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Understanding biophysicochemical interactions at the nano–bio interface

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Rapid growth in nanotechnology is increasing the likelihood of engineered nanomaterials coming into contact with humans and the environment. Nanoparticles interacting with proteins, membranes, cells, DNA and organelles establish a series of nanoparticle/biological interfaces that depend on colloidal forces as well as dynamic biophysicochemical interactions. These interactions lead to the formation of protein coronas, particle wrapping, intracellular uptake and biocatalytic processes that could have biocompatible or bioadverse outcomes. For their part, the biomolecules may induce phase transformations, free energy releases, restructuring and dissolution at the nanomaterial surface. Probing these various interfaces allows the development of predictive relationships between structure and activity that are determined by nanomaterial properties such as size, shape, surface chemistry, roughness and surface coatings. This knowledge is important from the perspective of safe use of nanomaterials.

At the interface between nanomaterials and biological systems, the organic and synthetic worlds merge into a new science concerned with the safe use of nanotechnology and nanomaterial design for biological applications. The 'nano–bio' interface comprises the dynamic physicochemical interactions, kinetics and thermodynamic exchanges between nanomaterial surfaces and the surfaces of biological components (for example proteins, membranes, phospholipids, endocytic vesicles, organelles, DNA and biological fluids). For this field to evolve, we must understand the dynamic forces and molecular components that shape these interactions. It is impossible to describe with certainty all the biophysicochemical interactions at play at the interface, but we are at a point where the pockets of assembled knowledge are providing a conceptual framework to guide this exploration. Here we explore such interfaces from the perspective of the forces governing colloidal chemistry and the adaptations that occur at biological interfaces. We focus on the biological interfaces that nanoparticles may encounter after suspension in a tissue culture or biological medium, and after interacting with cells (membrane surfaces, endosomal compartments, organelles and cytoplasm). We define how these interactions modify the fundamental forces that govern nanoparticle interactions under classical colloidal conditions and discuss the development of methods for probing the nano–bio interface.

Boundaries shaping the interface

The nano–bio interface comprises three dynamically interacting components: (i) the nanoparticle surface, the characteristics of which are determined by its physicochemical composition; (ii) the solid–liquid interface and the changes that occur when the particle interacts with components in the surrounding medium; (iii) the solid–liquid interface's contact zone with biological substrates (Fig. 1 and Table 1). In a given medium, the most important nanoparticle characteristics that determine the surface properties are the material's chemical composition, surface functionalization, shape and angle of curvature, porosity and surface crystallinity, heterogeneity, roughness, and hydrophobicity or hydrophilicity[1–3]. Other quantifiable properties, such as effective surface charge (zeta potential), particle aggregation, state of dispersion, stability/biodegradability, dissolution characteristics, hydration and valence of the surface layer, are determined by the characteristics of the suspending media[1], including the ionic strength, pH, temperature and the presence of large organic molecules (for example proteins) or detergents[4]. The particle characteristics contribute actively to the interactions with the medium through: (i) promoting the adsorption of ions, proteins, natural organic materials and detergents; (ii) double-layer formation; (iii) dissolution; or (iv) minimizing free surface energy by surface restructuring[5,6]. Many of these newly acquired particle properties or transformed states determine the forces that operate at the particle–medium interface with characteristic decay lengths[6] (Fig. 2). These forces include long-range forces arising from attractive van der Waals (VDW) and (generally) repulsive electrostatic double-layer interactions, plus short-range forces arising from charge, steric, depletion and solvent interactions[6,7] (Table 2). Media interactions (for example protein interactions) could also induce large-scale changes, such as nanoparticle dissolution, ion leaching, phase transformation and agglomeration (Fig. 3a).

Characterizing the solid–liquid interface is a key challenge in understanding the nano–bio interface. Although we often assume steady-state behaviour when assessing the bulk properties of suspensions (for example net charge, isoelectric point or average aggregate size), this approach must be adjusted when considering the nano–bio interface. The already metastable solid–liquid interface is subjected to an inhomogeneous and dynamic or transient environment that contributes to the formation of the nano–bio interface. Such interfacial inhomogeneity results from the distribution and distinct spatial localization of proteins, lipids and glycosylated structures on the surface membrane. Moreover, the interface is not at steady state. It undergoes continuous changes as a result of cellular housekeeping

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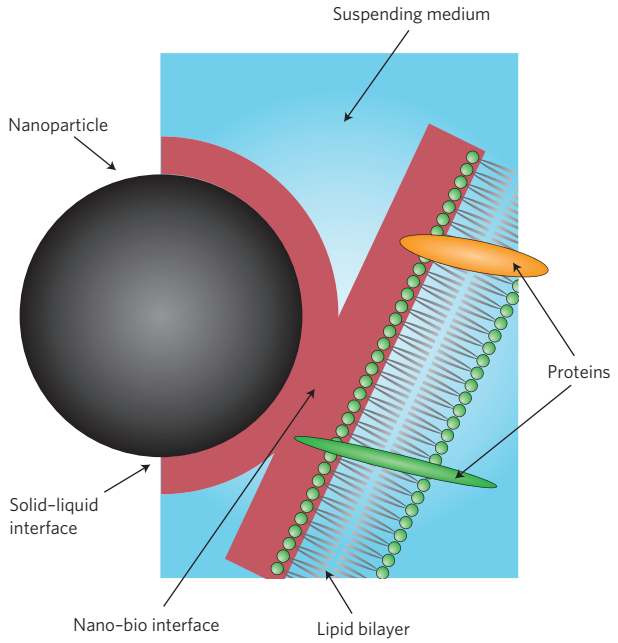

Figure 1 | Representation of the interface between a nanoparticle and a lipid bilayer. The main effects stem from material properties, modification of the surface properties of those materials through interactions with the suspending medium, and the dynamic interactions of the solid–liquid interface with biological molecules and cellular compartments. See Table 1 for details.

and environmental influences; for example, secreted cell products may further change a suspending medium's properties[8], and the nanoparticles may bind to these biomolecules in suspension or on the cell membrane. Likewise, the interacting biological components might be influenced by the nature of the particle, for example through binding to surface ligands, contact with hydrophobic or charged regions, changes in free surface energy inducing conformational changes or oxidant injury caused by reactive oxygen species (ROS)[1]. Particle wrapping by the surface membrane and cellular uptake will introduce the particles to new interfaces. Although interactions at cell surfaces and intracellular compartments involve large numbers of forces and molecular interactions, the successful (often anecdotal) use of nanoparticles to achieve measurable biological outcomes, such as subcellular imaging, indicates that it is indeed possible to probe the nano–bio interface experimentally. Although it may take some time to develop investigative methods to explore these interfaces in the same detail as has been accomplished in colloidal chemistry, the principles of the nano–bio interface are being investigated through new imaging techniques and biological approaches (Box 1).

Forces at the nano–bio interface

At first glance, the interactions between nanoparticles and cells seem to embody some of the same principles as those between colloidal particles. VDW, electrostatic, solvation, solvophobic and depletion forces still apply, but they require special consideration for events occurring at the nanoscale[6,9,10] (Table 2 and Fig. 2). For example, because nanoparticles contain relatively few atoms, their VDW forces are highly dependent on the positioning of their surface atoms and their standard bulk permittivity functions[10]. This complexity increases greatly when it comes to the interface between nanomaterials and biological systems. Consider two examples: the interaction of two silica particles, and that between a SiO_2 particle and a fibroblast cell.

Typical interactions between SiO_2 particles in water involve VDW, electrostatic and solvation forces (Fig. 2). VDW forces result from the quantum mechanical dance of the electrons; at any moment, their fluctuations produce a small but important dipole in the particle, thereby inducing a dipole moment in the atoms of the adjacent SiO_2 particle and causing an attractive force. The electrostatic force in the system results from surface charges that inevitably arise on the SiO_2 particles. In contact with water, silanol (Si–OH) groups dissociate to yield negative surface charges, which will generate, at least transiently, repulsive electrostatic forces between particles; under constant surface potential conditions, such repulsion might be mitigated by surface regulation. For SiO_2 particles, the sum of the attractive VDW and repulsive electrostatic forces yields the well-established Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloid science[6,7] (Fig. 2).

In biological fluids, the ionic strength is often about 150 mM, meaning that the electrostatic forces are most likely to be screened within a few nanometres of the surface. The high ionic strength also obscures the zero-frequency contribution of the VDW forces, whereas higher-frequency dispersion interactions remain operative. Moreover, solvation is another phenomenon that is important for inorganic and other hydrophilic nanoparticles (Fig. 2). The water molecules adhere to the particles with sufficient energy to form

Table 1 | Main biophysicochemical influences on the interface between nanomaterials and biological systems.

