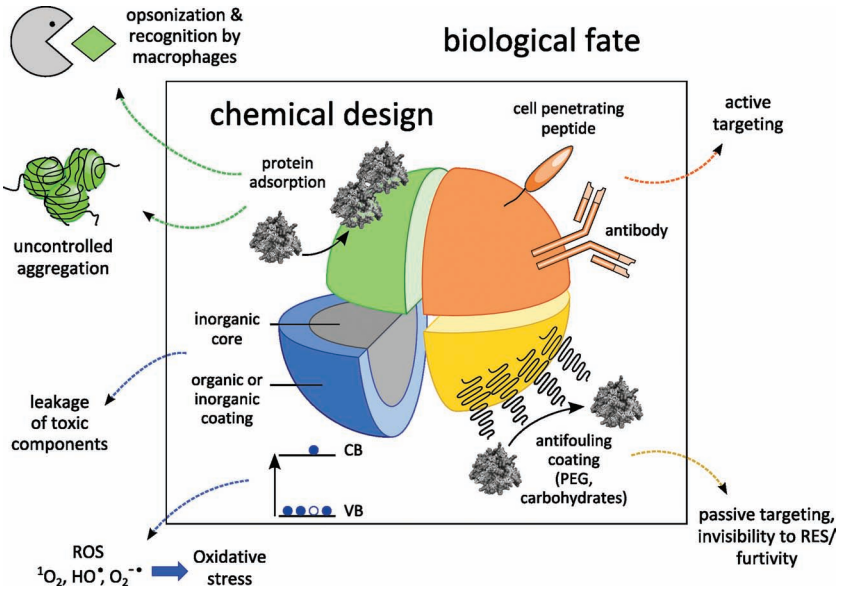

Figure 1. Schematic representation of the biological fate of engineered NPs in physiological media. The green scenario illustrates some potential adverse effects following protein adsorption; the orange scenario represents the functionalization of NPs with cell-penetrating peptides (CPPs) and antibodies; the yellow scenario emphasize the possibility of passivating NPs with suitable coatings; the blue scenario represents adverse effects such as NP dissolution and ROS generation.

2.3. Derivatization with Molecules of Biological Interest for Biofunctionalization

Once stabilization has been achieved in physiological environments by one or

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more of the aforementioned methods, NPs can be engineered into complex functional materials for a particular bioapplication. Nowadays, there is a wide variety of chemical methods to anchor molecules of biological relevance onto NPs, such as fluorescent tags, PEG chains, proteins, carbohydrates, DNA, peptides, siRNA, enzymes, antibodies, cyclodextrins, biotin, etc.[8,19,26] There are different approaches depending on the chemical groups present on the NPs and the ligand of interest. For instance, conjugation of NPs bearing carboxylates with amine-containing molecules/proteins can be achieved by classical carbodiimide cross coupling approaches;[27] linkage to sulfhydryl groups can be similarly achieved by way of maleimide-terminal ligands which are widely commercially available.[28] Copper(I) catalyzed azide-alkyne cycloaddition, a reaction from the click family, has been similarly employed in a number of gold nanoparticle conjugation strategies.[29] However, click cycloaddition in its copper(I) catalyzed form cannot be apply to the conjugation of quantum dots (QDs) without impairing the luminescence quantum yield. Indeed, Chan et al. demonstrated that Cu(I) ions can displace cations in the QD and subsequently quench the luminescence.[30] However, click cycloaddition can be performed in catalyst-free conditions when using a cyclooctyne derivative. This has been successfully used by Bernardin et al. in the derivatisation of CdSe/ZnS QDs.[31]

Different derivatization approaches allow for an extraordinary control over the arrangement of ligands onto NPs which can be used to tailor and predict how a nanomaterial of interest is for instance internalized by cells, among other biointeractions.[32] The pioneering work of Stellacci and coworkers showed that by patterning the coating of Au NPs the internalization process can be controlled.[33] The authors made "stripes" or defined hydrophobic/hydrophilic domains on monolayer protected NPs. Remarkalby, 'striped' NPs penetrate the plasma membrane without bilayer disruption, whereas the equivalent NPs but 'non-striped' are mostly trapped in endosomes.

The type of coating, the physico-chemical principle of stabilization, and the terminal groups control the interaction the nanomaterial not only with other NPs, but also with the surrounding biomolecules in physiological media.[34] This phenomenon has strong consequences for the performance of the nanomaterial (as a sensor, diagnostic tool, drug carrier, or more complex multitask systems),[11,35] as will be detailed in the next section.

