Multiple aspects of the interaction of biomacromolecules with inorganic surfaces

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Abstract

The understanding of the mechanisms involved in the interaction of biological systems with inorganic materials is of interest in both fundamental and applied disciplines. The adsorption of proteins modulates the formation of biofilms onto surfaces, a process important in infections associated to medical implants, in dental caries, in environmental technologies. The interaction with biomacromolecules is crucial to determine the beneficial/adverse response of cells to foreign inorganic materials as implants, engineered or accidentally produced inorganic nanoparticles. A detailed knowledge of the surface/biological fluids interface processes is needed for the design of new biocompatible materials. Researchers involved in the different disciplines face up with similar difficulties in describing and predicting phenomena occurring at the interface between solid phases and biological fluids. This review represents an attempt to integrate the knowledge from different research areas by focussing on the search for determinants driving the interaction of inorganic surfaces with biological matter.

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Abbreviations: AFM, atomic force microscopy; ANS, 1-anilino-8-naphthalenesulfonate; BSA, bovine serum albumin; CNT, carbon nanotubes; DC, circular dichroism; DSC, differential scanning calorimetry; ESR, electron spin resonance; FTIR, Fourier transform infrared spectroscopy; HEL, hen egg lysozyme; IEP, isoelectric point; MWCNT, multi-walled carbon nanotubes; NMR, nuclear magnetic resonance; PZC, point of zero-charge; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SDSL, site-directed spin-labeling; SEM, scanning electron microscopy; SERS, surface-enhanced Raman scattering; SWCNT, single-walled carbon nanotubes; TEM, transmission electron microscopy; TIRFM, total internal reflection fluorescence microscopy; Trp, tryptophan; UV, ultraviolet.

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1. Introduction

The phenomena occurring when an inorganic material meets a biofluid are extremely complex. Dissolution/precipitation processes, reconstruction of the surface, adsorption of ions, small molecules and macromolecules, and redox reactions may occur at once [1].

The comprehension of the molecular mechanisms of such processes is an intriguing challenge which rises interest in several different fields such as astrobiology, ecology, biology, biotechnology, engineering, and medicine [1–4]. In medicine a deep knowledge of the surface/biological fluid interface processes is needed for the design of new biocompatible materials for implants, drug delivery and nanodevices for diagnosis and therapy.

One of the main problems with implantable medical devices is to prevent adverse reactions as inflammation or infections. For this reason effort has been placed on creating surfaces which should promote the attachment of target tissue cells but preventing bacterial adhesion. The mechanisms of interaction of surfaces with tissues at a molecular level are however largely unknown [5], thus a predictive approach to the development of new materials is currently difficult to achieve.

The development of methods to manipulate matter at the nanolevel has recently lead to the production of several different kinds of inorganic nanoparticles (NPs) which may possibly find medical application in the future. At the same time the rapid diffusion of nanotechnological products opens new concerns on the possible adverse effects following the direct or indirect (i.e. through leakage in the environment) exposure of humans to NPs. The interaction of NPs with blood proteins is considered the most critical step determining the NPs biodistribution, toxicity and/or efficacy. Interaction of NPs with blood proteins has been in fact associated to thrombosis [6] or adverse responses of the immune system, macrophage uptake and elimination [7].

The definition of common determinants in cell/surface interface processes is difficult to achieve since inorganic surfaces and biomolecules, as well as the fluids wherein they interact are extremely variable entities.

Because of the specificity of its complex structure each single protein or nucleic acid molecules exhibits its own “personality” [8] and therefore interacts differently with surfaces. On the other hand, inorganic surfaces may expose to the solvent distinct functionalities or defects that may react or interact with solvent, solutes, biomolecules and cells. Such properties vary in abundance and topology not only in a way that depends on the kind of material, but also within materials having the same elemental composition but different structure or origin [9,10].

Large differences in chemical composition, ionic strength and acidity of biological fluids exist depending on tissues and cellular compartments [11]. Furthermore the composition of fluids varies as it depends on the cell responses to environmental stimuli. Biological compartments are open to matter and energy exchanges and therefore they never reach an equilibrium state. Kinetics are therefore relevant in determining which process prevails.

When the surface of a foreign body comes in contact with a biological fluid it is quickly covered by the medium components — particularly proteins — forming a complex layer which has been defined by Dawson and co-workers as “protein corona” in the case of particles. Such corona is a dual system, composed by a core of strongly bound proteins and an outer layer of fast exchanging molecules [12,13].

The establishment of such a corona is a competitive process driven by thermodynamic and kinetic factors, including the stability of the NP–protein adduct, the relative concentrations of proteins in the medium, the charge distribution on the NP as well as on the protein surface (which are both pH-dependent), the chemical nature and reactivity of the NP surface, the NPs abundance, the temperature [12].

Moreover the corona is expected to change depending on the nature and rate of “relaxation processes” as particles redistribute from one compartment or organ to another, as well as upon receptor-mediated endocytosis from the extracellular environment into the primary endosomal cavity, or from the cytosol to the nucleus [13].

Dawson has very clearly highlighted that the interaction of the cell with NPs is strongly driven by the protein corona, rather than by the NP itself because the cell “sees” protein-coated NPs rather than the bare NP surface. On the other hand, the formation of such a corona is heavily influenced by nature, size and shape of the NP, as well as by the composition of the medium [12]. Consequently, in order to foresee the cell reaction towards the protein-coated NP, it is essential to clarify the mechanisms that lead to the formation of the protein corona. On one side, this implies the need for a standardized multi-techniques approach for the characterization of the protein corona. On the other one, an accurate knowledge of the structural and chemical nature of the NP (and especially the NP surface) influencing the adsorption processes is needed as well [12].

A large number of coatings and surface decorations have been proposed to improve the biocompatibility of materials used in prosthesis and of NPs used in therapy and diagnosis. The description of the properties of these modified materials is beyond the scope of the present paper and we refer to other reviews for an extensive discussion of this kind of modified materials [5,14,15]. Here, we mainly focus on the interaction with bare inorganic surfaces. Note that coatings may be instable in fluids [16,17] and the long permanence in the body of functionalized surfaces may result in coating degradation [18,19]. Exposure of the internal inorganic core is therefore a realistic event at some stages.

This review tries to merge the information coming from different scientific areas in order to summarize the current knowledge concerning the behavior of proteins and nucleic acids approaching an inorganic surface as well as the physico-chemical determinants that drive such processes. In particular we will focus on those inorganic materials that have been proposed — or are currently used — as nanovectors or as excipients in pharmaceutical preparations.

2. Properties of inorganic surfaces

The importance of the surface in driving and modulating the biochemical fate of inorganic particles is now a solid and well-assessed key fact. The physico-chemical properties of inorganic surfaces play a crucial role in determining the interaction of a solid with biomolecules and hence with living matter [1,5,20–22]. Among the recent revolutionary advances in the field of surface science the
most fundamental discovery that has been made is that — in contrast to bulk properties which are derived from both chemical composition and structure — the properties of a surface depend on its own surface-specific composition and structure — which largely differ from bulk — and on nanotopography. Outside the fields of solid-state chemistry and engineering sciences, the fact that the bulk and the surface of a material are very distinct — though related — entities is not always enough stressed out. This consideration is indeed central in the study of all the complex physico-chemical aspects of the surface. The last atomic layer of a solid is a discontinuity point, an interface between the bulk structure (either crystalline or amorphous) and the surrounding medium (air, water, solvent, complex media like serum or blood). The surface of a solid is a complex and dynamic entity, that can actively interact with surrounding medium, e.g. undergoing protonation/deprotonation, adsorbing/desorbing moieties and biomolecules, being dissolved or assisting the precipitation of compounds.

Here we will focus on those surface features strictly ascribed to the chemical properties which are known to modulate the interaction with biomolecules: i) the hydrophobic or hydrophilic behavior; ii) the surface charge; iii) the sub-micrometric topography of the surface (nanotopography); iv) the surface curvature; v) the occurrence and nature of reactive sites; vi) the chirality; and vii) the dissolution/re-precipitation equilibrium.

2.1. Hydrophobicity/hydrophilicity

Defining the hydrophobicity and the hydrophilicity of a surface from a chemical point of view is not trivial. Macroscopically and qualitatively, the wettability of a material de-termines its hydrophilicity that can be measured evaluating its mirrored effect such as the contact angle of a water droplet. The more hydrophilic the material, the higher the wettability, the lower the contact angle. The contact angle is the resultant between adhesive (droplet-surface) and cohesive (droplet-droplet) forces. In other words, the tendency of a drop to spread out over a flat, solid surface (wettability) increases as the contact angle decreases. Down to the atomic scale, hydrophilicity originates in polar surface chemical functionalities (e.g. Si-OH, Ti=OH) or under-coordinated metal ions at the surface (Ti$^{3+}$, Al$^{3+}$, Fe$^{2+/3+}$) [23,24]. The abundance of such sites determines the degree of hydrophilicity/hydrophobicity, which is one of the determinants for biomolecules adsorption. The very same bulk material may show dramatic changes in hydrophilicity upon heating or surface alteration/functionalisation [25]. In general at the atomic scale there is a marked heterogeneity also in hydrophilicity, which can be evaluated from the strength of the interaction of each site with water molecules. The interaction of surface sites with water molecules is obtained by measuring the adsorption enthalpy as a function of coverage, whose chemical nature may be assessed by integrating data from microcalorimetry and infrared spectroscopy [23]. For example, detailed studies on the hydrophilicity of several quartz samples have been performed in the past [23] and therein) some of which have shown a large variation in cytotoxicity upon variation in hydrophilicity [26]. The occurrence of interstitial or lattice substituting ions such as Al$^{3+}$ or Fe$^{3+}$ largely modifies the hydrophilic behavior of SiO$_2$ surface.