Nanoparticle
Size, shape and surface area
Surface charge, energy, roughness and porosity
Valence and conductance states
Functional groups
Ligands
Crystallinity and defects
Hydrophobicity and hydrophilicity
Suspending media
Water molecules
Acids and bases
Salts and multivalent ions
Natural organic matter (humics, proteins, lipids)
Surfactants
Polymers
Polyelectrolytes
Solid–liquid interface
Surface hydration and dehydration
Surface reconstruction and release of free surface energy
Ion adsorption and charge neutralization
Electrical double-layer formation, zeta potential, isoelectric point
Sorption of steric molecules and toxins
Electrostatic, steric and electrosteric interactions
Aggregation, dispersion and dissolution
Hydrophilic and hydrophobic interactions
Nano–bio interface
Membrane interactions: specific and nonspecific forces
Receptor–ligand binding interactions
Membrane wrapping: resistive and promotive forces
Biomolecule interactions (lipids, proteins, DNA) leading to structural and functional effects
Free energy transfer to biomolecules
Conformational change in biomolecules
Oxidant injury to biomolecules
Mitochondrial and lysosomal damage, decrease in ATP

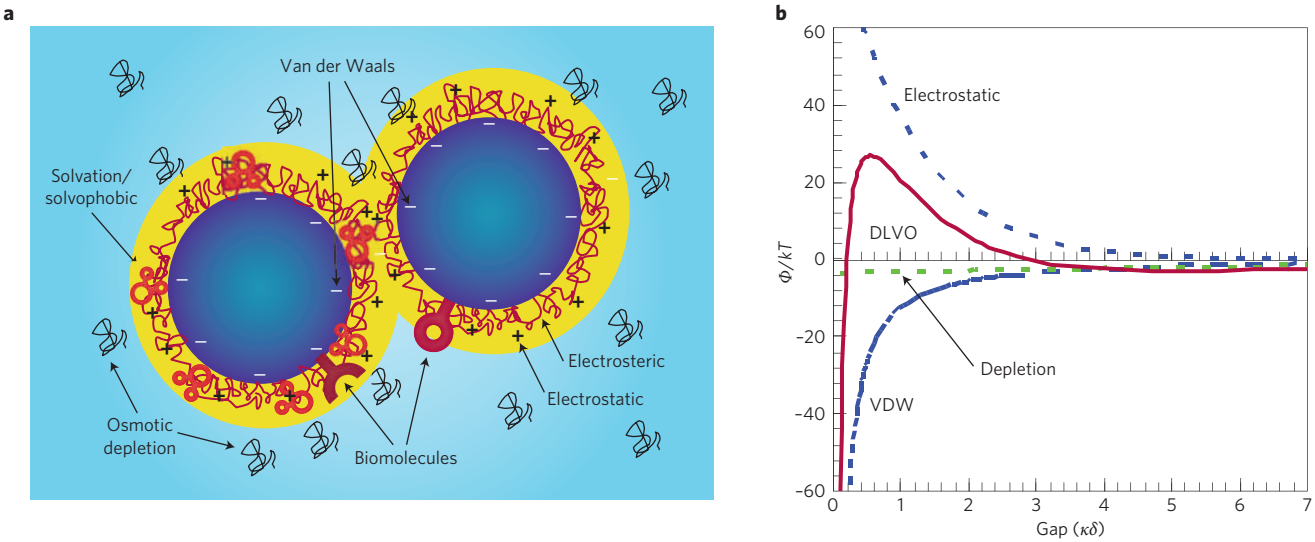

Figure 2 | Interactions between nanoparticles. a, Traditional forces for colloidal fabrication (for example electrostatic, VDW, covalent) and other important interactions (for example solvation, solvophobic, biomolecular, depletion) that occur when particles are suspended in biological media and come into contact with cells. **b**, VDW and depletion forces are attractive whereas the electrostatic forces are repulsive over a typical length scale. The DLVO theory in colloid science considers the sum of these forces. Φ, interaction potential; k, Boltzmann constant; T, absolute temperature; κ, inverse debye length; δ, separation distance. Modified with permission from ref. 7; © SPIE 2007.

steric bumper layers on their surfaces, making it difficult for pairs of particles to touch or adhere. Thus, solvation forces increase particle stability through 'hydration pressure' or 'hydrophilic repulsion'. Alternatively, rapid dehydration and aggregation will occur if the relative affinity of two interacting surfaces for water molecules is much lower than that between the water molecules themselves — a 'hydrophobic attraction' or 'hydrophobic effect'. All of these forces, which change little or slowly over time, can be predicted or estimated using known theories[10]. These experimental measurements also tend to be fairly consistent.

We now consider the example of a particle interacting with a fibroblast cell. Although forces similar to those described above are operative, important differences are introduced as the particle approaches the surface membrane. The first is that the cell has a non-rigid compliant membrane that can deform as a result of the fluidity and thermodynamics of the membrane[9]. This leads to a complicated set of interactions (see below). Even a relatively straightforward study — such as that of a particle interacting with a fluid mercury surface — introduces complexity of a nature that warrants extensive research[9]. A further complexity arises from the patchiness of the cell surface[11], where charge non-uniformity alters the energy of interparticle interactions to a great extent, even between two 'ideal' particles[12]. Cells have surface heterogeneity on the length scale of 10–50 nm because of the presence of surface proteins and other structures. Thus, a micrometre-sized particle interacting with a patchy cell surface would experience an averaging of the energy levels covering many heterogeneous patches. If a nanoparticle of size 10–50 nm were to interact with one patch at a time, however, the interaction energy would vary greatly depending on its precise location and its potential to roll over the cell's surface. In addition, multiple particles might form rafts that would show markedly different properties than those of single particles.

A third complexity arises because cells are not passive. By transporting ions or secreting proteins and other biological molecules, the cell conditions the surface of the SiO_2 particle, transforming it into something very different from the surface initially placed into the system. This phenomenon introduces the concept of a time-dependent, dynamic interface[8,13]. The possibility of endocytosis — the incorporation of a surface-bound nanoparticle into a cell by folding of the cell's membrane — increases the complexity of these interactions, making them difficult to predict theoretically. For particle phagocytosis (the active engulfment of particulates by professional phagocytes, including macrophages and dendritic cells[14,15]), attempts have been made to model the effect of specific receptor–ligand interactions together with electrostatic, steric repulsive and attractive VDW forces between the particle surface and the phagocyte cell membrane[16]. This approach considers that these interactions vary under experimental conditions, with contributions made by the cell types, their stages of differentiation, the composition of the culture medium and the pathways of cellular processing.

Finally, consider a conceptually infinite matrix of nanoparticles having: (i) diverse external shapes (for example spherical, cubic, triangular, tubular, hyper-branched, needle-like); (ii) internal crystallinity that, by design, gives rise to strong photonic, electronic, semiconducting, transport, sorption and catalytic properties; and (iii) external surface chemistry that produces selective binding reactions, temperature- or pH-dependent amphoteric or amphiphilic behaviour, and antimicrobial functionality. Without reference to specific examples, this system defines the vast matrix of nanomaterials currently being envisaged, created or studied by materials scientists, engineers and biomedical researchers. Any new findings for a particular nanomaterial, however groundbreaking at one moment, may be irrelevant a short time later if the same particle is produced through a different synthetic route or intentionally modified.

Protein corona

When nanoparticles enter a biological fluid (for example blood, plasma or interstitial fluid) they are coated with proteins that may undergo conformational changes, leading to the exposure of new epitopes, altered function and/or avidity effects[17–19]. The concept of the nanoparticle–protein corona is important in shaping the surface properties, charges, resistance to aggregation and hydrodynamic size of nanoparticles (Fig. 3). We note that the interactive nanoparticle surface might be pre-bound to chemical substances that reflect its prior history and could influence its protein adsorption kinetics. These pre-existing surface species could originate from: (i) residues from the manufacturing process or exposure to ambient gases; (ii) industrial chemicals and stabilizers used to prepare dispersions; or (iii) organic and inorganic constituents of biologically relevant buffers used in preparing laboratory stock solutions.

Table 2 | Main forces governing the interfacial interactions between nanomaterials and biological systems.

Force	Origin and nature	Range (nm)	Possible impact on the interface
Hydrodynamic interactions	Convective drag, shear, lift and Brownian diffusion are often hindered or enhanced at nanoscale separations between interacting interfaces	10^2 to 10^6	Increase the frequency of collisions between nanoparticles and other surfaces responsible for transport
Electrodynamic interactions	VDW interactions arising from each of the interacting materials and the intervening media	1 to 100	Universally attractive in aqueous media; substantially smaller for biological media and cells owing to high water content
Electrostatic interactions	Charged interfaces attract counter-ions and repel co-ions through Coulombic forces, giving rise to the formation of an electrostatic double layer	1 to 100	Overlapping double layers are generally repulsive as most materials acquire negative charge in aqueous media, but can be attractive for oppositely charged materials
Solvent interactions	Lyophilic materials interact favourably with solvent molecules Lyophobic materials interact unfavourably with solvent molecules	1 to 10	Lyophilic materials are thermodynamically stable in the solvent and do not aggregate Lyophobic materials are spontaneously expelled from the bulk of the solvent and forced to aggregate or accumulate at an interface
Steric interactions	Polymeric species adsorbed to inorganic particles or biopolymers expressed at the surfaces of cells give rise to spring-like repulsive interactions with other interfaces	1 to 100[a]	Generally increase stability of individual particles but can interfere in cellular uptake, especially when surface polymers are highly water-soluble
Polymer bridging interactions	Polymeric species adsorbed to inorganic particles or biopolymers expressed at the surfaces of cells containing charged functional groups can be attracted by oppositely charged moieties on a substrate surface	1 to 100	Generally promote aggregation or deposition, particularly when charge functionality is carboxylic acid and dispersed in aqueous media containing calcium ions

[a]Depending on the length of adsorbed or expressed polymeric species

Particles bound to proteins in biological fluids constitute an initial nano–bio interface that undergoes dynamic changes as the particles subsequently move onto or into cells. The kinetics of nanoparticle–protein association and dissociation, and concurrent exchange with free proteins in the media, have important roles in determining the particle's interactions with biological surfaces and receptors — and hence its fate. The lifetimes of particle–ligand complexes range from microseconds to days; they can be assessed quantitatively using various techniques (for example isothermal titration calorimetry, size exclusion chromatography, surface plasmon resonance spectroscopy or thioflavin T fluorescence spectroscopy).

Because the protein corona could shape the cellular interactions with nanomaterials, a key question is whether such protein interactions depend on particle composition (Fig. 3a). The nature of the particle surface (for example its hydrophobicity, size, radius of curvature, charge, coatings that exert steric or electrosteric effects; Fig. 3a) will control which biomolecules interact with the particles — and hence mediate their access to cells[17]. Several proteins are known to form transient complexes with nanoparticles; large variations in their dissociation rates establish preferential binding interactions between specific particle types and the biological fluids in which they are suspended. In addition, protein concentrations near the particles will influence their binding. For example, consider the dynamic corona of a nanoparticle in the blood: human serum albumin and fibrinogen might dominate the particle surface for short periods of time, whereas lower-abundance proteins with higher affinities and slower kinetics might ultimately displace them[17]. In contrast, in a lower-protein environment (such as bronchial or ocular fluid), lower-affinity binding proteins (such as albumin) may dominate the particle's surface. The proteins that bind most strongly to carbon nanotubes (CNTs), iron oxide particles, liposomes and polymeric nanoparticles are albumin, immunoglobulins, complement, fibrinogen and apolipoproteins. Complement and immunoglobulin binding leads to particle opsonization: that is, it promotes receptor-mediated phagocytosis[20]. This behaviour might be helpful in developing new vaccines or minimizing adverse health effects due to immune activation[20]. Although deliberate attachment of protein ligands (for example transferrin) can increase endocytic uptake, it is less clear how the spontaneous formation of a protein corona might contribute to tissue uptake *in vivo*. It is generally perceived that plasma protein binding is important in determining the *in vivo* organ distribution and clearance of carrier particles from the circulation. This behaviour could explain, for instance, why decreased protein absorption of injected polyethylene glycol (PEG)-coated particles leads to longer circulation times and altered biodistribution[20].