3. NP–Protein Interactions: Insights into the Protein Corona

Biomolecules adsorbed on the surface of NPs can be responsible for many of the toxicological effects of nanomaterials as well as for defining the fate of the newly formed hybrid NP. For instance, protein adsorption can lead to the nonspecific uptake of nanomaterials into cells by receptor-mediated endocytosis.[36] Indeed, when a NP is presented to a cell, what the cell actually sees is a patchwork made of all the different organic molecules shaping the outermost layer of the NP, which can be the result of synthetic design or of nonspecific biomaterial absorption.[37] Proteins are the most abundant biomolecules in biological fluids (blood, plasma, interstitial fluids, and cytoplasm) and will therefore be the main constituent of the biomaterial layer adsorbed around NPs in physiological media. This fact has been known for long time for planar surfaces. To be able to tune, optimize, or predict the performances of engineered nanoparticles for bioapplications, one needs to understand the driving forces and parameters of the formation of the so-called protein corona. This topic has been recently reviewed in a comprehensive manner by Walkey and Chan.[38] In the following, we highlight their main findings, but the reader is referred to the original publication for more detailed insights. We later exemplify how the protein corona can affect the bioapplications of nanoparticles.

Although the physico-chemical description and characterizations of the interactions of proteins with nanoparticles are still in their infancy, it now appears that these interactions are controlled by the surface chemistry and curvature of the nanoparticle. The interaction can proceed through electrostatic attraction, hydrogen bonding, or van der Waals interactions. Nanoparticles with hydrophobic or charged surfaces interact more readily and form more stable complexes with proteins than their hydrophilic neutral counterparts. They also tend to absorb more of them. The interactions of proteins with a NP of a given core material differ from those with extended surfaces. They are also subject to variations when the curvature radius is decreased. To date, there seems to be a general trend indicating that the lower the curvature radius, the lower the affinity of the protein for the NP. Several physico-chemical effects might be responsible for this observation. First, as the curvature radius is decreased, the contact area between the protein and the surface of the nanomaterial decreases, thus weakening the interaction. Second, it has been suggested that protein–protein interactions might act as an additional driving force for the adsorption of proteins on the NP surface. Therefore, lowering the curvature radius, which increases the deflection angle between adsorbed proteins, tends to decrease protein adsorption because of weaker cooperativity. In many bioapplications of nanoparticles, minimizing the adsorption of proteins is highly desirable. This can be achieved by coating the NPs with anti-fouling, hydrophilic, charge-neutral polymers, such as PEG, polysaccharide, or zwitterionic poly(carboxybetaine methacrylate).[18–20] The surface of the NP is then said to be 'passivated'. The development of anti-fouling polymers is an active area of research (Figure 1, yellow scenario).

One should bear in mind that, although trends for protein–NP interactions have emerged, counter-examples are not rare. It is to be expected that finer descriptions will emerge as the field matures. But for now, each supramolecular interaction between NPs and proteins is a nontrivial phenomenon determined by the intrinsically complex nature of NPs and proteins. It should be assayed systematically when designing a nanomaterial for a bioapplication.

For most proteins interacting with nanomaterials, adsorption is associated with some conformational change, the degree of which is highly variable and can span the entire range from overall structural integrity to full denaturation. Within this range, loss or impairment of biological activity can

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be expected. The extent of conformational distortion largely depends on the surface chemistry of the nanomaterial. As for the extent of the interaction, charged or hydrophobic nanoparticles tend to give rise to larger conformational changes than their hydrophilic neutral counterparts. Likewise, the larger the NP, the greater the distortion. However, it is important to acknowledge here that small enough NPs are capable of introducing local denaturation effects.[39] The structure of the protein also strongly influences the degree of the conformational change, with protein capable of strong internal stabilizing interactions such as disulfide or salt bridges, displaying superior resilience to distortion.

Importantly, as it strongly depends on the surface chemistry of the NPs, denaturation of a protein or impairment of its biological activity can be avoided or tailored by tuning the organic coating. Adsorption of proteases deserves special attention as some of them can be involved in the 'digestion' of endocytosed NPs. For instance, Chymotrypsin (ChT), whose enzymatic activity is well characterized, is particularly attractive for in vitro investigations. Based on amino acid-functionalized gold nanoparticles, Rotello and co-workers demonstrated how surface charge and hydrophobic side chains affect the binding affinity and denaturation of ChT. Although electrostatic interactions between NPs and ChT were proven to be the predominant driving force contributing to the complex formation, the hydrophobic interaction between the hydrophobic patches of the NP's ligands (receptors) and proteins enhanced the complex stability. Remarkably, control over association/dissociation as well as stabilization/denaturation of ChT onto NPs could be readily tailored by introducing diverse terminal derivatizations (i.e. tuning the organic coating).[40]

Recently, Au NPs of different sizes (5 to 100 nm) stabilized by citrate ligands were mixed with the most abundant plasma protein, namely human serum albumin (HSA).[41] The authors evidenced the formation of a protein corona via electrostatic interactions and hydrogen bonding. In contrast to previous examples, the structure of the adsorbed proteins was largely retained upon the interaction with the metal surface, although distortion increased as the curvature decreased, in line with the trends exposed earlier. Importantly, adsorbed HSAs became more resistant to complete thermal denaturation than free proteins.