Carbon-based nanoparticles, CNTs and fullerences are highly hydrophobic materials. However, they may be easily functionalized by inserting surface functionalities as hydroxyl (–OH) or carboxyl (–COOH) groups which impart to the surface a hydrophilic character.

2.2. Surface charge

We have previously clarified that bulk and surface of a material are very distinct entities. This consideration is central in the study of the surface charge. The chemical nature of the surface and the occurrence of polarized or charged moieties determine the electric nature of the surface. When any object with a charged surface is placed into an aqueous solution an electrical double layer — two parallel layers of charge surrounding the object — is formed on the surface. The first layer is made of ions adsorbed directly onto the surface due to specific chemical interactions with the surface, e.g. electrostatic forces, hydrogen bond, coordinative bond, and van der Waals interactions. The second layer loosely associated with the surface via the weak electrostatic forces, is composed of free ions which move in the fluid under the influence of electric attraction and thermal motion rather than being firmly anchored to the surface. This second layer is thus called the diffuse layer. The potential drop across the mobile part of the double layer responsible for electrophoretic phenomena is called ζ-potential [27]. Conventionally, the sign of this potential is reported accordingly to the charge of the surface generating it, thus being positive or negative for positively or negatively charged surfaces respectively. Being an interface measure the ζ-potential of a solid surface largely depends on the chemical nature of the surface interacting with it. Thus a ζ-potential value reported without defining the solution conditions is a virtually meaningless number. In aqueous media, the pH and the ionic strength of the solution are the most important factors that affect ζ-potential and should always be explicitly indicated.

In colloid science the determination of ζ-potential is commonly used to evaluate the stability of a suspension. A potential of 25–30 mV (positive or negative) can be taken as the arbitrary value that separates low-charged surfaces from highly-charged surfaces, hence instable from stable colloids [28]. The condition when the electrical charge density of a surface becomes zero is called point of zero charge (PZC). This point is usually experimentally determined by acid–base titrations while monitoring the electrophoretic mobility of the particles and the pH of the suspension (see below). From an electrophoretic perspective, the iso-electric point (IEP) and the PZC, i.e. the pH at which electrophoretic mobility is null, are generally considered equivalent. However, PZC and IEP have different meanings. According to Jolivet [29], when the surface is not charged, i.e. there are neither positive nor negative charges, the surface is best described by the PZC. When an equal amount of positive and negative charges are present, the IEP should be used instead.

When in the nanoscale, particles likely form agglomerates or are found in chemically stable aggregates. Under these circumstances the agglomeration and/or aggregation both in liquids or in air determine the actual particle size of NPs [30]. The role of important factors such as ionic strength, pH and particle surface chemistry which control dispersion of NPs was recently examined in a thorough work on some commercial and lab-synthesized titanium dioxide (TiO$_2$) and quantum dot samples [31]. In this study the difference between aggregation and agglomeration is discussed. Aggregated NPs are bound with hard, likely covalent bonds between primary particles. These sintering processes are likely to occur during NP preparation and cannot be avoided. Conversely agglomerated particles that are held together by van der Waals forces can be separated by overcoming these weaker interactions by several methodologies. The measurement of the ζ-potential can be of great usefulness in selecting the appropriate condition for manipulating a NP promoting or favoring aggregation to achieve different results. For instance, the dispersion of hydrophobic carbon-based nanomaterials in water may be largely improved by introducing a sufficient number of charged functionalities at the surface to generate repulsion among particles [32–34].

Besides being a well-known tool for the investigation of colloid stability the ζ-potential of inorganic solids is rapidly becoming an indispensable surface measurement to characterize NPs and to predict their behavior in terms of adsorption of biomolecules ([35] and ref therein). Recently ζ-potential measurements were also adapted to investigate the stability and the partial vs. full coverage of proteins adsorbed on different silica NPs [36].
The $\zeta$-potential of a solid measured during the acid–base titration of the surface functionalities reported as function of pH, i.e. the $\zeta$-plot, is a straightforward tool to characterize different NPs and to explain and predict their behavior in a biological fluid. Si, Ti and Fe oxide NPs of similar size exhibit largely different $\zeta$-plots (Fig. 1). PZCs are in the order silica < titania < hematite, clearly indicating the order of acidic strength of the hydroxylated surface functions (Si–OH $\rightarrow$ Ti–OH $\rightarrow$ Fe–OH). The value of $\zeta$-potential for biologically relevant pH values is highlighted. At physiological pH silica and titania are negatively charged, while hematite is positive (close to PZC). On the other hand at acidic pH – e.g. 4.5 inside phagolysosomal vesicles – silica remains negatively charged, while titania and hematite exhibit positively charged surfaces.

$\zeta$-potential is not only related to the type of material: Kosmulski dedicated a recent review [37] to remark that the $\zeta$-potential of a solid depends upon a number of material-specific features such as the chemical and crystallographic composition (commercial samples of the same material may largely differ in such characteristics), particle size, crystal lattice and defects, impurities, and the presence of thermodynamically unstable phases.

In a recent work specifically devoted to investigate the effect of some TiO$_2$ surface features on $\zeta$-potential [38] PZC was found to be a function of primary particle size. When primary particle size of lab-synthesized TiO$_2$ anatase NPs increased from 6 to 104 nm, the PZC decreased from 6.0 to 3.8 pH unit. At the same pH and ionic strength conditions an increase in size determines a decrease of $\zeta$-potential throughout the potential curve (at neutral pH, $\zeta$-potential decreases from $\sim$ 20 to $\sim$ 40 when particle size increases from 6 nm to 104 nm). This size-dependend mechanism is likely due to the differences in terms of acidity of surface Ti–OH. When the size of a NP increases, the acidity of surface hydroxyl increases, as indicated by the more acidic PZC reported for TiO$_2$. At the same time, the larger particles, being more acidic, will be more likely deprotonated in neutral or alkaline solutions, thus showing a more negative $\zeta$-potential. The opposite is expected to occur at acidic pH where positively charged species may account for this behavior. The chemical nature and the electrostatic charge of both rutile and anatase TiO$_2$ surface functionalities accounting for $\zeta$-potential and PZC have been investigated with theoretical considerations and experimental measurements by Pana-giotou and co-workers [39].

It is very hard, if possible at all, to draw general conclusions on the $\zeta$-potential trend with respect to surface and bulk features of NPs. There are however some well-assessed general rules that still apply to the PZC of metal oxides [40]: i) anionic impurities shift the PZC to more acidic values, cationic impurities to more basic values or toward the PZC of the impurity oxide; ii) even a partial oxidation and reduction of the material may shift the PZC toward that characteristic of the oxidation state produced, i.e. oxides at lower oxidation states have higher PZC; iii) an increase of the ionic strength of the solution produces a decrease of the $\zeta$-potential of the suspended particles.

### 2.3 Nanotopography

A smooth surface at the macroscopic level may be very irregular at the molecular level. Edges, kinks and steps are present to some extent in all crystals, and many solids have indented edges, irrespective of whether they are crystalline or amorphous. Exposed atoms or ions at these positions are more reactive, so that, in general, irregular surfaces behave differently from smooth ones, with the latter being more inert. Surface topographic parameters may play thus an important role to obtain effective material with high biocompatibility and good tissue interaction. Often the clinical success of an implant is related to an early osseointegration, i.e. the creation of a direct response is related to direct bone–implant relationship without the formation of an intervening connective tissue layer. Geometry and surface topography are crucial for the short- and long-term success of implants. Kim and Ramaaswamy [41] recently reviewed the latest electrochemical modifications performed on the native surface of metallic titanium. Titanium and titanium based alloys are commonly used for implantation in bone contact because of their mechanical properties as well as their excellent corrosion stability and osseointegration, both ascribed to the air-formed passive layer always covering the metal surface. Aluminum oxide is also used for implant due to excellent bioinertness and mechanical properties. However, all biomaterials require surface modifications to increase the biocompatibility and improve implant stability. Anodic oxidation is a common technique to obtain desired roughness, porosity and chemical composition of the oxide. Self-organized nano-titanium dioxide tubes are one of the most promising modifications, because the tubular structure acts as better scaffold for cell adhesion than the conventional flat surface. Cathodic electrochemical techniques, such as electrophoretic and cathodic depositions, are the other electrochemical pathway to surface modification. Deposition of nano-grained hydroxyapatite on titanium implant makes the implant surface a better substrate for cell interaction than the more coarse-grained [42]. Denis and co-workers [43] studied collagen adsorption as function of surface nanotopography and hydrophilicity. They found that the adsorbed amounts on rough substrata were similar to those found on smooth substrata, but the protein film morphology clearly depended on the substratum topography with elongated supramolecular assemblages formed on smooth but not rough substrata. The authors suggested that differences in the protein mobility may account for supramolecular rearrangement of protein where the collagen molecules would be relatively free to move and assemble on smooth surfaces while the nanoscale protrusions of rough substrata would inhibit the collagen mobility.

### 2.4 Curvature

It is now well assessed that size is a key factor in determining the interaction of inorganic particles with biological systems, thereof a cell-specific optimal diameter is relevant for promoting or avoiding cellular uptake [1]. However, when one refers to surface–bimolecules interaction the size of an object becomes relevant in terms of surface curvature. A large particle exposes to biomolecules a quasi-flat surface, while smaller ones exposing a surface with a high curvature are reported being able to host a larger amount of adsorbed molecules per unit surface area [44].

The curvature of the particles is reported to be a dominant factor for conformational changes occurring to proteins during adsorption [45]. The surface curvature can affect two distinct surface properties, the surface geometry and the type of surface functional groups. The
two effects will be discussed separately, even if they are mutually related and only well designed experimental settings can discriminate between them.

In the paper by Lundqvist and coworkers [46] large amorphous silica NPs (diameter 15 nm) affect the secondary structure of a carbonic anhydrase (HCA I) more than smaller ones (6 nm). Under these circumstances geometrical reasons account for the observed conformational changes, whereas the interaction energy is higher for a large interaction area as the sum of the interactions established with the solid surface is stronger than the energy associated to the conformational structure of the protein.