Proteins and organic substances increase the dissolution rates of particles of ZnO, CdSe, iron oxides, aluminium oxides and oxyhydroxides through at least two mechanisms[21]: aqueous complexation (that is, aqueous species complexing free ions released from the material's surface) and ligand-enhanced dissolution (that is, adsorbed natural organic material and organic acids extracting surface metal atoms from nanoparticle surfaces). The latter mechanism has been demonstrated for iron and aluminium oxides and oxyhydroxides, and is also likely to occur for ZnO.

Studying the reverse effects of particles on proteins is important for understanding potential biological injury due to such changes as fibrillation, exposure of new antigenic epitopes and loss of function such as enzymatic activity. In one of the best-studied experimental models, nanoparticles act as catalysts exposing protein interaction domains that induce aggregation through hydrogen bonding and the formation of disease-promoting fibrils[18]. For example, human β2-microglobulin fibrillation occurs on the surfaces of cerium oxide and copolymer nanoparticles and CNTs[18]; this formation of amyloid fibrils is a quantifiable process. It remains to be determined, however, whether these experimental conditions can be duplicated *in vivo*, where competitive binding in complex biological fluids may screen the nucleation surface. There is no evidence as yet that nanoparticle-induced protein fibrillation is involved in any disease pathogenesis. It is theoretically possible, however, that repetition of the same process in the brain could contribute to neurodegenerative processes (for example Alzheimer's disease). Although the exact trigger of protein unfolding at the particle surface is unknown, it might involve contact forces, such as the release of free surface energy through surface reconstruction. An example might be relaxation of the particle crystal structure through protein binding[5] (Fig. 3b). Similarly, it is possible that electron confinement or the formation of electron–hole pairs at the material surface could lead to cleavage of structural bonds or covalent cross-linking of protein SH domains.

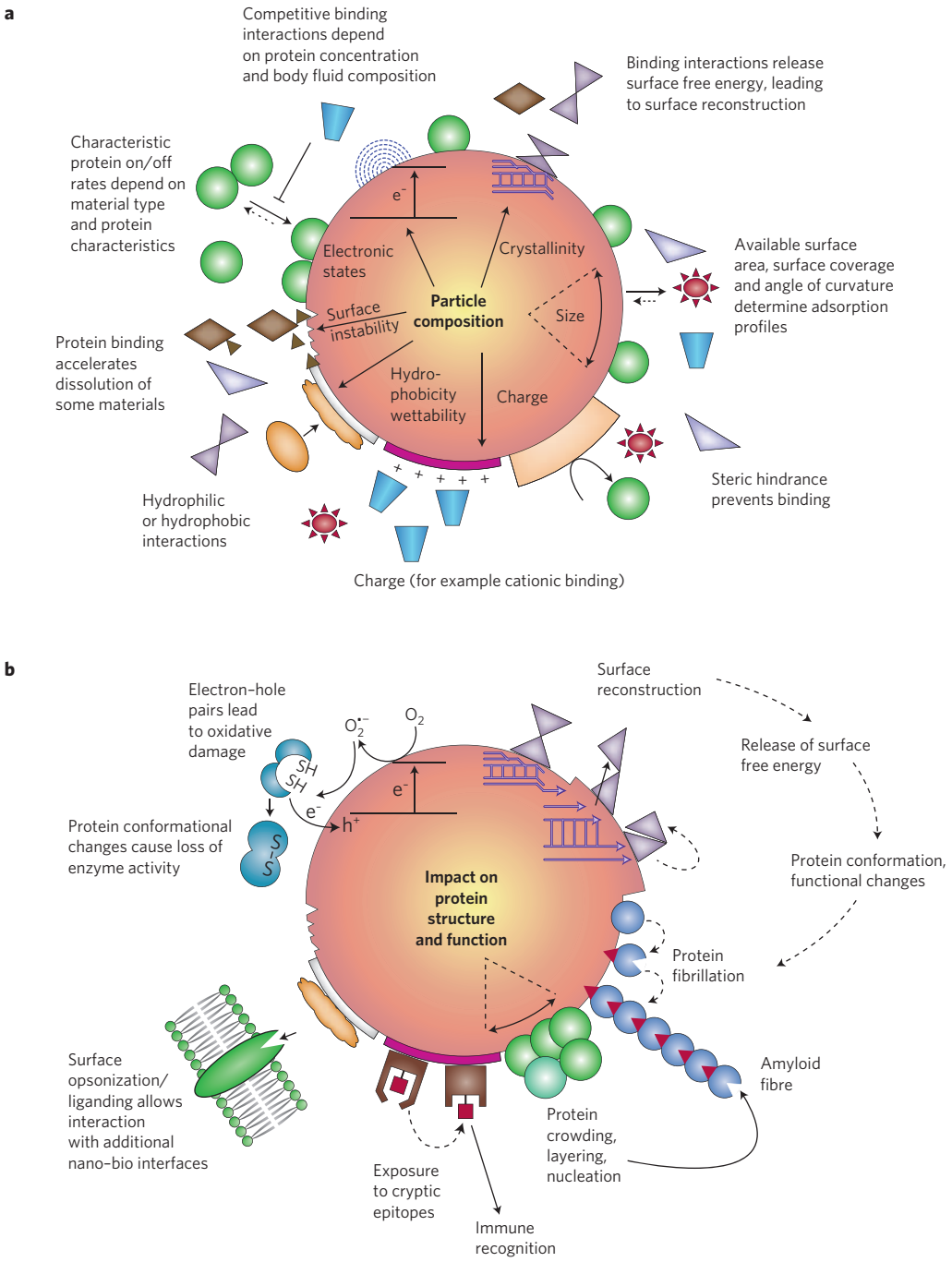

Figure 3 | Effects of protein corona surrounding a nanoparticle. The corona constitutes a primary nano–bio interface that determines the fate of the nanoparticle and can cause deleterious effects on the interactive proteins. **a**, Pre-existing or initial material characteristics contribute to the formation of the corona in a biological environment. Characteristic protein attachment/detachment rates, competitive binding interactions, steric hindrance by detergents and adsorbed polymers, and the protein profile of the body fluid lead to dynamic changes in the corona. The corona can change when particles move from one biological compartment to another. **b**, Potential changes in protein structure and function as a result of interacting with the nanoparticle surface can lead to potential molecular mechanisms of injury that could contribute to disease pathogenesis. The coloured symbols represent various types of proteins, including charged, lipophilic, conformationally flexible proteins, catalytic enzymes with sensitive thiol groups, and proteins that crowd together or interact to form fibrils.

For example, binding of chicken egg lysozyme to SiO_2 nanoparticle surfaces induces unfolding of a critical α-helix that disrupts the catalytic activity of the enzyme[3]. In addition, cross-linking of critical thiol groups in glycerol aldehyde phosphate dehydrogenase leads to a loss of function that can be quantified experimentally[22]. Along similar lines, when a protein containing cryptic epitopes is denatured on a particle surface, the exposure of new antigenic sites may initiate an immune response, which, if launched against a self-protein, could promote autoimmune disease (Fig. 3b).

Particle membrane wrapping

Particle adhesion to a cell-surface lipid bilayer is a prime example of an interface between nanomaterials and biological molecules that can be used for therapeutic drug delivery. Let us first consider the forces

Box 1 | New imaging requirements to probe the nano–bio interface.

To improve nanomaterial design, the nano–bio interface must be explored using new tools to probe dynamic biophysicochemical interactions. Previously, this interface was primarily the domain of practitioners of catalysis, colloid chemistry and related analytical fields. There are opportunities to extend established techniques to study interacting entities of nanoscale size that can form unexpected metastable intermediates. One approach is to adapt the catalyst characterization toolbox (for example specific surface area, crystal structure, bandgap energy). There are, however, considerable conceptual differences between the analysis of catalysts under severe conditions[83] and that of nanomaterials in biological environments. New methods must be developed, such as nanobiosensors that detect the production of ROS by using various redox enzymes aligned on nanoelectrode arrays[47]. Direct means of characterizing interfacial phenomena using traditional bulk material concepts (for example wetting, interfacial tension) remain important[16], but they must be integrated both theoretically and experimentally with studies of the nano–bio interface[30].

Imaging with scanning electron microscopy (SEM), transmission electron microscopy (TEM) and fluorescence microscopy has aided our understanding of the cellular uptake and processing of nanoparticles. Standard SEM and TEM approaches are useful for imaging electron-dense materials (such as metallic nanoparticles), but not soft materials (such as dendrimers and liposomes). Several advances, however, are improving the ability of TEM to study the interface between nanomaterials and the biological system. TEM cryomicroscopy is now used routinely to image unstained biomolecules and intercellular structures at the sub-nanometre level; when combined with data processing, it enables the molecular topographies of single biomolecules to be visualized in conformational states that are not accessible through X-ray diffraction[84]. It is now standard practice to obtain three-dimensional reconstructions of nanoscale biovolumes using different preparation strategies and eucentric tilting goniometers[85,86]. Recently, very high (sub-ångström) resolution, aberration-corrected TEM instruments have been developed to image directly the volumes and surface edge atomic structures of nanoparticles using both transmission and scanning transmission modes[86,87]; scanning TEM improves the contrast of biostructures when combined with energy-filtered TEM imaging[86,88].