Impairment of biological activity is likely to arise when large conformational changes occur but do not necessarily mean that the structural integrity is lost. Indeed, interaction with the NP surface can bury the active sequence of a given protein in the protein corona and hide it from the physiological environment. In addition, protein adsorption on the nanomaterials does not necessarily occur through interaction with the NP surface but can also stem from protein–protein interactions between free proteins and the primary protein corona. Burying of the primary binding protein or of the active domain of a secondary binder can result in the loss of biological activity.

In addition, it is important to stress the dynamic character of the protein corona, which reflects the instantaneous composition of the physiological environment surrounding the nanomaterial. As a NP traffics across different cell compartments, the protein corona is likely to be modified in structure or in composition. For instance, studies on the serum albumin model protein, i.e. bovine serum albumin (BSA), have shown that, under different pH conditions, this protein could undergo various conformational changes.[42] Therefore, trafficking from endosomes to lysosomes is expected to affect the overall structure of a given NP.

The protein corona not only affects the structure and biological activity of the adsorbed proteins but may also strongly influences the properties of the NP and hence its physical performance and biological fate. First, adsorption of a primary and secondary corona increases to a large extent the effective diameter of the nanoparticle in the physiological medium. For instance, the corona of 30–50 nm citrate-stabilized gold NPs has been reported to be 21–23 nm thick, evidencing in this case adsorption of multiple layers of proteins.[41,43] In contrast, other studies have pointed out the formation of only one saturated monolayer of proteins which depends on the NP geometry and NP-to-protein ratio.[44] Clearly, different concepts about the protein layer exist, and those depend on the NP model, the proteins, and the detection techniques used. In any case, the thickness of the protein corona increases the minimal interparticle distance compared to buffer conditions, a fact that has strong consequences on applications where optical coupling of NPs is required. The reverse situation can also be observed, namely, uncontrolled NP aggregation through interparticle bridging by proteins, possibly triggered upon protein denaturation.[38,45] Finally, adsorption of a protein corona can affect the physical properties of the individual NP itself, for instance by shifting its plasmon resonance[41] or altering its luminescence quantum yield.[46]

The aforementioned examples regarding protein-NP interactions illustrate how complex and diverse the structures resulting from interfacing NPs with proteins can be. The protein corona has a large influence on the performances of the nanomaterials as it impacts both its biological fate and its physical properties, as will be illustrated later on. Guidelines have been drawn to understand and sometimes predict the formation of this protein corona, although there is still much to do in this direction. More importantly, the chemical availability of various coatings allows for tailoring of this interaction and optimization of the bio-performances.

4. Toxicological Effects Related to Engineered NPs

In the following section, we aim at illustrating how the structure and composition of engineered nanoparticles may adversely affect the interplay between the nanoparticle and the biological system. We focus on both the fate of the NPs in the biological system and the fate of the biological system exposed to the nanomaterial.

4.1. Leakage of Toxic Material

Leakage of toxic components from the nanomaterial is possibly the simplest mechanism responsible for nanoparticle

cytotoxicity. The toxic components can be released from either the inorganic core, as toxic ions, or from the stabilizing coating shell, as surfactants, ligands, or polymers (Figure 1, blue scenario).[17,47]

The shape and composition of the inorganic material are determining factors in the dissolution or corrosion of the core. For instance, Au NPs are believed to be less prone to corrosion than their Ag counterparts, which may leak toxic Ag^+ ions. For this reason, Au NPs are often preferred over Ag NPs in surface-enhanced Raman spectroscopy (SERS) experiments, despite the superior enhancement abilities of the latter.[48] However, the sensitivity of Ag NPs to corrosion can be turned into an advantage in anti-bacterial applications.[49]

The dissolution of NPs can also be tailored synthetically by modifying the composition of the core material. For instance, doping ZnO NPs with Fe reduces their cytotoxicity.[50] Encapsulation into a robust inorganic matrix can also prevent dissolution of the core material. Following this principle, silanization helps prevent the release of toxic Cd ions from QDs.[51]