The effect of surface curvature on SiO2 NPs in the range 5–100 nm of diameter was tested on the main phase transition of phosphotidylcholine bilayers of different chain length around the particles [47]. The data reported by Ahmed and Wunder are based upon a phosphotidylcholine bilayer model in which as the SiO2 NP size decreases the difference in the curvature of the inner and outer leaflets of the bilayer increases. Flat surfaces and large particles (low curvature) adsorb lipids in a similar way. On highly curved surfaces (small particles) the lipids form bilayers that adopt morphology with outer polar heads being widely separated from the polymer segments that experience conformational changes is indicative of the bonding process; (b) when the cluster size is on the order of magnitude of the polymer, geometrical constraints force the scaling of the adsorbed monomer densities with the curvature. In this case, the thickness of the adsorbed polymer layer is similar to the thickness of the adsorbed layer on a flat surface; (c) when the cluster size is on the same order of magnitude as the thickness of the adsorbed layer, the curvature is too high to support an energy gradient between the adsorbed layer and the bulk. As a result, the adsorbed polymer chains will extend outwardly from the surface of the cluster in order to decrease the density gradient and the clusters are enveloped by a mixture of adsorbed polymer chains that extend outward into the solution and entangled polymer chains from the solution.

Beside geometrical considerations the physico-chemical properties of the surface may be highly dependent on surface curvature. Curvature-induced surface modification reflects upon many other biologically relevant macroscopic features, such as hydrophilicity, z-potential, strength of hydrogen bonding. As reported above the silica surface terminates with hydroxylated functions attached to Si atoms named silanols (=Si–OH). Silanols can be broadly classified as i) isolated, ii) interacting, and iii) geminal. Siloxane bridges (Si–O–Si) are formed upon condensation of two adjacent silanols [49]. The presence of surface silanols and siloxanes and their nature (structure) are determined via diffuse and reflected FT-IR and FT-NIR analyses. In a study on amorphous silica powders with different diameters in the range 8–260 nm, the amount of isolated silanol (IR adsorption band at 3750 cm⁻¹) was shown to decrease as the particle diameter increases, almost disappearing in the case of the largest NP (260 nm). In contrast, the ratio of absorption of the hydrogen-bonded silanol IR band to that of the isolated group increases as the particle diameter increases [50]. Generally speaking, the curvature of silica NPs determines the relative abundance of isolated vs. interacting silanols and thus influences the dispersibility of silica in water suspension.

Theoretical considerations on the adhesion of an organic polymer (oligothiophene) on large-scale atomistic models of nanostructured titania surfaces were recently carried out by Melis [51]. Adhesion of the polymer appears to depend upon the local curvature and roughness. The interaction between titania and the polymer was reported to be dominated by electrostatic interactions. The adhesion forces on the curved surface were very different from the case of a planar surface. Better adhesion was achieved on flat or poorly curved surfaces and the molecular strain induced by a highly curved surface had a detrimental effect on adhesion. The authors discuss the binding force on flat surface, which is dominated by the Coulomb contribution [52] and report that, at the surface of nanostructured titania, a covalent contribution larger than that on a flat surface is expected.

2.5. Reactive sites

The surfaces of both covalent and ionic solids exhibit defective sites generated by the interruption of the bulk structure of the solid. Freshly ground, abraded, indented, and defective surfaces may expose surface charges, dangling bonds (i.e. surface-bound radicals) and poorly coordinated ions which may react with biological molecules. In some cases such reactions may cause detrimental effects on cells [59] and ref therein) as discussed in Section 5.

Crystalline – but not amorphous – silica dusts are known to be inflammatory and fibrogenic when inhaled [53]. Silica-derived reactive oxygen species (ROS) that are generated at the particle surface are deeply involved in the mechanism of toxicity of quartz [54] and ref therein). Two cooperative particle-derived ROS-generating mechanisms are commonly reported. One is mediated by the presence of dangling bonds on the quartz surface, the other is related to the occurrence of redox-reactive transition metals exposed at the surface. The first mechanism involves the presence of homolytically ($Si^\bullet$, $SiO^\bullet$) and heterolytically generated ($Si^{+\bullet}$, $SiO^{-\bullet}$) reactive sites, which originate from the grinding processes used to produce quartz dusts, that promptly react with molecular oxygen originating several surface radical species, i.e. $SiO^{\bullet\bullet}$, $SiO^{\bullet\bullet\bullet}$, $Si^{+\bullet}$–$O^{2-\bullet}$–$O^{2-\bullet}$. The second mechanism involves the presence of transition metal traces, typically iron [55], at the particle surface. Both dangling bonds and metal impurities are not present on synthetic amorphic silicas (precipitated or pyrogenic silicas) which appear therefore completely inactive in generating free radicals [56]. Metals exposed to the surface may react via radical mechanisms or electron transfer reactions with biological relevant molecules, such as $H_2O_2$, $C_2H_2$ and ref therein) as discussed in Section 5.

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Theoretical considerations on the adhesion of an organic polymer (oligothiophene) on large-scale atomistic models of nanostructured titania surfaces were recently carried out by Melis [51]. Adhesion of the polymer appears to depend upon the local curvature and roughness. The interaction between titania and the polymer was reported to be dominated by electrostatic interactions. The adhesion forces on the curved surface were very different from the case of a planar surface. Better adhesion was achieved on flat or poorly curved surfaces and the molecular strain induced by a highly curved surface had a detrimental effect on adhesion. The authors discuss the binding force on flat surface, which is dominated by the Coulomb contribution [52] and report that, at the surface of nanostructured titania, a covalent contribution larger than that on a flat surface is expected.
the free radical release by means of the spin trapping technique on three kinds of iron containing particulate: two asbestos fibers (chrysotile and crocidolite, Fe ca. 5% and 27% respectively); an iron-exchanged zeolite (Fe ca. 4%) and two iron oxides (magnetite and hematite). The authors give evidence for three different kinds of iron sites: one acting as hydrogen atoms abstractor, the other as a heterogeneous catalyst for hydroxyl radical release, the third one related to catalysis of hydrogen peroxide disproportionation. In both mechanisms of free radical release, the Fe-exchanged zeolite mimics the behavior of asbestos whereas the two oxides are mostly inert. Conversely magnetite turns out to be an excellent catalyst for hydrogen peroxide disproportionation while hematite is inactive also in this reaction.

Even if iron contamination is involved in many detrimental reaction of mineral surface, a recent study clarified that the high toxicity of some very pure quartz sample (DQ12) is not related to metal contamination but arises from the presence of undissociated silanols with potential to form strong hydrogen bonds, rather than being due to radical surface reactions. The reactivity of these surfaces is believed to interact in such a strong manner with biomolecules to bind and disrupt cell membranes and/or proteins [64].

Reactivity mechanisms also originated by metal impurities are partially involved in the biological detrimental reactivity of carbon nanotubes. Most commercial CNTs contain ultrafine metal particles (e.g., Fe, Ni, Y, Co, and/or Mo) derived from the original growth catalyst or support. It has been reported that carbon nanotubes containing iron as impurity were able to induce intracellular ROS. When acid treated to remove metal contaminants, the cellular ROS generation did not occur, suggesting that the metal catalyst contaminants are responsible for the ROS generation [66]. On the other hand, cell-free experiments using multiwall carbon nanotubes (MWCNT) in aqueous suspension determined that MWCNT do not directly generate either oxygen or carbon-centered free radicals whereas are able to quench such reactive species [67–69].

In the case of semiconducting materials, reactivity may arise following the charge separation occurring in the bulk of the oxide leading to the promotion of an electron in the conduction band and to the formation of a hole in the valence band. When the charge carriers reach the surface of the solid they entail reduction (e–) and oxidation (h+) reactions with the surrounding medium [70].

$\text{TiO}_2$ is a well-known photocatalyst. Under UV illumination it produces highly reactive radical species such as $\text{O}_2^–$ (e– transfer to O2) and HO• (hole interaction with water) [71].

$$\text{Ti}^{4+} + \text{O}_2 \rightarrow \text{Ti}^{3+} + \text{O}_2^–$$

$$\text{h}^+ + \text{OH}^– \rightarrow \text{HO}•$$

Superoxide radicals may be oxidized to singlet oxygen by electron holes [72].

For $\text{TiO}_2$ NPs entering inside the body no reactivity is expected in the absence of illumination. However it was recently reported that the generation of free radicals from uncoated rutile and anatase surfaces also occurs in the dark in the presence of hydrogen peroxide or organic molecules [73]. In the case of titania, the crystalline phase largely modulates the surface reactivity, anatase being more reactive than rutile under illumination [73,74]. Photoreactivity of titania also depends upon the size of particles and method of synthesis [75].

Other materials (e.g., quantum dots or fullerences) [76,77] have been reported to generate free radicals under UV illumination.

A size effect is generally observed for particles in the nanometric range. By reducing the particle size down to nanometers the disorder of the structure increases and the generation of defects affects the physical properties of the material and possibly (e.g. Au and Fe NPs) surface reactivity [20,78]. However, this is not a rule since it strictly depends upon the type of material.

2.6. Chirality

Biomolecules generally rely on chirality to induce site-specific molecular recognition as the basis for their correct function. Proteins have mostly evolved to recognize specific substrates, or be recognized by specific receptors and is not surprising that asymmetry plays a significant role in recognition processes. Addadi’s and her coworkers’ work has over the years elegantly clarified that the asymmetry of organized surfaces, such as chiral faces of calcium tartrate crystals, is selectively recognized by some proteins, antibodies and eventually differentiated in terms of cell adhesion [79]. Experimental studies have been devoted to the adsorption of organic molecules on right- vs. left-handed quartz, which is the most common acentric mineral [80]. Although the chirality of quartz crystal seems not to account for homochirality of living matter [81], intrinsically chiral mineral and metal surfaces have been reported to act as enantioselective agents in several reactions [82,83]. Surfaces of metals with achiral bulk lattice symmetry can become chiral by cutting a single crystal along specific high Miller index planes. Intrinsically chiral surfaces of some common minerals with both chiral and achiral bulk symmetry, such as α-quartz or calcite respectively, have been investigated with regard to the designing of catalysts with high yield in terms of enantiomeric excesses. Many more details on this topic can be found in the Mallat, Orglmeister and Bailey extensive review on the asymmetric catalysis at chiral metal surfaces [84].