An understated problem is the need to resolve nanoscale particles in very large volumes of biomaterials. One approach is correlative microscopy: using optical techniques to identify targets, transferring the sample and grid coordinates to a TEM and automatically navigating those targets to obtain high-resolution images, while maintaining the sample in a frozen, hydrated state throughout[89–91]. An alternative technique uses a dual-beam instrument — an ion beam to cut a cross-section in the bulk biomaterial and SEM to record it. By automating the cutting and recording of the image, data can be processed to provide tomographic representations of the volume[92]; this approach can be used in the fully automated analyses of bulk materials with site-specific targeting, even extended to cryo-frozen natural biological materials, where the structure is recognized through the different rates of sublimation of its cellular components. This technique has been used to remove artefact-free, thin lamellae of frozen tissue from specific sites for TEM cryomicroscopy[93].

Fluorescently labelled nanoparticles and corresponding imaging techniques (for example confocal microscopy) have several potential problems: label instability, altered physicochemical properties and photobleaching from laser exposure. Ideally, new imaging techniques will be developed to visualize local populations of nanoparticles at nanometre resolution in real time within cells without structural damage. A promising development is live cell confocal microscopy, which is ideal for high-resolution imaging of movement through intracellular environments, including endo-exocytosis, vesicle tracking, particle transport and nuclear-cytosol membrane mechanisms[94].

Many technologies are emerging to resolve the nano–bio interface. For example, if the cantilever is vibrated independently at a slightly different frequency, the atomic force microscope tip reveals the accompanying contrast in acoustic impedance as nanoscale heterogeneity[59]. Surface-enhanced Raman scattering (SERS) is another technique being used increasingly for bioimaging of cells and intact animals[95]. It measures the enhanced Raman scattering of molecules adsorbed on metal surfaces (which may be nanotextured). With enhancement factors as high as 10^{15}, this technique is sensitive enough to detect single molecules (PEGylated Au and Ag nanoparticles, for example). Recent tumour imaging with radiolabelled SWCNTs suggests that SERS may be a promising molecular imaging technique in living subjects[96,97].

Developing new techniques will take time. Meanwhile, there is tremendous growth in the development of nanomaterials with enhanced performance characteristics. Many 'legacy materials' have already found widespread commercial use — some for decades — without a full understanding of their reactivity. Although industry desires the safe, widespread use of nanotechnology, it will not occur unless surface characterization is possible using simple and accessible laboratory equipment. Static and single-point measurements are insufficient; the intermediary regimen must accommodate the dynamic and metastable states present at the nano–bio interface. Rather than performance-related, single zeta-potential measurements, broader potentiometric titrations must span the acid/base characteristics of adsorbates, coatings and substrates—as is done in catalysis and colloidal science[85,86]. Other candidate techniques for such a regimen include measuring: (i) particle size distributions in water and biological fluids; (ii) thermal weight losses to evaluate hydration; and (iii) altered spectroscopic responses (in, for example, Fourier transform infrared or energy-dispersive X-ray spectroscopy) of test specimens washed with water, acid and base.

and interfacial phenomena that determine nanoparticle contact and membrane wrapping[23,24]. Particle adherence and engulfment at the adhesion site require specific and nonspecific binding interactions to overcome the resistive forces that hinder particle uptake (Fig. 4 and Table 3). The particle 'wrapping time' is a quantitative expression of the integration of these forces, which can be expressed mathematically according to a series of variables (for example particle size or shape, energy of the system, rate of receptor diffusion, and elasticity of the cell membrane)[23–25]. Among the most effective specific binding interactions are those of surface ligands that allow the material to interact with complementary molecules or receptors on the cell membrane. These interactions result in receptor-mediated endocytosis, a process that has considerable implications for therapeutic drug delivery[24]. To accomplish ligand-mediated uptake, the membrane receptors must diffuse to the adhesion site to assist the formation of a critical number of interactions (Fig. 4). Receptor diffusion constitutes a resistive force with an optimal rate constant to achieve wrapping[23]. A limited number of receptors and a decrease in the bond elasticity factor of the ligand–receptor interaction could also restrain particle wrapping. Ultimately, these cooperative interactions must generate sufficient thermodynamic energy to overcome the elastic recoil of the membrane (another resistive force).

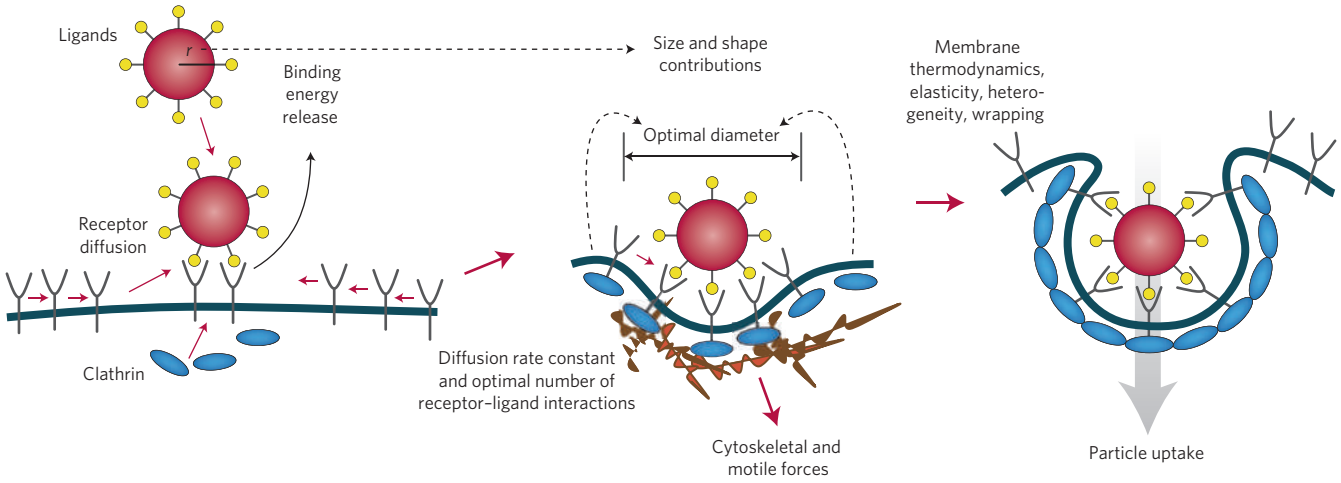

Figure 4 | Nanoparticle wrapping at the surface membrane. For particle uptake to occur, specific (ligand–receptor) and nonspecific (for example hydrophobic, Coulombic) binding interactions must decrease the free energy at the contact site to overcome resistive forces (see Table 3). The blue ellipses represent clathrin, an example of an endocytic component that engages in energy-dependent uptake of particles. The red spheres represent nanoparticles with attached ligands (yellow dots). These ligands bind to the Y-shaped membrane receptors.

Table 3 | Forces that resist and promote nanoparticle wrapping at the surface membrane.

Promotive forces	Resistive forces
Specific binding: ligand–receptor interactions	Stretching and elasticity of cell membrane
Nonspecific binding: particle surface characteristics	Thermal fluctuations of cell membrane
Free energy release at contact site	Receptor diffusion to adhesive front
Optimal particle size and shape	Hydrophobic exclusion of polar surface by surface membrane
Energy-dependent membrane and cytoskeletal components, motile forces (for example in the formation of a clathrin cage that binds to cytoskeletal proteins)	Stretching of receptor–ligand bonds, bond elasticity factor

Ligands do not necessarily need to be of biological origin; they can comprise chemical moieties, metallic sites, polymers or surface functionalities that promote binding afinity, potentially resulting in either endocytosis or direct penetration of the surface membrane. An example of the latter mechanism is that of gold nanoparticles featuring amphipathic compounds in striped arrangements on their surfaces[26], which allow the particles to slip through the intact cell membrane without causing damage. This behaviour is strikingly similar to that of amphipathic cell-penetrating peptides (CCPs), the α-helices of which feature hydrophilic residues on one side and a hydrophobic surface on the other[26]. The CCPs penetrate cells presumably by means of their cationic groups initially interacting with negatively charged residues on the surface membrane, leaving their hydrophobic regions free to access the membrane's hydrophobic interior[27]. Such a mechanism provides an effective strategy to transfer nanoparticle carriers through 30-Å-thick surface membranes that use hydrophobicity as an effective barrier. In addition to the attachment of CCPs, polymeric substances (for example the polycationic polyethyleneimine (PEI) and polyamidoamine) can be used as abiotic ligands to promote direct cellular entry[28]. If the cationic density is not controlled, however, these interactions may compromise the cell membrane's integrity, potentially leading to hole formation, membrane thinning and/or erosion and, thereby, cytotoxicity[28].