Organic coatings can also be responsible for cytotoxicity. For instance, gold nanorods have been believed for a while to be cytotoxic while later studies indicated that the actual cytotoxicity stems from the surfactant used for their anisotropic growth, namely cetyltrimethylammonium bromide (CTAB).[36,52] Common strategies to avoid leakage of toxic organic components involve surface cross-linked coatings such as silica shells, or wrapping in cross-liked polymers.[21b]

4.2. Production of ROS

Inorganic nanoparticles can produce reactive oxygen species (ROS), such as singlet oxygen, radical hydroxide, or superoxide, through photochemical or Fenton-like reactions.[53] These ROS in turn trigger oxidative stress in the biological system (Figure 1, blue scenario).

In vitro, this phenomenon needs to be taken into account when monitoring cellular analytes or parameters as this brings about cellular defense mechanisms that can interfere with the measurements. Hence, prior to SERS measurement of intracellular potential via a redox probe adsorbed on gold nanoshells, Auchinvollet et al. checked that the SERS substrate did not induce ROS production compared to pristine cells using independent ROS fluorimetric assays.[54]

In vivo, ROS production is thought to be the main mechanism responsible for nanotoxicity affecting the blood, spleen, kidney, respiratory system, liver, or immune system (immunotoxicity).[55] Sequestration of NPs by phagocytic cells in the organs of the reticuloendothelial system (RES) makes organs such as the liver and spleen major targets of oxidative stress.[56] Other organs exposed to high blood flow such as the kidneys and lungs are also subject to oxidative stress.[57]

4.3. Interaction with Organelles and Organelle Impairment

In the context of in vitro studies, cationic NPs are generally observed to be more toxic to cells than their neutral or anionic counterparts.[33,,36,58] This phenomenon may be due to their higher degree of cell interaction and/or internalization compared to neutral and negatively charged NPs,[24,,58,59] although the 'big picture' seems to be more complex.[60] A variety of mechanisms have been proposed to explain the observed cytotoxicity of cationic NPs. These are largely dependent on the NP model, cell line, concentration of NPs, and possibly other experimental variables. Among distinct cytotoxic processes, there are examples for hole formation, membrane thinning, and/or erosion due to the strong interaction of cationic NPs and the cell membrane.[58,60,61] The proton sponge effect by which acidic endosomal compartments can be damaged have been also widely proposed as leading to toxicity. This is typically followed by mitochondrial injury[58,62] and also by membrane depolarization.[58,63] In general, cationic nanomaterials, once introduced into the circulatory system, have been shown to strongly interact with red blood cells, destabilize cell membranes, and cause cell lysis.[55a]

NPs coated with a variety of cationic molecules such as polyamidoamine (PMAM) and polypropylenimine (PPI) dendrimers of different generations, cell penetrating peptides, amine molecules, polyethyleneimine (PEI), and diethylaminoethyl-dextran, to name a few, have been proven to induce defects in lipid membranes.[60,61,64] A recent work of Lin et al. addressed the interaction of NPs with model lipid membranes by molecular dynamics (MD) simulations.[65] Their simulation results reveal that the sign as well as the surface charge density on NPs determine their fate, i.e., repulsion of, adhesion to, or penetration into lipid membranes. Cationic NPs are shown to strongly adhere to and penetrate into the membrane but more critical is the fact that at high surface-charge density, cationic NPs are able to disrupt the membrane and introduce defects. Although these simulations do not take into account the presence of biomolecules in the environment (e.g. protein coronae), which are likely to absorb in such cationic surfaces, they are in excellent agreement with previous equivalent experimental works.[33,58,61] Disruption of the cellular membrane can lead to exchange of the medium between extracellular fluid and cytosol, whichmay cause acute cytotoxicity.[60] To add complexity to the big picture, the work of Xia et al. showed that cationic particles exert differential toxicity on different cell lines (i.e. either viable or apoptotic and/or necrotic features) upon NP uptake.[66]

4.4. Impairment of Biomolecule Functions

As explained earlier, the adsorption of proteins onto NPs is paired with conformational changes, which can lead to loss of biological activity and impair the cell machinery. Cathepsins are proteases found in lysosomes and endosomes of different mammalians cells which are mainly involved in protein degradation and antigen presentation (for adaptative immune response).[67] In a recent work, the ability of a number of Ag and Au NPs of different sizes and coatings to distort cathepsin activity to different degrees was confirmed, even when the cytotoxicity of the type of NP was not an issue.[68] Although these results should be considered with caution because they were performed with one cell line and a limited set of NPs,

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misregulation of cellular cathepsin activity can be a critical issue as it may alter the adaptative immune response.