Chirality may also be observed in engineered single-walled carbon nanotubes (SWCNT). SWCNT are made of a single graphene sheet rolled up to form tubes which may have distinct structures [85]. Depending upon the method of synthesis chiral helical tubes may be formed albeit a mixture of SWCNT having different diameter and chirality are generally obtained [86]. The preparation of enantiomically pure SWCNTs has raised interest in the past years since enantiomers exhibit distinct electronic and optical properties [85,86].

2.7. Dissolution/re-precipitation equilibria

Solid materials may be constituted by atoms bound through i) polar or apolar covalent bonds (e.g. elemental carbon, silicon dioxide or titanium oxide); ii) ionic bonds (typically alkaline, alkaline earth, e.g. NaCl, CaCO3); and iii) metallic bond (e.g. Au, Ag). When an ionic or covalent material is immersed in water, the solubility depends on the balance between reticular free energy and energy of solvation of the ions and related entropy changes. In the case of metals in the elementary form both the tendency to be oxidized in aqueous media and the solubility of the oxidized form have to be considered. Biological fluids contain several chemical species which can form stable coordination compounds. Therefore the solubility in biological fluids of metal-containing materials is generally higher than in pure water [87,88]. A similar effect has been reported also for silica [89].

The dissolution of a solid in biological fluids is relevant for at least two reasons: a) metal ions in the solid may be progressively released and become involved in many reactions and b) the chemical nature of the surface and sub-surface layers are progressively modified. The metal mobilized may then be a direct or indirect source of damage for biomolecules, such as lipids, proteins or DNA [1,88]. The ion-depleted material may in turn re-acquire the same ions, or other ions which are similar in size and charge, from the surrounding solution. The structure and composition of the surface inorganic solids may therefore largely be modified during the permanence in biological fluids.

The crystal structure and surface morphology determine to what extent ion depletion occurs, and which are the chelators more appropriate to extract a given ion from a particular solid. Many cases of detrimental interactions with cells induced by solubilized ions instead of surface-driven mechanism have been reported, e.g. for iron oxides, Ag and ZnO NPs ([11] and ref therein).
Different bulk structure (crystalline vs. amorphous, type of crystalline phase or insertion of doping elements) may induce large differences in terms of solubility or rate of dissolution of an inorganic compound. As an example, the dissolution rate of amorphous silica is much greater than quartz in similar conditions. A large variability exists among the solubility of crystalline silica particles with different lattice symmetry [49]. The higher solubility of amorphous silica by respect to crystalline forms is one of the various factors claimed to play a role in the much lower toxic response elicited [90–93].

Finally, as far as nanometric particles are less thermodynamically stable than their larger counterparts and expose to the fluids a larger surface area per unit mass, the dissolution kinetic is expected to be much faster than that of bulk material [78].

### 2.8. Techniques to evaluate physico-chemical surface properties

The surface chemistry of a solid and particularly the properties that govern the response to bio-interactions, can be studied by several techniques, many of which are now virtually able to give a response at the atomic resolution. Among the many possible ways to organize these techniques, we chose to discuss them with regard to their direct or indirect interaction with the surface. Direct tools include imaging techniques, such as scanning and transmission electron microscopy (SEM and TEM), atomic force microscopy (AFM), and elemental techniques, such as X-ray photoelectron and Auger electron spectroscopy (XPS and AES) and secondary ion mass spectrometry (SIMS), just to name a few. Indirect methods gather information about the surface analyzing the broader effect of some relevant surface features (e.g. z-potential/electrophoretic mobility) or the interaction with a molecular probe for specific surface sites (e.g., adsorption enthalpy measure — calorimetry; ESR spectroscopy coupled with spin-trapping technique; FTIR and Raman detection of site-specific vibrational probes).

Scanning electron microscopy (SEM) is largely the most used technique to study surface topography. When a high-energy electron beam (1–30 keV) is spotted across a sample surface, low-energy secondary electrons are generated. Due to their very low energy (<50 eV), secondary electrons may escape only from the uppermost surface layers (ca. 5 nm) of an object and are hence able to provide high spatial resolution (up to 0.5 nm) and impressive long-depth-of-field images of the sample surface. The main difficulties that arose in SEM imaging are due to sample preparation. The sample has to be conductive per se or coated with a conductive thin layer (usually C, Au, Pt). Normally SEM microscopes operate under high vacuum condition (ca. 10⁻⁶ Torr) to prevent electron beam to be deviated by gas molecules. These operating conditions pose particular difficulties when analyzing biological samples generally electrically insulators and water-rich. Variable pressure SEM microscopes overcome to these limitations by allowing non-conductive and partially hydrated samples to be imaged but a general loss in spatial resolution is observed. Very low-energy electron emission source (Field emission gun) can be operated to achieve better performance on poorly conductive samples.

When an electron beam interacts with an element, X-rays are also emitted. For this reason, SEM are often coupled with X-ray analyzers (energy-dispersive or wavelength dispersive X-ray spectroscopy, EDS or WDS) that allow to obtain information on the elemental composition of the sample. However, opposite to secondary electron, X-rays have high-energy (in the keV range) and can be produced several microns below the surface. Thus the information provided describes the bulk composition of the material rather than its surface, which has to be investigated with surface specific techniques (see below). The spatial resolution of the X-ray spectroscopy coupled with SEM is however high enough to allow the elemental mapping of a surface with a submicron resolution. Similar to SEM, TEM uses an electron beam to create an image of the sample. However, in TEM the electrons are forced to pass through the sample which has to be thin or ultra-thin. TEM samples will have a thickness that is comparable to the mean free path of the electrons that travel through the samples, which may be only a few tens of nanometres. TEM may operate in bright field or in dark field. The bright field imaging mode is the most common mode of operation for a TEM. In this mode the image, thus the contrast, is formed directly by occlusion and absorption of electrons in the sample. Denser regions will appear darker, while regions with less density (both in terms of crystal lattice or atomic number) or with no sample in the beam path will appear brighter. The bright field image is virtually a two dimensional projection of the sample down the microscope optic axis. The very short wavelength of electrons used in this case as beam of electromagnetic radiation makes it possible to achieve a very high spatial resolution which makes TEM capable to attain an atomic resolution. The dark field imaging mode uses the electrons diffracted by one or more crystallographic planes of the sample. In this mode, the image is formed by the scattering of specific crystal planes and the zones where there is no sample remaining dark. Bright and dark field images can be combined obtaining a so called high-resolution image (HR-TEM). Although TEM is intrinsically a bulk technique, the necessary preparation of thin cross-sections of massive samples often permit to obtain fundamental information about many surface characteristics, in particular elemental composition, contamination, defects, and crystal lattice modification, as well as surface amorphization.

The most recently developed but today widely used imaging technique with atomic resolution is the atomic force microscopy (AFM). AFM is a scanning probe microscopy (SPM), a branch of microscopy that forms images of surfaces using a physical probe that scans the specimen. The AFM probe consists of an oscillating silicon cantilever with a sharp tip (few nanometers wide) at its end. When the tip is brought into proximity of a sample surface, several different forces are established between the tip and the sample and the cantilever is deflected. The cantilever deflection or the modification of its natural oscillation frequency can be followed with a laser spot reflected from the top surface of the cantilever on an array of photodiodes. The AFM can be operated in a number of modes, depending on the application. Topographical analysis can be readily performed by scanning the surface maintaining the tip at a constant height, thus exerting a constant force between the tip and the sample. This is obtained with the aid of a piezoelectric tube that can move the sample in the z direction for maintaining a constant force, as well as along x and y axes. The resolution along z can be sub-nanometric, whereas AFM lateral resolution is typically of the order of 1 nm. Besides static mode (also called contact), a variety of dynamic (or non-contact) modes where the cantilever is vibrated exist. Although the lateral resolution of AFM is comparable with SEM and TEM imaging technique, the extraordinary resolution on z and the possibility to operate in complex media (water, but also buffers or solvent) make AFM often the most suitable technique for surface imaging. Furthermore, the silicon probe can be variously functionalyzed making AFM an even more powerful tool to characterize surface properties [94,95]. Many reviews exist on the topic and can be used for further reading [96,97].

X-ray photoelectron spectroscopy, also known as electron spectroscopy for chemical analysis (ESCA), is used to determine quantitatively the atomic composition of a material and the chemical properties of its elements. During XPS experiment, the sample is irradiated with an X-ray beam that interacts with the electronic shell of the material atoms determining the emission of some electrons, thus called photoelectrons. The kinetic energy of these electrons, subtracted by the X-ray photon energy and some instrumental parameters (the work function), corresponds to the binding energy of the electron with the nucleus and is therefore directly representative of the atomic number of the element (Z). Furthermore, element in
different oxidation state (e.g., Fe$^{2+}$ and Fe$^{3+}$ or S$^{2-}$ and SO$_2^{2-}$) exhibit different electronic shell and produce differentiable XPS signals. The low energy of photoemitted electrons makes them impossible to escape from inner material layers, thus the XPS signal is representative of the first 10–100 Å. One of the most interesting features of XPS spectroscopy is the large spectrum of elements detected, when compared to other electronic spectroscopies. Virtually only He and Ne are not emitting photoelectrons in a sufficient amount to allow determination. Elements from Li (Z = 3) and above can be quantitatively analyzed by means of XPS. Unfortunately, the limit of detection is in the order of part per thousands. Detection limits of parts per million (ppm) are possible, but require special experimental conditions. As for the other electron involving techniques, ultra high vacuum conditions are required. This is the primary drawback for analyzing biological samples. For most applications, XPS is in effect a non-destructive technique that measures the surface chemistry of any material.