Nonspecific attractive forces that promote cellular contact and particle uptake result from such intrinsic nanomaterial characteristics as surface charge, hydrophobicity and roughness (Fig. 5). Surface charge plays an important part in particles' interactions with charged phospholipid head groups or protein domains on cell surfaces[29]. Although both are important, cationic surface units generally exert stronger effects than their anionic counterparts. When considering the role of hydrophobicity in cellular uptake, the picture is complicated by possible hydration of the particle surface and/or the cell membrane. This situation leads to a series of dynamic interactions, the outcomes of which are determined by the strengths of the bonds formed between water molecules and the material surface relative to the bonds formed between the water molecules themselves (Fig. 2). Although attempts have been made to liken this process to the wetting of material surfaces or the formation of film tension between solid and liquid surfaces, the actual dynamics of a colloidal particle meeting a phagocytic cell's surface involve more than just classical wetting[16]. The film-tension model suggests that particles that are more hydrophobic than the surface membrane are more readily engulfed than their less-hydrophobic counterparts[16]. Computer simulation has revealed that the surface membrane uptake of hydrophobic C_{60} agglomerates is thermodynamically favoured because of the hydrophobicity of the interior membrane space[30]. Once these agglomerates have pushed past the lipid head groups on the membrane, they assemble in the interior membrane space where the individual carbon cages become dispersed[30]. Whether this occurs in *in vivo* biological settings remains to be determined; particles possessing hydrophobic surfaces tend to agglomerate and are therefore removed early on by the reticuloendothelial system (see below). For less-hydrophobic materials, a continuum may exist in which a particle's ability to contact a surface membrane is determined by their relative hydrophilicities. Note, however, that all of these attractive forces can be abated or negated through steric hindrance. Examples are attachment of polymers (PEG, carboxymethyl cellulose), electrostatic repulsion (such as attachment of surfactants) or electrosteric repulsion (such as attachment of anionic triblock copolymers or poly(aspartic acid))[31]. The application of such surface coatings may be important when considering a material's safety (for example decreased

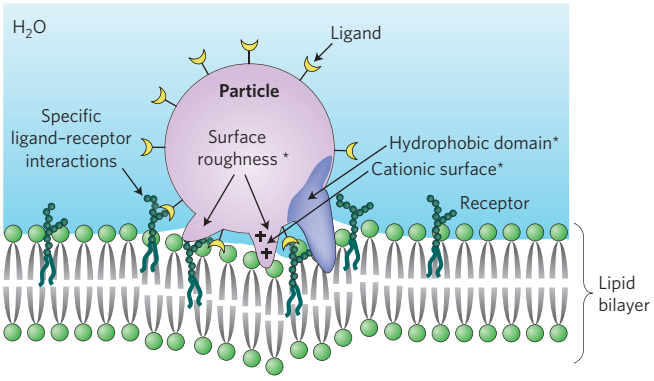

Figure 5 | Representation of receptor-mediated uptake. This is a specific biological mechanism for particles interacting with the surface membrane and undergoing cellular uptake. The intrinsic nanoparticle characteristics that promote surface binding (roughness, hydrophobicity, cationic charge) generally lead to nonspecific binding forces (marked by asterisks) that promote cellular uptake. In contrast, specific receptor–ligand interactions generally lead to endocytic uptake. A combination of nonspecific binding forces on the surface of spiked particles can lead to direct penetration of the surface membrane without the need to involve endocytic compartments[26].

bioavailability; preventing uptake of nanoparticle drug carriers by monocyte-phagocytic cells in the liver and spleen). Surface effects are also greatly altered by nanoscale surface roughness (that is, local protrusions or depressions with radii smaller than that of the particle)[32]. Indeed, small-radii surface asperities dictate the strength of nanoparticle–cell interactions. Simulations of nanoparticles interacting with synthetic membranes suggest that nanoscale surface roughness greatly minimizes repulsive interactions (for example electrostatic, hydrophilic), thereby promoting adhesion, which might translate into easier engulfment by cells.

Other characteristics of particles' surfaces — size, shape and radius of curvature — also affect their cellular uptake[23–25] (Fig. 4). For every particle that is capable of cellular entry, a threshold radius (r_{th}) exists below which cellular uptake is reduced; a larger optimal particle radius (r_{opt}) accelerates wrapping[23–25]. Whereas values of r_{opt} of about 15 and 30 nm have been deduced for cylindrical and spherical particles, respectively, optimal wrapping of transferrin-coated gold nanoparticles occurs at about 50 nm (refs 24, 25). These values suggest that the number of contact sites between the membrane and particle surface influences the free energy for particle wrapping. This concept explains why transferrin-coated Au nanoparticles undergo slower wrapping than do corresponding naked particles, and why at least six 14-nm-diameter transferrin–Au particles are required to form a cluster that undergoes uptake[25]. Particles larger than r_{opt} have a longer wrapping time because of slower receptor diffusion kinetics[23,33]. Transferrin has been investigated extensively as a potential lead for delivery of therapeutic agents that suffer from poor pharmacokinetics[34]. Coating particle surfaces with other ligands will affect the wrapping time based on variations in size, ligand density, receptor diffusion and free energy changes.

It had been suggested that rod-shaped and cylindrical nanoparticles experience longer wrapping times than spherical nanoparticles because of the greater thermodynamic forces required for their engulfment[33], but this notion was recently challenged by Gratton *et al.*, who found that the internalization of rod-like, high-aspect-ratio cationic PEG hydrogel particles occurs more rapidly and efficiently than those of other particle shapes at the same volume and size[35]. Particle shapes and aspect ratios are important features when considering nanomaterial safety and nano-carrier design. For example, the high aspect ratios of CNTs make them particularly critical biohazards[36]. Non-degradable multi-walled CNTs (MWCNTs) that are relatively stiff and capable of forming fibrous structures create problems for tissue macrophages, which specialize in the degradation and removal of foreign particulates[36]. These macrophages may, therefore, struggle to incorporate relatively long (>20 μm) and stiff MWCNTs into phagosomes. As a result, the stiff tubes may protrude beyond the limits of the cell membrane, a phenomenon known as 'frustrated phagocytosis' because, in an attempt to destroy the CNTs, harmful oxygen radicals and hydrolytic enzymes are deposited in the surrounding medium, leading to chronic inflammation[36]. If sustained for long periods, chronic inflammation in the pleura or peritoneum could lead to additional mutagenic events and ultimately the formation of mesothelioma. Although no current evidence suggests that MWCNTs actually lead to mesothelioma, asbestos fibres induce these malignancies through such a mechanism[36]. Table 4 lists the most prominent experimental mechanisms of nanomaterial toxicity. From a therapeutic perspective, mathematical modelling reveals that disc-like, cylindrical and hemispherical particles substantially outperform spherical particles in terms of evading uptake by phagocytic cells, flowing through capillaries and adhering to blood vessel walls[23,37].

Interactions during endocytosis and in organelles

Despite remarkable advances in nanoscience, relatively little is known about the intracellular fate and function of nanoparticles. This area of study is particularly important for developing effective and safe nanoparticle drug delivery systems. Much of our current knowledge was derived from studies of relatively small numbers of fluorescent or radiolabelled nanomaterials (for example block copolymers, liposomes, dendrimers or quantum dots). Most of these studies have addressed intracellular fate by focusing on endocytic pathways or the impact on a limited number of cellular organelles, with few attempts to link the cellular responses to the physicochemical properties of the engineered nanoparticles. Moreover, little research has been conducted on the material properties that determine subcellular targeting.

Nanoparticles elicit a wide range of intracellular responses depending on their physicochemical properties, intracellular concentrations, duration of contact, subcellular distributions and interactions with biological molecules. Endocytic pathways include pinocytosis, the formation of caveolae and clathrin, and caveolae/clathrin-independent uptake, each of which depends on distinct components and mechanisms[14]. Although the cell types and their states of differentiation can determine the choice of pathway, the physicochemical properties and surface reactivities of nanoparticles are also important. Particle size and shape are important parameters from the viewpoint of the space available in these endocytic compartments. Although it has been suggested that particle size might determine caveolar versus clathrin-coated uptake, inconsistent results have highlighted the difficulties encountered when experimentally modifying one physicochemical parameter at a time. It is often suggested that particle sizes of less than 120 nm are preferred for endocytic uptake, but scientific data supporting this notion are sparse. Although limited studies in tumour cell lines suggest a relationship between particle size and endocytic mechanism[25,38], most of them suffer from poor nanoparticle characterization and a reliance on relatively nonspecific inhibitors to block endocytic uptake. One study revealed that internalization of microspheres with diameters of less than 200 nm involved clathrin-coated pits; when their size was increased, the uptake shifted to caveolae-mediated internalization, which became the predominant pathway for 500-nm particles[38]. These data conflict, however, with other estimates of diameters of 50–80 nm and 120 nm for caveolae and clathrin-coated pits, respectively; such obvious discrepancies confirm that a considerable gap exists in our understanding of such systems[35]. Surface ligand attachment plays a part in specifying the endocytic compartment. For

Table 4 | Mechanisms of nanomaterial cytotoxicity and potentially useful safe design features.

Nanomaterial	Cytotoxicity mechanism	Possible design features to mitigate toxicity	References
TiO_2	ROS production mediated by electron–hole-pairs Glutathione depletion and toxic oxidative stress as a result of photoactivity and redox properties Nanoparticle-mediated cell membrane disruption lead to cell death; protein fibrillation	Cap with surfactants, polymers or complexing ligands Coat with low-molecular-weight antioxidants (for example ascorbate, glutathione, thiols), enzymatic scavengers (for example superoxide dismutase, or its mimics) Alter the chemical composition that, for example, eliminates crystalline states and material defects	61,62
ZnO	ROS production Dissolution and release of toxic cations Lysosomal damage Inflammation		21
Ag	Dissolution and Ag^+ release inhibits respiratory enzymes and ATP production ROS production Disruption of membrane integrity and transport processes		63,64
Au nanoparticles and nanorods	Disruption of protein conformation	Modify charge, size, hydrophobicity Induce steric hindrance	26,65,66,
CdSe	Dissolution and release of toxic Cd and Se ions	Coat the toxic core with biocompatible polymers or inorganic shells	67
SiO_2	ROS production by surface defects and impurities Protein unfolding Membrane disruption	Induce particle aggregation Modify the size and/or surface charge Modify the surface with aluminium lactate or polyvinylpyridine *N*-oxide	68
Fe_3O_4	ROS production and oxidative stress Liberation of toxic Fe^{2+} Disturbance of the electronic and/or ion transport activity in the cell membrane	Functionalize with biocompatible shell Coat with a polymer (for example alginate, chitosan)	69–71
CeO_2	Protein aggregation and fibrillation	Modify the material to exert antioxidant effects through altered electronic states	21
MWCNT	Frustrated phagocytosis causes chronic tissue inflammation and DNA oxidative injury	Modify MWCNT structure to reduce stiffness Reduce aspect ratio to yield fibre strands <5 μm Make tubes easier to phagocytose (for example, γ-irradiation may reduce the stiffness) Use alternative structures (such as multi-walled carbon nano-onions)	36, 72
SWCNT, MWCNT	Generation of ROS due to the metal impurities trapped inside CNTs Pro-inflammatory effects due to oxidant injury	Functionalize the surface with antioxidant or metal complexing agent Coat with a polymer or shell to prevent metal ion release Purify to remove metal contamination Attach ligands to promote receptor-mediated phagocytosis N-dope	51,73,74
	Granulomatous inflammation due to hydrophobic CNT aggregation Interstitial pulmonary fibrosis due to fibroblast-mediated collagen production	Improve the dispersion of CNTs using steric hindrance or electrostatic repulsion through appropriate surface coating/functionalization	75
Fullerenes	ROS production (spontaneous or photoactivated) Hydrophobic surface increases aggregation but promotes intramembranous localization	Decrease hydrophobicity by coating/functionalizing fullerene surface with anionic head groups	76
Cationic nanospheres and dendrimers	Membrane damage, thinning and leakage Damage to the acidifying endosomal compartment by the proton sponge effect that allows entry into the cytosol (Fig. 6)	Reduce cationic charge density Replace cationic head groups with amphiphilic head groups Reduce nonspecific cellular interactions through conjugation with appropriate moieties	28, 46, 77
Co/Ni ferrite nanoparticles, magnetic metallic nanoparticles	Liberation of toxic cations	Coat with end-grafted polymer, carbon, difunctional phosphonic and hydroxamic acids Encapsulate in a polymer coat or liposome Create a core–shell (gold or silica) structure	78
Al_2O_3	ROS production Pro-inflammatory response	Coat or functionalize the surface	79, 80
Cu/CuO	DNA damage and oxidative stress		81, 82
MoO_3	Membrane disruption		63