Alteration of biological activity can trigger defense mechanisms from the cell, such as an inflammatory response. Recently, Minchin and co-workers[69] showed that negatively charged NPs (polyacrylic acid-coated gold NPs) can strongly bind to (forming a 'hard' protein corona) and consecutively induce unfolding of fibrinogen, a plasma protein. As a result, a chain of activation (receptor Mac-1) and corresponding signaling pathway (NF-$\alpha\beta$) trigger the release of inflammatory cytokines.

4.5. Recognition by the Immune System, and Complement Activation and Opsonization

A major fate of nanomaterials in physiological media is opsonization, which typically leads to phagocytosis of the opsonized body by macrophages.[55,70] Opsonization is mediated by the recognition by macrophages of plasma proteins called opsonins. As proteins, opsonins also take part in the formation of the protein corona and are therefore responsible for the opsonization of the NPs.[38,71] This immunological response leads to rapid clearance of the nanomaterial from the blood stream and accumulation in the liver and spleen.[55a]

In the process of secondary protein corona formation, proteins adsorb onto nanoparticles via protein–protein interactions. These protein interactions can be specific, meaning that they would occur in the absence of the nanomaterial, or nonspecific. The secondary nonspecific binding events are driven by electrostatic or hydrophobic interactions between free proteins and charged or hydrophobic protein domains on the corona that have been exposed as a result of conformational changes. These interactions do not occur in the native system in the absence of the nanomaterial. The resulting complexes are detected as an abnormality by the biological system and therefore trigger an immune response.[38,72] Importantly, NPs decorated with anti-fouling coatings that minimize protein adsorption (as described in Section 3) can reduce the interaction with the phagocytic system.[19,73]

5. Limitations Derived from Interfacing Sensing Nanoplatforms and Physiological Environments

In the following, we review the limitations of two relevant and widely used bio-sensing nanoplatforms, SERS and Förster resonant energy transfer (FRET), related to the complexity of biological systems.

5.1. SERS

SERS is a spectroscopic technique by which the Raman signal of an analyte is strongly enhanced when sitting in close proximity to the surface of a nanostructured noble metal (Au, Ag).[74] Enhancement factors (EFs) are strongly localization-dependent and drop dramatically when moving away from the surface of the metal. In general, isolated nanoparticles do not provide EFs high enough to be responsible for the observed SERS signal of a nonresonant analyte. The signal then stems from 'hot spots', gaps of a few nanometers within agglomerates of NPs. Some nanoparticles displaying sharp apexes or strong roughness give rise to EFs comparable to that of coupled nanoparticles and can even enable single molecule detection.[75]

Intracellular SERS experiments mainly focus on two objectives: (i) the measurement of intracellular analytes of endogenous or exogenous nature or, alternatively, of cell chemical parameters (pH, redox potential); (ii) the tracking of cellular moieties or organelles through their labelling with SERS-encoded substrates.

Due to the narrow bandwidth of Raman signals, intracellular SERS allows for the detection of various biological species having specific vibrational peaks such as DNA, lipids, or specific amino acids within proteins.[76] When recorded with high spatial resolution, SERS spectra can be exploited to map the distribution of molecular species.[77] Exogenous molecules attached to or adsorbed onto SERS substrates can also be detected. Their SERS spectra can be exploited for instance to monitor the kinetics of uptake or metabolization of a drug within a cell.[78] The most advanced intracellular SERS applications involve the measurement of cell parameters through the use of SERS substrates encoded with molecular probes that are strongly adsorbed onto the substrate. The SERS spectra of these probes are sensitive to the targeted parameters and can therefore be exploited for the ratiometric quantitative determination of the parameters of interest, e.g. pH values,[79] or intracellular redox potentials.[54]

Alternatively, intracellular SERS can be used as a labelling technique for a given cell moiety. This strategy was followed by Rector and co-workers to track the fate of an internalised IgE receptor.[79b] Their sensor consisted of a SERS substrate encoded with a pH-senstive Raman reporter and bearing 2,4-dinitrophenol-L-lysine (DNP) ligands. The DNP ligands could be recognised by DNP-specific IgE which in turn associated with IgE receptors on the cell membrane. The SERS encoded substrate allowed for the monitoring of the traffic of IgE receptor within the cell. In addition, the pH-sensitive SERS spectrum enabled the identification of the compartment in which the receptor stood (endosome or lysosome).