Complementary to XPS measures many quasi-surface analytical techniques exist that involve either X-rays or electron as excitation or information source. Among them, Auger electron spectroscopy is of particular interest for surface analysis. Auger electrons in fact are generated when a high-energy electron beam is focused on a material surface. As for secondary electrons used in SEM imaging, the low energy of Auger electrons (50 eV–3 keV) has a short free path in a solid and is therefore related to the uppermost surface or quasi-surface atomic layers (few nanometres). For this reason, however, AES has to be operated under ultra-high vacuum conditions. As in XPS, AES measures the kinetic energy of the emitted Auger electrons, which is characteristic of the element present at the surface. Some modern electron scanning microscopes have been specifically designed for coupling with Auger spectrometer; these scanning Auger microscopes (SAM) can produce high resolution, spatially resolved surface chemical images, since Auger electrons can be discriminated from secondary electrons on a kinetic basis, being Auger more energetic than secondary electrons. Besides surface information, depth profiles are often obtained with AES coupled with a sputtering device able to dig into the surface with a subnanometric resolution. Opposite to quantitative elemental methods using X-rays (such as EDS, WDS or X-ray fluorescence), AES is sensitive to the lighter elements and Auger peaks can be detected for elements as light as lithium (Z = 3), which is the lower limit for AES sensitivity. Neither H nor He can be detected with this technique. The very low Auger yield for elements with Z > 50 strongly limits AES to identify heavier elements. Beside atomic number, there are several factors that can limit AES applicability. The most common limitation is often due to charging effects in non-conducting or poorly-conducting samples. Since excitation source of AES is conceptually identical to SEM, poorly conducting sample will gain a net polarity at the surface during the experiment. However, in this case, a conductive surface coating is obviously not applicable. Both positive and negative surface charges severely alter the yield of electrons emitted from the sample and hence distort the measured Auger peaks.

In this brief overview of surface techniques, it is worth mentioning the currently most sensitive surface analysis technique, secondary ion mass spectrometry (SIMS), which is able to detect elements present in the parts per billion range. When a solid surface is bombarded with high energy ions (primary ions), new ions from the sample are formed (secondary ions) and are sputtered away from the sample surface. The secondary ions can thus be detected and analyzed by means of a mass spectrometer, such as a time-of-flight (TOF) or a quadrupole mass analyzer. The erosion of the sample by an accessory ion beam can also provide a depth profile of the sample, and the ion beam, opportunely focused up to a 50 nm lateral resolution, can be scanned over the surface to provide an elemental image. SIMS is of particular interest when high sensitivity measures for dopants and impurities are required. It is also the reference choice for depth profiles with simultaneously excellent detection limits and depth resolution (subnanometric). Using mass detectors, SIMS can analyze virtually any element of the periodic table, and can be fine-tuned to discriminate among isotopes, including H.

The most external atomic layer of a solid is a discontinuity point, an interface between the outlying bulk structure and the surrounding medium. Structural ligands of surface atoms are generally replaced by molecular water or hydroxyl groups. The degree of ligand loss, and thus of coordination, varies with the location of the ions at the surface (e.g., extended surfaces, edge or corner positions). It is well-known that such poorly coordinated ions play a determinant role in the surface reactivity. Nature and abundance of the surface active sites may be evaluated by adsorption of suitable probe molecules from the gas phase on the material surfaces deprived of the adsorbed adventitious molecules by a standard treatment in vacuum [98–102]. The coordinative unsaturation created in such way can then be filled by a suitable probe molecule. Both the adsorption/desorption enthalpies and the vibrational features of the probe depend on the characteristics of the surface centers where it is adsorbed, while the total amount adsorbed evaluates the abundance of surface active ions. The vibrational features of adsorbed probe are practically monitored by means of FTIR spectroscopy and the stepwise adsorption/desorption enthalpies of the process can be followed by a conventional adsorption microcalorimetry.

Many well-established surface site-probe couples have been thoroughly investigated: Fe$^+$–NO, Al$^+$–CO, Cu$^+$–CO$_2$, Brønsted acid–NH$_3$ or pyridine, noble metal (Au, Pt)–CO [57, 61, 62, 64, 98, 103–106].

The information which can be obtained from FTIR spectra and microcalorimetry using molecular probes concerns: (i) the abundance and nature of Brønsted and Lewis acidic or basic groups; (ii) the occurrence structural defects; (iii) the nature and location of framework cations.

Among indirect surface properties measurements, ζ-potential is becoming more and more important in understanding and prediction of solid surface–biomolecule interaction [37], with a particular regard for NP studies [31]. ζ-potential can be measured using several complementary techniques. A complete report on such techniques is beyond the purposes of this review and only the most commonly applied electrophoretic light scattering (ELS) and electroacoustic phenomena measurements will be discussed. When an electric field is applied across a dispersion, charged particles move toward the electrode of opposite polarity. This phenomenon is called electrophoresis. If a laser beam is passed through the sample undergoing electrophoresis, the scattered light from the moving particles will be frequency shifted. By measuring the frequency shift, the electrophoretic mobility can be determined given the laser wavelength and the scattering angle. ζ-potential can then be calculated from the electrophoretic mobility. It should be noted that in the surrounding electrical double layer there is a notional boundary (slipping plane), within which the liquid moves together with particles. The measured ζ-potential is the potential at this slipping plane. ζ-potential is not exactly the surface potential (surface charge), but is the potential of practical interest in dispersion stability because it determines the interparticle forces [37, 107]. Electroacoustic phenomena measurements are based on the generation of and electric signal following the propagation of ultrasound through a heterogeneous fluid, such as dispersions or emulsions. This electric signal is called colloid vibration potential/current (CVI) and can be used for characterizing the ζ-potential of various dispersions and emulsions. At the opposite, when an electric field propagates through a suspension an ultrasonic acoustic wave arises. The so-called Electric Sonic Amplitude (ESA), the inverse of CVI effect, can be detected by an acoustic transducer behind the electrode. The generated sound wave is at the same frequency as the applied electric field and its amplitude simply correlates with the dynamic electrophoretic mobility of spherical
particles hence with its $\zeta$-potential. A review of modern electro-acoustic methods for measuring $\zeta$-potential in non-diluted samples is available [108].

The nature and occurrence of reactive surface centers can be investigated by provoking specific chemical reactions with suitable target molecules. Fenton-like reactivity using hydrogen peroxide as a probe has been extensively applied for the characterization of reactivity of iron-bearing mineral surfaces [57,65]. Formic, ascorbic, linoelic acid, glutathione, and cysteine were also used to investigate surface reactivity of various quartz samples, TiO$_2$, cobalt–tungsten carbide, indium–tin oxide [69,72,109–111] The surface-driven reaction on a specific target molecule unveils very informative details on the coordinative and oxidative state of reactive centers, as well as their abundance. Different methods have been proposed to evaluate the oxidative potential of particulates [112]. Among the various methods, spin-trapping technique, coupled with electron spin resonance (ESR) spectroscopy, is probably the most informative procedure to assess such relevant surface features which impart radical reactivity to a material or particulate. In a spin trapping experiment [113], the radicals generated from a target molecule is stabilized by a covalent bond with a “spin-trapping” molecule. The stable radical adduct generated is analyzed by ESR. The intensity of the ESR signal is proportional to the amount of the radical species in solution. Among the different probes that have been developed to evaluate the oxidative potential of particles [58,112,114,115] ESR is the only available technique that allows to unveil the chemical nature of the radical formed and to discriminate among different radicals simultaneously generated. The ESR signal can be fitted with appropriate simulation software and the spectroscopic resonant parameters (the hyperfine splitting-constants) may be usefully used to identify the chemical nature of the radical adduct formed.

3. Adsorption of proteins

Among the different processes occurring at the interface between surfaces and biological fluids the interaction with proteins is the most relevant in the response of the tissues/cells to the xenobiotic entity. At the same time this process is the most complex to describe mainly because of its dynamic nature. In the case of nanoparticles the surface/biofluid interface may be described as a protein corona, i.e. a layer characterized by a slowly established core, with relatively stable composition, and an outer layer made of fast-exchanging proteins, whose composition is strongly influenced by the biological environment and is subjected to relaxation processes. Such corona is time-dependent [1,116], as Leo Vroman showed in 1962 with his work on the adsorption of blood serum proteins onto an inorganic surface, and it is the result of both thermodynamic and kinetic factors, as it implies competition between proteins for the adsorption sites. The proteins with higher mobility absorb first and are subsequently replaced by less motile proteins with a higher affinity for the surface. The process may take several hours to get to equilibrium [116] and the outcome is influenced by the environment which may change significantly whenever the particle moves from one biological compartment to another.

The two main factors that drive protein competition for the solid surface are: i) the relative concentration of the proteins in the biological environment; ii) the stability of the protein–particle adduct. The adduct stability ultimately depends on the intermolecular forces that dominate in the mutual interaction and are influenced by the type of proteins involved [117] and by the chemical properties of the solid surface [46,118].

The adduct stability reflects on the entropy vs. enthalpy balance. Enthalpic changes are usually related with the formation/disruption of a number of chemical bonds either between protein and surface or within the protein molecule after adsorption or following the redistribution of charged groups (ions) when the electrical double layers around the protein molecules and the sorbent surface overlap; conversely, entropic changes are usually related with the release of bound water from the solid surface or with the presence of denaturation/structural rearrangement phenomena within the protein molecule [8].

A significant factor that may influence the final composition and aspect of the protein corona is the size of NPs with respect to the size of proteins. This may influence the molecular crowding on the surface as well as the establishment of protein–protein interactions that may affect the conformation of the adsorbed proteins.