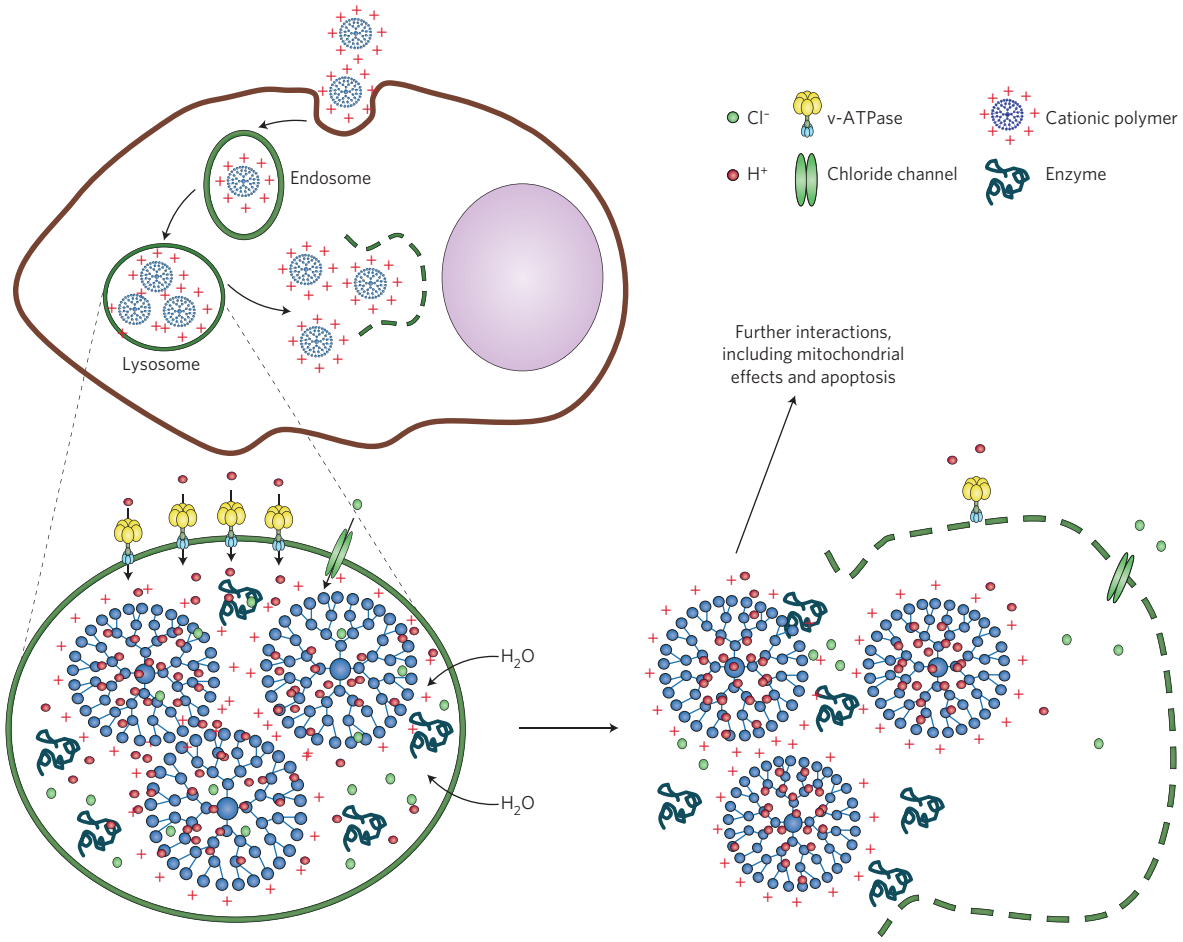

Figure 6 | Schematic of the proton sponge effect leading to lysosomal damage and the induction of cytotoxicity by cationic nanoparticles. Cationic (for example PEI-coated) particles bind with high affinity to lipid groups on the surface membrane and are endocytosed in the tight-fitting vesicles. Once these cationic nanoparticles enter into an acidifying lysosomal compartment, the unsaturated amino groups are capable of sequestering protons that are supplied by the v-ATPase (proton pump). This process keeps the pump functioning and leads to the retention of one Cl^- ion and one water molecule per proton. Subsequent lysosomal swelling and rupture leads to particle deposition in the cytoplasm and the spillage of the lysosomal content.

example, the binding of transferrin ligands to transferrin receptors triggers endocytosis by means of clathrin-coated pits[34]. Caveolae are abundant in many mammalian cells (for example adipocytes, endothelial and smooth muscle cells, fibroblasts) but rare in others. Caveolae-mediated endocytosis has emerged as an important entry mechanism for a number of viruses (such as SV40); adopting viral coat proteins that allow caveolar entry and transport might favour this pathway. An additional difference between caveolae and clathrin-coated pits is that internalization by way of caveolae is not a constitutive process — it requires cellular stimulation[39].

Although the phagocytosis uptake mechanism favours larger particles (>0.5 μm), many nominally nanoscale materials that agglomerate to this size range (during manufacture or when dispersed into aqueous solutions of biological interest) are capable of being phagocytosed. Throughout the community, debate rages regarding the effects of the sizes of primary particles, aggregates (strongly bonded or fused particles) and agglomerates (loosely bonded particles or aggregates acting under weak forces such as VDW, or simple physical entanglement)[6,40]. Toxicity studies demonstrate that the hydrodynamic diameters of certain test materials (such as TiO_2, ZnO or carbon black) are significantly greater in phosphate-buffered saline (PBS) than in water, and that their sizes are often significantly larger than the quoted primary particle size[41]; SiO_2 particles, however, are not affected in this manner. A systematic study of TiO_2 particles revealed that ionic strength and pH also influence the particle diameter in biological systems[42]. In the field of geochemistry[5,43], the sizes of nanoscale ZnS particles are affected by the presence of extracellular proteins — they grow to the micrometre range when cysteine is present. Moreover, relaxation of metastable nanoparticle surface states (by aggregation, agglomeration, Ostwald ripening or orientated attachment) can influence the ultimate particle size and assessable surface area (Fig. 3b). If the surface area of nanoparticles is indeed the driver of their bioactivity, controlling agglomeration in biological media may be an important approach to increasing their bioavailability or limiting their spread as a means of improving their safety. Sager *et al.* found that diluted alveolar lining fluid obtained through bronchoalveolar lavage improved the dispersion of carbon black and TiO_2 nanoparticles over that in PBS; this effect is mimicked by the addition of DPPC (dipalmitoylphosphatidylcholine) and albumin at concentrations found in bronchoalveolar lavage fluid[41]. Improved dispersion of carbon black nanoparticles significantly increases the inflammatory response of rats after intratracheal instillation; similar observations have been made for CNTs[44,45].

Additional evidence exists that surface functionalization or passivation affects nanoparticle phagocytosis. For example, the attachment of PEG, poloxamer and poloxamine polymers prevents phagocytosis[14,31]. This occurs partly because steric hindrance promotes particle dispersion and partly because of the camouflaging of the surface properties[14,31] (Fig. 2), such as charge and surface ligands that lead to receptor- and non-receptor-mediated uptake[14]. In