SERS substrates can be cytotoxic (Figure 1, blue scenario) and, therefore, the first key to meaningful SERS measurements of biological activity is to ensure that the SERS probe does not largely impair the normal functioning of the cell on the timescale of the measurement. In the same line, when measuring endogenous analytes or cell parameters, it is important to make sure that the SERS substrate or integrated probe does not affect the target of the measurement.[54]

Cellular mapping of the distribution of analytes has been routinely done through the use of molecular fluorophores or genetically encoded fluorescent proteins. SERS imaging is advantageous compared to fluorescence imaging because of the narrow signal bandwidth that enables multiplexion. However, SERS can have a major disadvantage compared

to molecular probe based techniques: When using smooth SERS nanoparticles (nanospheres, nanorods), which are the most common type of substrate for intracellular measurements, the obtaining of a SERS spectrum requires the formation of agglomerates. These agglomerates, let alone the SERS substrates themselves, are generally not evenly distributed throughout the cells and, therefore, not all cell areas are probed with equal probability.[76] Accordingly, the absence of an analyte signal in a cell compartment does not necessarily mean that the analyte is not present. This difficulty may be overcome by cross-referencing SERS mapping with TEM imaging to map the areas that are rich in agglomerates and by enlarging the random sample size. However, in most intracellular mapping, it is not enough to identify a given analyte or to co-localise two target species throughout the cell. One aims to quantify the relative concentrations. This task is rendered tedious by the distribution of EFs throughout agglomerates within the cell. Indeed, the protein corona might increase the NP-to-NP distance in the agglomerates and either strongly affect the EF within the hot spot or prevent the formation of hot spots altogether (Figure 1, green scenario).[38] The reverse effect can also be observed. Denatured proteins within protein coronas can cross-link NPs and lead to uncontrolled agglomerates of unknown EFs. An elegant strategy to overcome both the issues of mapping and of reproducible EFs over the cell involves the use of nanoparticles displaying sharp apexes that provide strong enough EFs to give rise to decent SERS spectra as single NPs. This has been achieved by Brust and co-workers using SERS-encoded gold nanostars.[16] Another strategy is based on the building of agglomerates made of smooth NPs prior to cell exposure. This idea has been recently developed by Kotov and co-workers, who synthesised controlled assemblies made of a central gold nanorod and surrounding gold nanospheres of various topologies (end, side, and satellite).[80] Using these nanostructures, intracellular lipids were detected with sensitivities that depended on the type of assembly (**Figure 2**).

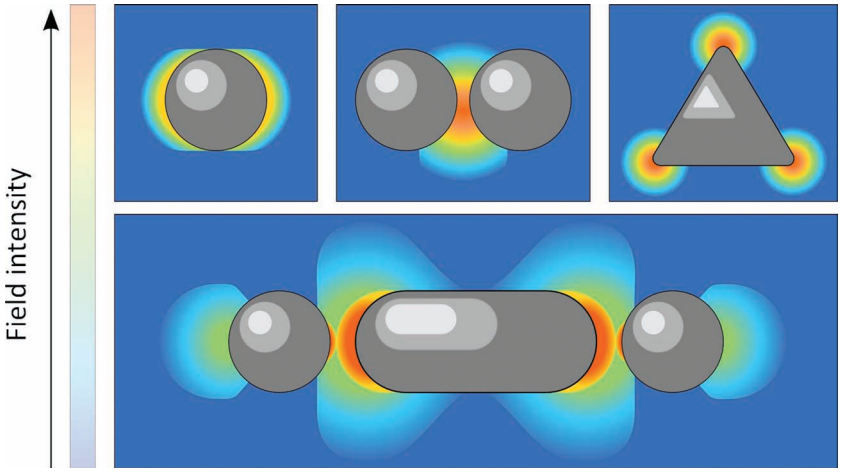

Figure 2. Electromagnetic field intensity maps for a spherical plasmonic NP, a dimer, a prism as an example of anisotropic NP and an 'end assembly' as described in reference 80.

Quantification can also be a hassle due to the complexity of the intracellular environment. Difficulties arise when attempting to quantify a given protein in a physiological environment because of the presence of a large amount of proteins surrounding the SERS substrate.[76] Any protein present within a few nanometers of the SERS substrate surface will see its spectrum enhanced. Hence, the resulting SERS spectrum is expected to be very complex as it will display not only the vibrational signature of the protein of interest, but also the contribution of all other proteins within the protein corona. Moreover, as proteins are made of similar building blocks, namely amino-acids, they display very close spectral signatures that can lead to peak overlaps, further complicating the spectrum. Advanced deconvolution techniques are then required to assess the presence of the targeted protein and further quantification is expected to be challenging. The targeted protein is not even guaranteed to see its Raman spectrum enhanced since it might never reach the metal surface. Indeed, proteins with different affinities compete for adsorption on the nanomaterial surface. Hence, if the targeted protein has a relatively weak affinity for the SERS substrate compared to other surrounding proteins, and/or if its relative concentration is low, it might never be detected. The same observation holds relevance for other physiological molecules of interest, such as lipids or sugars which might never reach the hot spots if they cannot efficiently compete with the protein corona.