Despite the amplitude of factors that influence the process of formation and the composition of the protein corona and the consequent difficulties in predicting the outcome of the interactions between NPs and biological fluids it’s worth to mention that several attempts of building models for such process have been already made. Dell’Orco et al. [119] propose a dynamic model for the prediction of time evolution and equilibrium composition of the protein corona that forms when polymer NPs come into contact with human plasma. The model is based on affinity, stoichiometry and rate constants. Similarly, Raffaini et al. [120] employ computational methods to predict the behavior of albumin in the presence of carbon nanotubes or C$_{60}$ fullerenes. Other theoretical studies on proteins interacting with different surfaces have been reviewed by Gray [121].

In this section we will focus on three phenomena that occur after protein adsorption on the solid surface and that may heavily influence the cell response: i) the alteration of surface properties of the solid due to protein coverage; ii) the structural modifications induced by the adsorption on the protein fold; iii) the changes of protein activity related with structural modifications or selective orientation of the proteins onto the NP surface. Fig. 2 reports a summary of the events that may occur after protein adsorption.

A number of studies have been devoted to the different aspects of the subject. Our aim is to provide an overview on the kind of modifications that may occur as well as a survey of the available methods for characterizing such changes.

3.1. Alteration of the surface properties of the solid

The first consequence of the interaction of a solid surface with biological fluids is the alteration of surface properties of the solid.

The coverage of a solid surface by proteins implies important changes in the surface charge distribution, in the $\zeta$-potential (in case of NPs) and in the accessibility of a number of chemical functions that affects the ability of the solid to establish H-bonds, electrostatic or hydrophobic interactions, etc., that is to say the ability of the surface to interact with the chemical environment.

A progressive modification in the surface charge of silica NPs as a result of increasing protein coverage of the surface has been reported by various research groups [122–124] and recently by some of us [36]. Fig. 3A illustrates some data reported in this latter study on the behavior of hen egg lysozyme (HEL) at the surface of silica NPs. By increasing the protein concentration in the supernatant, the amount of HEL adsorbed onto the silica surface gradually reached the theoretical monolayer (Fig. 3A). A high degree of coverage of the surface was confirmed by the $\zeta$ potential values which showed a gradual decrease of the negative surface charge of silica, which became even positive at the highest degree of coverage (Fig. 3B). Note however that HEL would hardly completely mask the silica surface in complex media because the adsorption of HEL on silica is partially reversible [36] thus HEL is likely to be displaced in vivo by any other protein with a higher affinity for the surface.

Any modification of surface charge is particularly relevant in NPs studies as it reflects on both the stability of the colloidal suspension and the biological response.

Colloidal instability may concern both NPs (e.g. particle aggregation, flocculation, precipitation, etc.) and proteins (through protein aggregation, clustering, fibrillation, etc.) and heavily influences the initial biological response to NPs [13].

Experimental evidence suggests that formation of a protein corona improves colloidal stability. A study on polysterene NPs by Walczyk...
et al. [12] remarks that — on the only base of the low surface charge — the protein-coated NPs would not be expected to be colloidal and assigns the observed stability (which is comparable to that of bare particles) to the presence of the protein corona. Similarly, a study on gold NPs by Casals et al. [125] shows that AuNPs instantaneously aggregate when dispersed in protein-free cell culture medium, while they are stable upon addition of serum to the solution. This is also taken as an indication that NPs protein coating is faster than NPs aggregation in those experimental conditions [125].

This protein-mediated ability to stabilize nanomaterials has even been exploited for NPs assembly [126] as is the case of biotin-streptavidin [127] and antigen–antibody [128] interactions. Anisotropic particles such as nanowires, nanotubes and M13 viruses have been also assembled through biomolecular recognition mechanisms [129]. Interestingly, even the structural stability and instability of proteins upon binding may be exploited to modulate NPs spacing, as reported by the group of Rotello [130] in their study on chymotrypsin (which denatured upon binding) and cytochrome c (which retained its native structure).

Deguchi et al. [131] show that fullerenes C₆₀ can be stabilized by adsorption of proteins in the physiological environment. They found that the salt-induced coagulation of C₆₀ was suppressed by the presence of human serum albumin (HSA) in a molar ratio-dependent mode. Similarly, CNTs aqueous suspensions are stabilized by proteins [132].

Schulze et al. [133] report that ZrO₂, CeO₂ and TiO₂ particles exposed to Dulbecco’s Modified Eagle’s Medium (DMEM) were strongly agglomerated. Nevertheless a deagglomeration effect was found with ZrO₂, CeO₂ upon enrichment of the medium with FCS (Fetal Calf Serum) where deagglomeration was scaled with the protein content. In some cases interaction of particles with proteins lead to a destabilization of the suspension. Agglomeration was observed in NP suspensions following interaction with fibrinogen [134]. In this case, bridges of proteins between NPs were observed by SEM. Similarly, Xu and coworkers [135] observe that cytochrome c, DNase II or hemoglobin tend to form coralloids with SiO₂ NPs by bridging between them.

The impact of the interaction with a solid phase on protein stability will be discussed in a further section. Nevertheless in discussing the alteration of surface properties of the protein–solid particle system is worth to mention here that NPs have been reported to influence protein fibrillation processes [136–138]. This is likely related with the conformational perturbations associated with protein binding to solid surfaces, whose effect can be to bring protein domains that are normally buried inside the protein towards the surface. It’s worth to mention that formation of protein fibrils as a result of conformational changes is a relevant phenomenon for a number of diseases, such as amyloidosis, Alzheimer, etc.

Lynch et al. [136] show that the fibrillation rate of β-2-microglobulin — an amyloidogenic protein — is increased in the presence of polymer NPs. As the resulting fibrils are not associated with the NP, a mechanism implying pre-concentration of protein on the NP surface and subsequent detachment of the pre-fibrillar aggregate, which migrates in the solution phase, has been proposed. Similar findings have been reported by Bellezza and coworkers [137] on myoglobin (Mb) with phosphate-grafted zirconia NPs. Conversely, C₆₀ hydrated fullerene has been reported to display anti-amyloidogenic ability as it inhibits fibrillation of the amyloid β25-35-peptide [139]. If confirmed this finding may have important implications in the development of NP-based therapies against neurodegenerative diseases.

Another important effect which may follow protein adsorption is the inhibition of the ability of inorganic surfaces to react with biomolecules or to generate free radicals.
Ground quartz dusts are highly reactive surfaces and are able to generate free radicals or to directly react with organic molecules (see paragraph 2). A useful method to monitor such reactivity consists in evaluating the amount of carbon-centered radicals generated by a definite amount of powder in an aqueous suspension of a model protein by means of the ESR/spin trapping technique[89,110]. In Fig. 3C the ESR signal obtained by a commercial quartz dust is reported (spectrum a). When pre-incubated with a solution of bovine serum albumin (BSA), which rapidly adsorbs onto silica surfaces in an irreversible mode, the signal is completely suppressed (spectrum b) suggesting that the reactive sites have been completely covered by protein molecules. However upon removal of the adsorbed BSA by enzymatic degradation the ability to generate radicals was restored (spectrum c). This experiment recalls what may happen when a particle comes into contact with proteins in extracellular fluids and is subsequently phagocytised by macrophages. Proteinases inside the cell may degrade the protein corona and hence restore the original reactivity of the dust. In panel D, Fig. 3 a scheme of such process is depicted.

3.2. Structural modifications induced by the adsorption on the protein fold

Adsorption onto a surface may bring about important structural changes in the protein. These are strictly dependent on the mode of interaction of the protein with the surface which, in turn, is influenced by the physico-chemical nature of the solid surface and by the intrinsic properties of the protein [46]. The final outcome results from a balance between the extrinsic and intrinsic forces involved in the process.

The degree of conformational change and the rate at which the proteins undergo conformational changes after adsorption on a solid surface are influenced by a number of parameters:

i. The intrinsic protein stability
ii. The characteristic of the sorbent surface
iii. The surface curvature
iv. The protein concentration on the surface
v. The pH and the ionic strength

In the following lines, we will try to discuss each one of these factors, through meaningful examples taken from the literature.

3.2.1. The intrinsic protein stability

This parameter deals with the intrinsic resistance of the protein to undergo conformational changes in the presence of a solid surface. Following a classification by Norde [8] proteins may be divided in two groups: hard, which designates relatively rigid structures that tend to maintain their original conformation and soft, which indicates those that readily undergo conformational changes or deformation [140,141].
The hardness and softness character of a protein is particularly relevant as one considers that adsorption of proteins on a solid surface normally results in conformational heterogeneity of the surface-bound proteins that implies the presence of a population of protein structures with different degrees of perturbation [46,142,143]. Wu et al. [143] while commenting on the extent of unfolding of globular proteins, underline that highly flexible proteins (e.g. β-casein) adsorb much faster and spread at the interface to a greater extent than very rigid and compact ones [144] whose tertiary structure should undergo conformational changes in order to spread at the interface. Protein hardness and softness [46,145] depends on structural determinants (such as the number of disulfide covalent bridges within the protein, the presence and distribution of salt bridges in the protein structure, the presence of stabilizing sites such as metal-coordination sites that may both influence the spatial arrangement of the protein domains and exert a charge stabilization effect, etc.) and on thermodynamic factors (the balance between enthalpic and entropic contributions, that finally defines the amplitude of conformational heterogeneity). Larsericsdotter [145] reports that the structural heterogeneity induced by the adsorption of RNase and lysozyme on silica particles is decreased by calcium ions; in fact, co-adsorption of bivalent cations has been reported to prevent effectively charge accumulation in the adsorbed protein layers which seems to be a main reason for structural heterogeneity. Similarly Bilstien et al. [146] have shown that the protein’s structural stability is a key factor in determining the rate of conformational change for different mutants of human carbonic anhydrase II. Further, Wu et al. [117] report that the extent of unfolding for lysozyme (positively charged) is comparable to the values for β-lactoglobulin (negatively charged) [143] in spite of favorable electrostatic interaction with the silica surface and assign this peculiar behavior to the presence of disulfide bonds stabilizing the tertiary structure of both proteins.