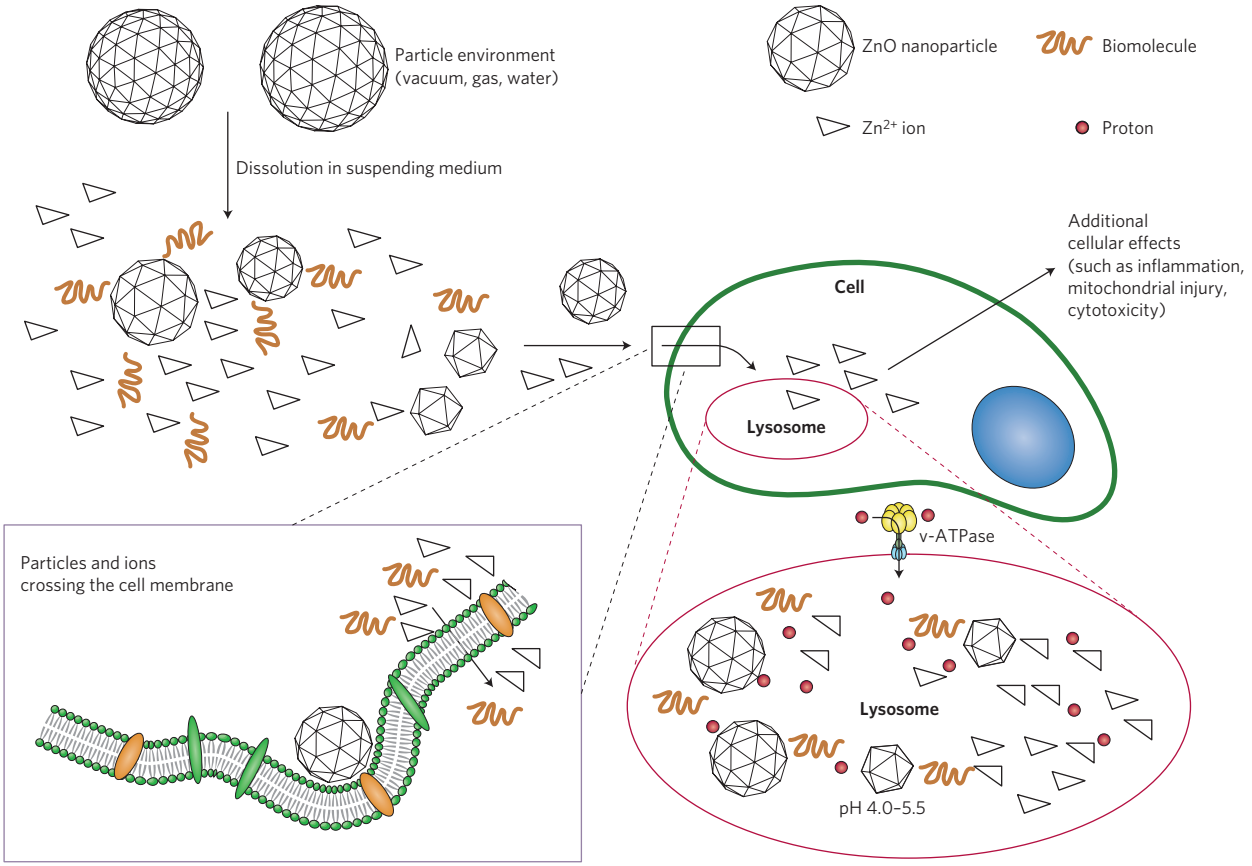

Figure 7 | Influence of ZnO on lysosomal function. ZnO dissolution through interactions at sequential nano–bio interfaces in the extracellular environment and the acidifying lysosome generates cellular toxicity through the release of toxic Zn^{2+} ions. Release of Zn^{2+} in the lysosome and the intracellular environment can induce a series of harmful cellular outcomes, such as lysosomal damage, mitochondrial perturbation, ROS production, excitation of pro-inflammatory cytokine and chemokine production.

addition, particle interactions with blood and other body fluids can lead to surface modifications that promote phagocytosis[14], including opsonization through the attachment of immunoglobulins and complement products that promote phagocytosis by means of Fcγ and complement receptors[14,20]. Phagosome-mediated nanomaterial uptake plays important parts in determining nanomaterial degradation in the body and in nanoparticle biodistribution and safety studies. Biopersistent nanomaterials that resist degradation may impart significant toxicity and granulomatous inflammation (as for MWCNTs).

In addition to entry into endosomal compartments, engineered nanoparticles exert important effects on other organelles[21,46,47]. Particularly noteworthy is the impact on lysosomal function, which is key to nanoparticle use as a delivery device, as well as for nanomaterial toxicity. Here, we briefly discuss two examples. The first relates to the potential of amino-labelled polystyrene, cationic dendrimers and polymers (for example PEI) complexed to DNA (polyplexes) to enter the lysosomal compartment. The lysosomal proton pump, which is responsible for acidification, is key to understanding the proton sponge hypothesis, which posits that unsaturated amines on the material surface are capable of sequestering protons[21] (Fig. 6), keeping the pump going and leading to the retention of one Cl^- anion and one water molecule for each proton that enters the lysosome. Ultimately, this process causes lysosomal swelling and rupture, leading to particle deposition in the cytoplasm. Thus, although the cargo may be delivered to the cytosol, spillage of the lysosomal content and triggering of intracellular Ca^{2+} release may lead to toxicity[21]. This process includes triggering of the Ca^{2+}-regulated permeability transition pore in mitochondria[21], potentially leading to decreased ATP production and cellular apoptosis. Spillage of lysosomal enzymes can also lead to the activation of Bid and Bax proteins and pro-caspases[21]. Nanoparticles functionalized with polycationic polymers can deliver anticancer drugs and/or DNA/siRNA into the cytosol, based on the endosomal escape mechanism to achieve tumoristatic effects[48]. Lysosomes are also involved in nanoparticle dissolution, as illustrated by the toxicity of ZnO (ref. 23). In addition to the interfacial interactions that promote ZnO dissolution in body fluids and tissue culture media, particle uptake into the acidifying endosomal compartment accelerates dissolution. Moreover, lysosomes have important roles in cellular zinc homeostasis; release of excess Zn^{2+} ions to the cell can induce cytokine production and cytotoxic effects[21] (Fig. 7). This phenomenon may explain the clinical manifestations of metal fume fever, which can follow the inhalation of ultrafine ZnO (ref. 49). The dissolution of ZnO and other metal oxide particles in the lung releases Zn^{2+} ions which are responsible for initiating acute inflammation, which presents with an acute onset of fever and chills. Typically, however, the illness is of short duration (24–48 h), presumably because of the particle dissolution.

In vivo evidence

The nature of the interface between nanomaterials and biological systems affects the *in vivo* biocompatibility and toxicity of nanoparticles. One example is the effect of the chemical composition, shape and surface characteristics on the *in vivo* responses of single-walled carbon nanotubes (SWCNTs). Mercer *et al.* tracked the distribution of gold-labelled SWCNTs after pharyngeal aspiration[50]. During pulmonary exposure, pure SWCNTs are poorly phagocytized by alveolar macrophages; instead, they migrate to the interstitial space in the

Box 2 | Safe design of materials at the nano–bio interface.

An understanding of the hazardous properties of nanomaterials is essential if they are to come into contact (intentionally or otherwise) with humans and the environment. Nanomaterial safety strikes at the heart of the debate over whether nanotechnology should proceed unchecked or be regulated to prevent biological harm. So it is fitting to ask whether understanding the interface between biological systems and nanomaterials will introduce master design features that can be used to control the exposure, bioavailability and biocatalytic properties of nanomaterials. Although there is no single design feature that currently fits this description, we are just beginning to identify some possible approaches (Table 4). It is important to note that redesign of any of these properties may affect nanoparticle performance characteristics (for example electrical conductivity, thermoconductivity, magnetic properties) that are essential for product development. Accordingly, the optimal design process is one of multiple compromise.

One clear route to mitigate toxicity is to take advantage of the tendency of nanoparticles to aggregate in natural and biological media. For example, the degree of aggregation of TiO_2 nanoparticles determines their cytotoxic effects on fish cells under ultraviolet radiation[62]; DNA strand cleavage correlates with particle dispersion, which is higher in distilled water than in PBS. Design features that promote particle aggregation must take into account the competing effects of natural organic materials (such as proteins); for example, although steric hindrance of a nanoparticle's coating might decrease its cellular uptake and bioaccumulation, it might paradoxically increase transport and environmental exposure by preventing aggregation. Therefore, potentially competing principles (aggregation, dispersal, transport, bioavailability) must be analysed in detail to formulate the best design strategy. One approach could be to use natural nanoparticles to aggregate and immobilize engineered nanomaterials at disposal sites. Alternatively, we may design better-dispersed particles if the principal requirement is for their wide dissemination to clean up environmental spills. In that case, it may be better to use a biodegradable material that does not have intrinsic toxicological potential and does not bioaccumulate.

Surface coating is another design feature being used to improve nanoparticle safety by preventing bioreactivity. For instance, TiO_2, ZnO and Fe_2O_3 nanoparticles in cosmetic formulations (such as suntan lotions) are often coated with a hydrophobic polymer such as poly(methylvinylether)/maleic acid to reduce direct contact with the human skin[61]. An extension of this principle uses the steric hindrance of polymers and detergents to decrease cellular uptake. Antioxidant chemicals (for example oligomeric proanthocyanidins) have been used as coating agents for particulates that generate ROS. We must recognize, however, that many coatings are environmentally labile or degradable — an initially non-toxic material may become hazardous after shedding its coat. An important design feature would, therefore, increase the stability of coating materials or design them to prevent adverse biological responses.

Coating nanoparticles with protective shells is an effective means of preventing the dissolution and release of toxic ions[67], while also providing a physical barrier against cellular uptake. Suitable shell materials include biocompatible organic or inorganic substances (for example PEG–SiO_2, gold, biocompatible polymers)[70]. Modification of surface charge is another approach to reducing toxicity[65,66]. For example, layer-by-layer coatings of polyelectrolytes on gold nanorods decrease their cellular uptake by modifying their surface charge and functionality. For the safe design of materials that form biopersistent fibres (for example CNTs), it is important to consider their aspect ratios, hydrophobicity and stiffness[36]. Chemical functionalization of short (<5-μm) MWCNTs can provide stable dispersions of individual tubes in physiological media, allowing their safe use as imaging and drug delivery devices[56]. The high rate of urinary excretion of these materials provides an additional safety margin, as demonstrated in the biomedical development of potentially toxic nanomaterials such as quantum dots[98]. Experimental evidence suggests that long, rigid CNTs should be avoided for *in vivo* applications; their functionalization with small hydrophilic groups is a safety feature allowing the formation of stable dispersions with high excretion rates[98,99].

The oxidative state of nanomaterials at the interface is another potential design feature that can be used to mitigate cytotoxicity. The toxicity of hydrophobic C_{60} derivatives can be modulated by increasing their hydration through surface functionalization[76]. It has been theorized that increasing the solubility of fullerenes through specific functional groups might lower the formation of ROS.

alveolar wall, where, after close contact with fibroblasts, they directly stimulate cellular proliferation and collagen production[50]. These bioresponses are dependent on the SWCNTs' high aspect ratios[50]. These results may explain why the rapid and progressive interstitial fibrotic response to SWCNTs can proceed in the absence of robust macrophage activation or the presence of prior pulmonary inflammation[51]. *In vitro* assays confirm that macrophages fail to recognize pure SWCNTs as foreign materials because neither nanotube uptake nor the production of ROS and cytokines occurs[51]. Modification of the SWCNT surface can, however, markedly alter these biointeractions. For example, when SWCNTs are suspended in 10–15% serum before their introduction to macrophages, the *in vitro* uptake is substantial[45], possibly because the SWCNTs adsorb albumin from the suspending medium and the accompanying change in protein structure leads to scavenger receptor uptake in macrophages.