In several intracellular SERS experiments aimed at monitoring exogenous analytes or Raman reporters, the molecule of interest is adsorbed onto the SERS substrate prior to its internalisation by the cells.[78,79] The Raman-active molecule can be either physisorbed (as, for example, Rhodamine 6G or Nile Blue) or more firmly anchored to the SERS substrate through a thiol or amine coordination to the metal.[81] Physisorbed dyes are usually chosen because, as resonant molecules in the visible range, they provide very intense SERS spectra with high signal-to-noise ratios. However, their SERS signals rapidly vanish due to displacement of the Raman label by the protein corona (Figure 1, green scenario).[81] In contrast, coordinated molecules (examples so far were nonresonant) display intracellular SERS signals that are stable on a 24 h timescale. Therefore, to monitor cell parameters such as pH or redox potential, it is advisable to use thiol- or amine-bearing probes. In addition, the use of ratiometric probes appears particularly appealing since they enable quantification independent of the amount of probe or of the EF, provided that the latter gives rise to a sufficient signal-to-noise ratio.[82]

5.2. FRET

Förster resonant energy transfer has long since been exploited for the sensing of biomolecular recognition processes using organic dyes and green fluorescent protein (GFP) derivatives. The transfer of quantum dots (QDs) into aqueous solutions

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has enabled their integration into new FRET set-ups where the QDs are mostly exploited as donors. This research field has been highly active in the past ten years because of the advantageous optical properties of QDs over conventional molecular fluorophores.[83] However, FRET sensing of biomolecules or bio-events using nanomaterials faces challenges inherent to the interaction of biomaterials with NPs. These can be foreseen at two developmental stages: (i) the optimisation of the sensor 'in synthetico', that is, in buffers and relatively simple analytical media; (ii) the implementation of the sensor 'in physiologico', that is, in physiological conditions (in protein-rich environments, such as plasma or cells).

The protein corona and the related conformational changes induced by it have tremendous impact on FRET sensing since they can affect the recognition mechanism involved in the formation or dissociation of the FRET pair (cf. Section 3 and Figure 1, green scenario). To the best of our knowledge, there is no general trend and each case should be examined carefully. For instance, Dezhurov et al. have observed that BSA retains its folding capability upon recognition of oleic acid once supported on QDs.[84] This enabled the sensing of oleic acid through the modulation of FRET efficiency between the QD and the dye-labelled BSA upon binding. This result is in contrast with the loss of substrate ability of siRNA adsorbed onto up-converting nanocrystals (upCNs) for RNA-ase, observed by Jiang and Zhang (**Figure 3**).[85] This impairment of activity was turned into an advantage because it prevented degradation of the siRNA prior to delivery into the cytoplasm. To avoid impairment of biological activity of a ligand, recognition unit, or enzyme, several strategies have proven successful. Introducing rigid or long spacers into the linkers between the NP and biomolecules prevents their strong interaction, thus preserving biological activity.[86] Conjugating the nanocrystals with an anti-fouling coating (Figure 1, yellow scenario) also provided good results.[87] However, the advantages of long spacers or anti-fouling coatings for preserving bioactivity may come at the price of reduced optical performance. Indeed, the accumulation of conjugation-coating spacer and biomolecules can lead to rather high donor-to-acceptor distances and therefore poor FRET efficiencies that limit the sensitivity of the sensor. This is particularly true for sandwich immunoassays, where the recognition of the target necessitates assembly of two antibodies, which are rather heavy proteins (Figure 1, orange scenario). This constraint puts more pressure on finely tuning the spectral properties of donor–acceptor pairs to maximise the Förster radius. Several materials look appealing to address this issue. First, aptamers can be considered as lighter alternative recognition units,[88] although their comparatively lower complexation constants can lead to cross-talk in multiplexed assays. Second, as acceptors, gold nanoparticles tend to give rise to larger Förster radii than conventional dyes.[89] Finally, the use of a lanthanide complex as a donor and a QD as an acceptor enables maximal spectral overlap between their respective emission and absorption spectra and consequently provides Förster radii on the order of 10 nm.[87]