Albeit the overall structural stability of proteins is definitely an important determinant in the adsorption of proteins, differences in the resistance to conformational changes among the different domains of proteins should be considered in describing the process at a molecular level.

3.2.2. The characteristics of the sorbent surface

A huge number of papers show that the chemical nature of the sorbent surface has a strong influence on the adsorption outcome, in terms of affinity and structural distortion of the protein.

Zoungrrana [147] reports that adsorption of α-chymotrypsin on positively or negatively-charged polystyrene surfaces decreases the enzymatic activity at a different extent suggesting that electrostatic interactions between the protein residues and the solid surface play a key role in determining the degree of unfolding.

Electrostatic interactions appear to be important also in the case of silica particles. Xu et al. [135] report that the stability constant of adducts of negatively charged silica particles with cytochrome c, deoxyribonuclease and hemoglobin increases with increasing numbers of positively charged Lys and Arg residues. The role of these residues in the interaction with the surface was confirmed by the involvement of the polar residues of cytochrome c located near positively charged residues in H-bonding to silanols in silica. Similarly, a study on the adsorption of lysozyme onto silica [148,149] shows that the amount adsorbed decreases as the pH decreases. This posits in favor of a mechanism of adsorption dominated by coulombic interactions [148]. It is generally accepted that the hydrophobicity/hydrophilicity degree of the surface plays an important role in the affinity of proteins and in the extent of surface-driven unfolding. Irreversibility is strictly related to consistent surface-driven conformational changes that occur mainly when protein adsorbs through hydrophobic interactions [8,142].

Adsorption of lysozyme onto silica NP was shown to result in a marked loss of α-helix content for larger surface coverage [148]. The same protein adsorbed onto polytetrafluoroethylene (PTFE) surfaces underwent a biphasic conversion of α-helix to β-sheet, with an initial quick phase followed by a much slower conversion [150]. Interestingly, Norde [8] highlights that apolar surfaces may induce a higher degree of ordered structure in the adsorbed proteins, in that they promote H-bonding between peptide units in the protein–surface contact region, although this cannot be taken as a general rule.

3.2.3. The surface curvature

The surface curvature has been shown to influence both the composition of the protein corona and the conformational state of the adsorbed protein.

Lynch et al. [136] underline that − departing from the simple limiting case of flat surfaces − as the particles become smaller and approaches the size of the adsorbed proteins, the composition and organization of the protein layer changes dramatically. According to Lundqvist [46] this is related with the fact that a large interaction area between the protein and particle (typical of larger particles) affects the secondary structure more than a small one, because of the higher interaction energy. Nevertheless foreseeing the trend of such changes is not straightforward, as the effect of curvature is also strongly dependent on the nature of the adsorbed protein.

Several examples of opposite behaviors that are paradigmatic of such dependence are available in the literature. Vertegel et al. [148] and Lundqvist et al. [46], in their work on the adsorption of lysozyme and human carbonic anhydrase on silica NPs show that these proteins retained more native-like structure on smaller particles. Oppositely, a higher curvature results in a more destabilized protein in the case of RNase adsorbed onto silica [151]. A similar behavior has been reported for albumin adsorbed on silica particles, whereas fibrinogen follows the opposite trend. Roach et al. report that, although both proteins showed similar trends in binding affinity and saturation, the loss of secondary structure of fibrinogen is more important in the presence of particles with higher surface curvature [45]. Besides, Karajanagi et al. [152] report that soybean peroxidase on single-walled carbon nanotubes retained more of its native structure and activity than chymotrypsin, which exhibited a nearly complete loss in activity and structure. In conclusion the surface curvature modulates the degree of unfolding of proteins, however, a common trend may not be found since the structure of an adsorbed protein is not independent from the nature of the protein itself.

A protein stabilization effect exerted by highly curved surfaces has been reported by Asuri et al. [126]. They report that single-walled carbon nanotubes (SWNTs) may stabilize proteins in strongly denaturing environments to a greater extent than flat supports. The provided explanation is that lateral interactions between adjacent adsorbed proteins contribute to protein deactivation in harsh environments and that these unfavorable interactions are suppressed on highly curved supports such as SWNTs relative to flat surfaces [126]. Similarly, the highly curved surface of C60 fullerenes have also been reported to enhance enzyme stability [136].

3.2.4. The protein concentration at the surface

Molecular crowding, which depends on the abundance of proteins approaching the surface, is another factor that seems to have an influence on both the extent and the kinetic of unfolding of adsorbed proteins. Wu et al. [143] in their investigation on β-lactoglobulin adsorbed on silica particles remark that the protein unfolded to a greater extent at low surface concentration; in addition, as compared to higher surface concentration, the process was faster and kinetic curves were different. The energy barrier for unfolding in a crowded surface seems to be higher; the rationale behind this finding lies in protein–protein interactions that would predominate in these conditions, whereas, at low surface concentration, proteins mainly interact with the solid surface. At high surface concentration the unfolding behavior is affected by the interaction between protein molecules, due to limitation of the physical space and to the energy barriers that arise from the interaction
with other nearby protein molecules; such barrier also decreases the unfolding rate.

A similar behavior has also been reported in the case of lysozyme adsorption onto silica nanoparticle [117].

3.2.5. The pH and ionic strength

As discussed in the Introduction section, pH and ionic strength of biological fluids may largely vary in the body [11] and this is highly relevant for protein adsorption phenomena onto solid surfaces.

Both the pH and ionic strength of the medium have been reported to display an effect on the folding state of adsorbed proteins. pH can modulate the protonation state of all the ionisable groups in the protein–solid dual system. So it may influence the surface charge distribution of the solid phase and the protonation state of aminoacids involved in H-bonding, cumblic interactions, etc. It is well known that pH has a strong effect on the H-bonding network of proteins and this may result in protein destabilization [153]. Changes of ionic strength are known to influence protein stability and may result in aggregation or protein precipitation.

A strong effect of pH on the secondary structure of lysozyme adsorbed on silica NPs is reported by Vertegel et al. [148]. They report that the α-helix content decreases from 40% (in the free enzyme in solution) to respectively 8 and 5% for lysozyme adsorbed on 100-nm particles at pH 6.9 and 5.0.

As for the ionic strength, higher values of this parameter were found to induce a much higher extent of unfolding at higher surface concentration, in the case of the adsorption of β-lactoglobulin onto silica NPs [143]. According to Wu et al. [117] at higher ionic strength the electrical double layer in the vicinity of the adsorbed protein molecule is compressed. As a result, the electrostatic interaction between adsorbed protein molecules is reduced and this may improve unfolding. Interestingly, such an effect of ionic strength was strongly dependent on the surface concentration and it was not significant at lower concentration, where the average distance between adsorbed protein molecules is far greater than the interaction range of the double layers of different protein molecules [117].

Another important issue concerns the dependence of the amount of adsorbed protein on ionic strength, as it may provide information on the nature of interaction forces that dominate the establishment of the protein–NP system. In a study on the adsorption of Cytc, DNase II and Hb to silica NPs, Xu et al. [135] find that the amount of bound protein decreased sharply with increasing ionic strength. This is taken as evidence that electrostatic attraction is the main interaction force between the protein and the nano-SiO₂ particle. The double electric layer on the nano-SiO₂ particles adsorbed Na⁺ so the positively charged side groups of proteins were repelled. The induction, orientation and dispersion forces between proteins and nano-SiO₂ become stronger in a high salt medium owing to polarization of the double electric layer. However, once again, this mechanism cannot be generalized as a different behavior is found in the case of the adsorption of HSA and nano-TiO₂ [154].

The understanding of the conformational changes undergone by proteins upon binding to a surface implies the necessity of grasping information on two different aspects: i) to get a detailed description of such structural changes at a molecular level of resolution; and ii) to clarify the mechanism that underlies such changes.

In order to face these problems, both traditional and new investigation approaches needs to be used.

Traditional approaches include the techniques normally used in protein stability and conformational studies, such as Trp fluorescence, circular dichroism (CD), differential scanning calorimetry (DSC), etc. Nevertheless, these approaches need to be somehow refreshed as soon as they are applied to heterogeneous systems (solid plus protein dispersed in liquid media) instead of homogeneous media.

Unraveling the molecular details and the behavior of such heterogeneous systems requires the development of synergistic approaches in terms of characterization techniques. A multi-disciplines and multi-disciplinary approach is required, able to put together two distinct fields of expertise (protein structure and function, on one side; the characterization of solid surfaces and particles, on the other side), to combine their experimental strategies and to merge their disciplinary viewpoints. This strong challenge will influence the future perspectives for the employment of NPs in health sciences.

A number of studies have been performed on different proteins and different materials with the aim of highlighting the influence of adsorption on protein conformation. As expected, a huge variety of cases has been collected, due to the number of factors that influence these complex phenomena and to the peculiar individual character of each protein. It is almost impossible to extrapolate a common trend and a systematic exploration of different combinations of proteins and materials appears necessary, in order to build a huge reference database.

Conformational changes induced by protein adsorption are often a stepwise process. This is clearly shown by Karlsson et al. [155] in a study on the kinetics of conformational changes of differently denatured mutants of human carbonic anhydrase II (HCAII) adsorbed on silica NPs. By using a combination of circular dichroism (CD), Trp fluorescence and ANS (1-anilino-8-naphthalenesulfonate) fluorescence spectroscopy, Karlsson et al. show that the process takes place in a progressive mode. At first, HCAII binds to the solid surface and structural changes occur afterwards. The disruption of the active site occurs first and it is followed by the rest of the tertiary structure; half-lives are reported for each step. The stability of HCAII mutants affects the rate of conformational rearrangements (i.e. the kinetic of denaturation). The less stable the structure, the more firmly the protein is associated with the surface and the more quickly the structure is unfolded. Interestingly, the extent of conformational changes is not influenced by protein stability as all mutants unfold to the same extent, i.e. a molten globule state. According to the authors this is taken as an indication that surface-driven denaturation differs from chemical denaturation, as the first deals with stability at the surface and the second with global stability. According to Karlsson, this is because the protein interacts in a specific orientation with surfaces, while in chemical denaturation the protein is immersed in the denaturating medium.