Particle size and surface area are important features affecting *in vivo* bioreactivity. For example, on an equal mass–dose basis, ultrafine particles cause greater pulmonary inflammation in rat models than do fine particles of the same composition. When the dose is normalized to the equivalent total particle surface area delivered to the lung, the difference between ultrafine and fine particles no longer exists. *In vitro* studies support the hypothesis that particle surface area is an important factor affecting nano–bio responses. In these studies, the *in vitro* potency of particles to stimulate cytokine production by lung epithelial cells on a particle surface area is predictive (on a cell surface area basis) of the *in vivo* inflammatory potency, which correlates with the response of the deposited particle surface area with respect to the alveolar surface area[52]. Recently, Warheit *et al.* questioned the importance of particle surface area in pulmonary reactivity[53], but notably their sample used for intratracheal instillation of ultrafine TiO_2 was highly agglomerated, having an effective diameter identical to that of the fine TiO_2 sample. Sager *et al.* reported that when efforts are made to improve the dispersion of carbon black and TiO_2 samples, ultrafine particles are at least 40 times as potent as fine particles on a mass–dose basis[41]; the effect is no longer significant when the dose is expressed in terms of the total surface area of delivered particles. A further demonstration of the importance of agglomeration size is the intratracheal instillation of poorly and well-dispersed ultrafine TiO_2 and carbon black in rats[54], where pulmonary inflammation one day post exposure was threefold greater for more-dispersed nanoparticles. Therefore, an increase in the delivered nanoparticle surface results in greater interactions with pulmonary cells. We must, however, also consider that the surface reactivity can contribute independently to particle

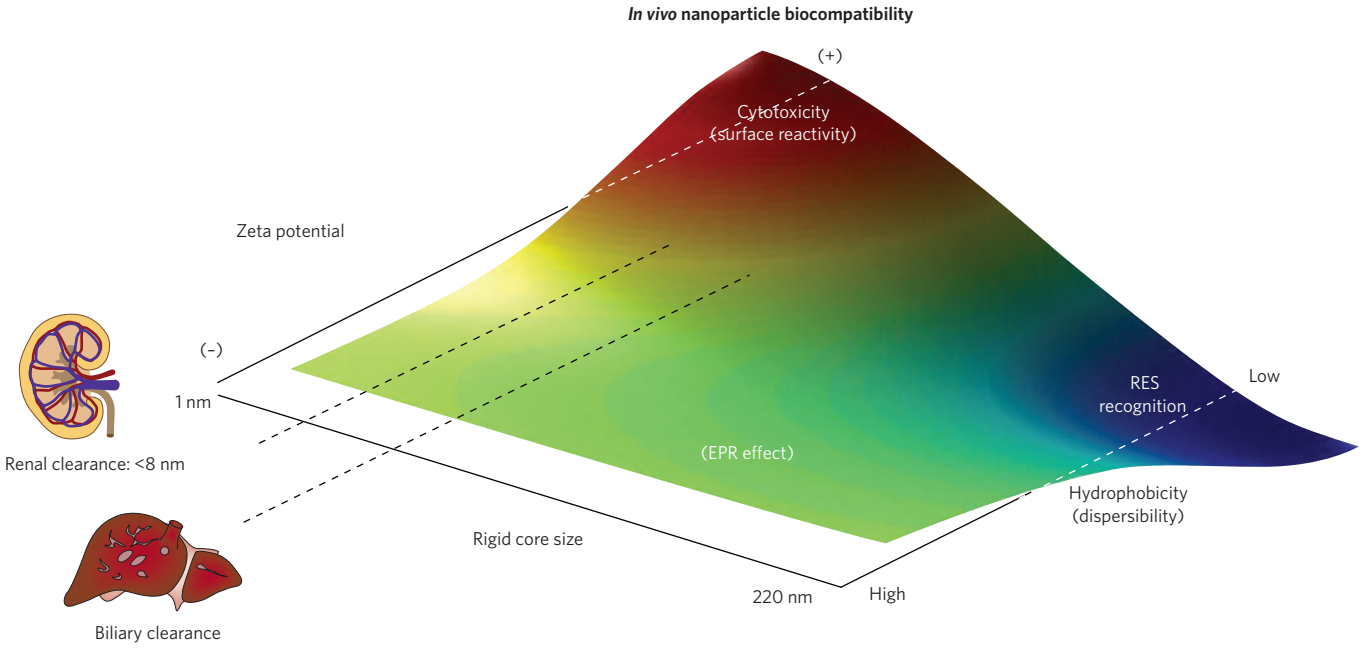

Figure 8 | Physical characteristics of nanoparticles determine *in vivo* biocompatibility. The three-dimensional phase diagram displays the qualitative biocompatibility trends revealed after *in vivo* screening of around 130 nanoparticles intended for therapeutic use[57]. The main independent particle variables that determine the *in vivo* biocompatibility (colour spectrum) are size, zeta potential (surface charge) and dispersibility (particularly the effect of hydrophobicity). Biocompatibility is reflected in the colour spectrum, with red representing likely toxicity, blue likely safety and blue–green–yellow intermediate levels of safety (in the same order). Cationic particles or particles with high surface reactivity are more likely to be toxic (red hue) than the larger relatively hydrophobic or poorly dispersed particles, which are rapidly and safely (blue hue) removed by the reticuloendothelial system (RES). Particles that promote enhanced permeation and retention (EPR) effects—and are therefore optimal for chemotherapeutic drug delivery to cancers — generally have mid-range sizes and relatively neutral surface charges. Figure courtesy of Scott McNeil.

toxicity; the reactive surface area may therefore constitute a more accurate measure of particle toxicity. This situation is illustrated by the increased inflammatory response profile of high-toxicity quartz relative to that of low-toxicity TiO_2 or carbon black nanoparticles, after correcting for their surface areas[52]. Moreover, blocking newly reactive quartz surfaces with aluminium ions reduces their inflammogenic potential. Although it is difficult at present to quantify reactive surface areas accurately, studies of the pro-oxidative potentials of ambient ultrafine particles under abiotic and cellular conditions and how they relate to pro-oxidative pathogenic events (for example atherosclerosis) in animals are examples of *in vitro* toxicological measurements that are predictive of *in vivo* disease outcomes[55,56]. Table 4 and Box 2 provide additional examples of potential design features to improve nanomaterial safety.

The biophysicochemical characteristics of nanoparticles also determine their *in vivo* biocompatibility in the field of nanotherapy (Fig. 8). Having assessed more than 130 different nanoparticle types, including fullerenes, metal oxides, polymers, liposomes, dendrimers, quantum dots and gold colloids, the Nanotechnology Characterization Laboratory (NCL) at the National Cancer Institute in Maryland has observed that hydrophobicity, size and surface charge are the main parameters influencing nanoparticle biocompatibility[57]. Hydrophobic nanoparticles generally have short (seconds to minutes) *in vivo* half-lives because they are rapidly removed from circulation by cellular elements of the reticuloendothelial system, particularly in the liver and spleen. Although this behaviour could limit the bioavailability and utility of particles as drug carriers, some *in vivo* applications rely on rapid uptake by macrophages to carry nanoparticles to lymph nodes or sites of inflammation (for example for magnetic resonance imaging of the liver by paramagnetic iron nanoparticles). The importance of particle size is corroborated by data revealing that the lungs, gastrointestinal tract and skin are quite effective barriers against the uptake and spread of nanoparticles. For example, TiO_2 and polystyrene nanoparticles do not penetrate normal human skin, whereas small quantum dots can penetrate broken skin to lodge in other tissues and organs[58]. When deliberately injected into the bloodstream, however, nanoparticle size has an important effect on the rates and routes of clearance from the body. For example, the kidneys can excrete particles smaller than 8 nm (Fig. 8). On the other hand, the liver and spleen can trap particles larger than 200 nm; the liver clears anything greater than about 200 nm in size, but that clearance from the bloodstream does not necessarily imply excretion, as the bile duct is only ~30 nm in diameter. Kupffer cells hold onto the particles until the liver degrades them. Nanoparticles varying in size from 30–40 nm to a few hundred nanometres can undergo passive accumulation at tumour sites through the enhanced permeation and retention (EPR) effect. This phenomenon refers to the enhanced permeability of tumour vasculature, which when coupled with decreased lymphatic drainage allows those materials to accumulate in the vicinity of the leaky vasculature. Finally, the importance of particle charge is illustrated by NCL studies of the *in vivo* and *in vitro* nanomaterial safety of a series of nanoparticles of relatively constant size, but varying zeta potential[57]. Cationic particles are more cytotoxic and more likely to induce haemolysis and platelet aggregation than are neutral or anionic particles[59]; this trend has been confirmed in studies of the ability of cationic polystyrene beads to induce cytotoxicity, vascular leakage and inflammatory infiltrates in the lungs of exposed rats and mice[46]. This toxicity mechanism may also explain the occurrence of acute pulmonary oedema and bronchiolitis obliterans in humans exposed to cationic spray paint particles[60].

Conclusion

The interface between nanomaterials and biological systems comprises a dynamic series of interactions between nanomaterial surfaces and biological nanoscale or nanostructured surfaces. These interactions are shaped by a large number of forces that

could determine whether the nanomaterial is bioavailable and may participate in biocompatible or bioadverse interactions. The interactions determine such processes as the formation of the protein corona, cellular contact, particle wrapping at cell surfaces, endocytosis and intracellular biocatalysis. Probing these various interfaces demands new ideas and imaging techniques. We expect that a better understanding of the mechanisms of nanoparticle injury, together with appropriate stewardship, will allow us to make more informed decisions about the design features and steps that should be taken for the safe use of nanotechnology.

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