Another difficulty in optimising the sensitivity of the FRET sensor stems from the distribution of FRET configurations within the sample, which originate from the distribution of donor-to-acceptor ratios and the distribution or relative orientations and distances between them.[84,89] Control over stoichiometry or geometrical parameters is especially difficult to achieve when there is significant nonspecific interaction between the biomolecules and the nanocrystals (Figure 1, green scenario). However, anti-fouling coatings seem to overcome this issue, and control over donor–acceptor stoichiometry has been reported using this strategy (Figure 1, yellow scenario).[87]

As protein-rich environments have very different physico-chemical properties (optical index, polarity, etc.) compared to buffer solutions, the photophysical performances of each component of the FRET pair are likely to be modified. Indeed, Hildebrandt and co-workers observed both dynamic and static quenching of QDs upon introduction into various protein-rich media, in agreement with the formation of a protein corona.[46] Importantly, the extent of dynamic and static quenching varied depending on the composition of the medium, on the size, composition and surface coating of the QD. No general trend could be formulated, leading to the conclusion that 'not every QD is alike'. Hence, the photophysical properties must be re-evaluated for each medium and each nanocrystal sample.

Much like for the SERS assays, nanocrystal-based FRET sensors can see their performances brought down by competitive adsorption of one part of the donor–acceptor construction and proteins. For instance, attachment of polyhistidine-containing peptides tagged with a Tb complex to CdSe/ZnS core–shell QDs has been shown to be unsuccessful in plasma, while the reverse was true in TRIS- or BSA-containing buffers (tris(hydroxymethyl) aminomethane (TRIS) and bovine serum albumin (BSA)).[46] The polyhistidine moiety, which has a strong affinity for the Zn-rich QD shell, is also present in plasma proteins in rather abundant concentration. Competitive adsorption of these histidine-rich proteins was thought to be the main cause of poor FRET performance in plasma. A stronger linker, possibly crosslinked around the NP surface, might circumvent this limitation.

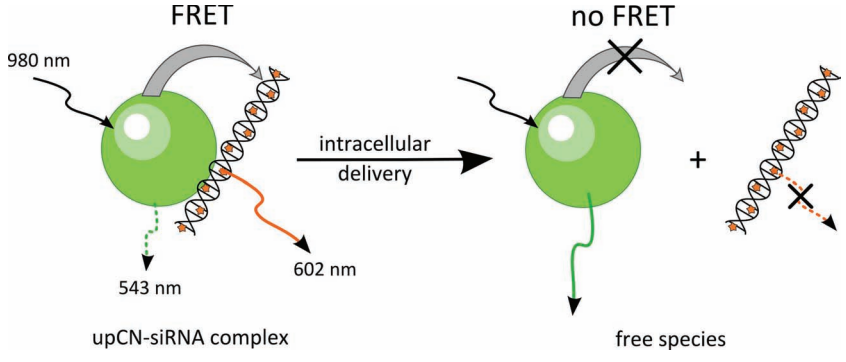

Figure 3. FRET scheme between siRNA and up-converter nanocrystals (upCN) used in reference 85 to monitor the intracellular delivery of siRNA.

The protein corona, even if it does not displace components of the FRET edifice,

can prevent successful FRET events by sterically hindering the pairing of donor and acceptor. The use of FRET assays in which a preformed donor–acceptor pair dissociates upon recognition of the analyte, or alternatively in which the FRET path is broken, might overcome this hindrance,[85,90] although the intracellular assembly of a donor–acceptor pair from initially free components has also been reported.[91]

6. Conclusion

Despite the need for standard protocols for assessing the toxicity and bioperformance of each new functional nanomaterial, these are still scarce or still under development. Interfacing engineered nanomaterials with biological fluids and predicting their biological fate are not trivial issues, since the interplay between nanomaterials and biological components is dictated by a complex scenario of interactions. The structural integrity and function of nanomaterials can be greatly compromised by the presence of components of physiological fluids. The most relevant parameters in the NP design are: synthesis of the inorganic core (composition, size, shape, and structure, e.g., solid or hollow), stabilization (i.e. derivatization techniques towards enhanced colloidal stability in physiological media) and, lastly, functionalization with molecules of biological relevance for enhanced bioperformance. Any new nanomaterial has to be critically analyzed, as many adverse effects can be triggered accidentally (e.g. toxicity, allergy, genotoxicity, etc.) and/or the performance of the nanomaterial (e.g. as a therapeutic or diagnosis agent, or nano-biosensor) can be compromised. However, some trends concerning physicochemical descriptions are emerging and these 'nano' attributes can be accessed synthetically, enabling tailoring for specific purposes and partly preventing adverse effects such as cytotoxicity, opsonization, leakage of toxic NP components, nonspecific NP–protein interactions, distortion of biological relevant proteins.

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