A study on the adsorption of Cyt c, DNase II and hemoglobin onto silica NPs [135] reports distinct behaviors. In Cyt c the fractions of α-helix and β-turn decreased, whereas the β-sheet fraction increased with increasing SiO₂ particle number. Conversely, the fractions of α-helix and β-turn in DNase II and Hb increased with increasing SiO₂ particle number and a decrease of the β-sheet fraction was found. According to Xu et al. [135] the rationale behind this finding is that the β-sheeted sheet areas of DNase II and Hb bound directly to the SiO₂ particles and the press-and-pull interactions twisted the sheets, thus inducing the emergence of an α-helix-like structure.

When protein is adsorbed on a surface, rearrangement phenomena may occur: these may be associated with partial protein refolding. This is what Wu et al. report in the case of lysozyme and β-lactoglobulin adsorbed onto silica NPs [117]. These authors found that a comparison of the kinetics of tertiary conformational changes of the two proteins indicates that, the structural perturbation is faster and more extended in lysozyme as compared to β-lactoglobulin during the first phase of adsorption. Nevertheless, in a further step lysozyme was able to undergo a refolding process that was absent for β-lactoglobulin.

The initial rapid unfolding of lysozyme upon adsorption seems to be associated with the rapid decoupling of the α and β domains of lysozyme, with consequent disruption of the tertiary structure, loss of α-helix and, increase in β-turn and random coil [150]. This results in a ‘molten globule-like’ structure in which α-helix is transformed into β-sheet, thereby resulting in a refolding phase [117]. Thus at least for lysozyme the adsorption process occurs in three phases: an initial unfolding phase, that occurs as the proteins interact with the surface, followed by partial
renaturation (second phase) likely associated with protein reorientation on the surface. The third phase is characterized by a further slow unfolding step, which drives the system towards the equilibrium.

Another aspect of conformational changes is the degree of reversibility of the induced modifications upon protein desorption from the surface.

The case of BSA interacting with synthetic chrysotile nanocrystals, investigated by Sabatino et al. [156], is a typical example. This study shows that BSA modifications are driven by surface interaction with chrysotile. Once BSA is desorbed back into the solution its structure rearranges, without getting back to the native conformation. A significant amount of β-structures is found in the desorbed molecules. According to Sabatino, β-structures are more involved in the surface adhesion process adsorption and their increase is related to the extent of protein unfolding that exposes to the solvent protein regions that are normally buried in the interior. Sabatino et al. [156] suggest that this change in secondary structure is driven by the formation of surface-mediated hydrogen bonds with the polar aminoacidic side-chain groups.

3.3. Changes of protein activity related with structural modifications or selective orientation of the proteins onto the surface

The issue of protein conformation is important as it reflects on the protein behavior. This is especially dramatic in the case of enzymes, whose catalytic activity is strongly dependent on a correct protein folding and hence may be seriously influenced — even completely disrupted — as an effect of the interaction with a surface. It may also be relevant for proteins involved in molecular recognition processes or in the interaction with receptors. Such kind of processes requires that the protein or specific protein domains maintain their conformation in order to bring about the expected effect. Even when the general fold of the protein and the conformation of the active site is not compromised by the adsorption process there is still another issue that may influence protein functionality, namely the orientation of the molecule onto the solid surface. In the case of enzyme, the interaction with the surface may hinder the entrance of the substrate channel or interfere with the diffusion of substrates within the active site. In addition, if a protein involved in molecular recognition processes exposes to the solid surface the epitope that needs to be recognized by a receptor or a molecular partner, the related process will be impaired [13].

As in the case of conformational changes the effects of adsorption on the functional behavior of proteins are extremely varied. Foreseeing a general common trend is unfeasible as too many factors concur in determining the final exit.

Important conformational changes recorded upon adsorption of lysozyme onto silica NPs reflect on the enzyme activity: about 60% of the native lysozyme activity is retained when the α-helix content is reduced to ~30% of the initial value. Further loss of secondary structure results in a much sharper activity decrease [148]. As for the orientation of the protein [157] it has been shown that lysozyme adsorbed onto flat silica surfaces exposes the largest positively charged patch on its surface (which contains 7 of the 17 basic aminoacidic residues of lysozyme) towards the negatively charged silica surface. As the active site of lysozyme lies on the opposite side [158] one can speculate that the moderate loss in activity is due to the distance existing between the structurally perturbed region and the catalytic site. Nevertheless further activity loss is found, as the perturbation spreads over the whole structure. Conversely, both cytochrome c and Dnase II were reported to be inactivated up to 75% upon adsorption on aggregated nanometric SiO₂ [135].

Activity measurements performed on RNase [151] show that the enzyme retained 90% of its intrinsic activity upon adsorption on 4 nm silica NPs, whereas a net 30% loss was found with 15 nm particles. This is clearly related with the higher degree of conformational integrity of protein molecules associated with smaller particles (see Section 3.2).

The degree of hydrophilicity/hydrophobicity of the solid surface influences the functional perturbation of some proteins, as it reflects on the kind of interactions that a protein can establish with the solid phase which, in turn, may affect its conformation. Noinville et al. [159] report that the activity of α-chymotrypsin is more affected when the protein interacts with hydrophobic surfaces because of greater driving force for unfolding.

In addition, as protein concentration at the surface has been shown to influence the kinetic of unfolding, an effect on their activity is expected as well. Wu et al. [117] in their investigation on lysozyme adsorbed onto silica particles found that the immobilized enzyme retains its activity only at high packing densities.

Although this review does not deal with coated-particles, it is worth to mention the study by Rotello and coworkers [130,160] who showed that, by controlling the surface chemistry, it was possible to achieve distinct levels of interaction of chymotrypsin with CdSe NPs, implying enzyme inhibition with denaturation or with retention of structure [130].

In the case of enzymes with dual catalytic functions, adsorption may modulate the balance between them. A study by Shang et al. [161] on Cyt c shows that the heme environment is perturbed by the adsorption of the protein onto a solid surface and this increases solvent accessibility of the heme. This reflects on the peroxidatic activity, which is usually weak in native Cyt c: a size-dependent increase of peroxidase activity was found in Cyt c adsorbed on silica NPs of different size.

As previously mentioned, a loss of activity upon protein adsorption has not always to be expected. Several reports have shown that using some NPs as solid supports does not necessarily imply important activity loss [143]. This is especially true in the case of biochemically functionalized silica NPs with enzymes, that have been successfully employed for cell membrane staining and other applications [143,146].

As far as the issue of protein orientation is concerned, it is worth to mention the potentialities of labeling techniques, that allows collecting topological information on the interaction between the protein and the surface.

Site-directed spin-labeling (SDSL) is a labeling technique developed by Hubbell et al. [162] that relies on the conjugation of a paramagnetic spin-label on specific sites of a protein, usually exploiting Cys residues as the anchor-point. Cys may be introduced by site-directed mutagenesis. Such approach is useful to investigate protein dynamics at a level of molecular details [163,164]: changes of the ESR spectrum as a consequence of conformational transitions of the labeled protein allow evaluating a number of parameters related with the anisotropy degree of the paramagnetic probe motion and to measure interatomic distances.

This approach has been applied by Jacobsen et al. [165] on a series of T4 lysozyme mutants adsorbed on quartz surfaces, with the aim of evaluating changes of the local environment of distinctly labeled sites, spread over the whole lysozyme structure, upon protein adsorption. Based on the comparison of ESR spectra, the authors were able to show that the C-terminal part of the protein is actively involved in the adsorption process.

An ESR study by Nicholov et al. [166] on human serum albumin labeled on the Cys34 residue allowed establishing the degree of conformational distortion of the protein environment around the labeled site, thus providing information on the orientation of HSA on three distinct solid supports.

In a recent study [36] we employed SDSL to investigate the interaction of lysozyme and ribonuclease with nanostructured silica particles. We were able to conclude that the lysozyme region containing the spin-labeling site adhered quite flatly to the silica surface, whereas RNase adsorption resulted in a label-containing cavity that was almost inaccessible from the outside, but still allowed a certain degree of mobility to the spin-label trapped inside. These details allowed concluding that both proteins adsorb preferably on one side of the molecule but each enzyme exhibited a peculiar adsorption mode.
3.4. Techniques to evaluate protein adsorption, unfolding and orientation

A number of techniques and experimental approaches have been applied either separately or in synergy to investigate NPs contact with proteins.

A thorough characterization of the protein-NP system needs distinct approaches, depending on the aspect of the system that one aims to focus on. Different techniques are needed for characterizing the analytical composition of the protein corona and the dynamic aspects related with its formation (Table 1), as compared to the changes in protein fold brought about by protein adsorption as well as the orientation of proteins onto the solid surface (Table 2).

In this section we will try to report a schematic overview of the available techniques and the kind of information they can provide.

Size exclusion chromatography allows separating NP-associated proteins from complex mixtures such as plasma and other biological fluids. It allows estimating the exchange-rate of proteins adsorbed on NPs because only slow-exchanging proteins, with a residence time several times longer than the separation time, are recovered in the same final fraction as NPs. Conversely, very fast exchanging proteins elute at the same time as isolated proteins. A study by Cedervall et al. on the adsorption of plasma proteins to N-isopropylacrylamide (NIPAM)/N-tert-butylacrylamide NPs shows a dependence of the exchange rates on NPs' hydrophobicity, as apolipoprotein A-I co-eluted with the particles, whereas HSA and fibrinogen eluted much later [118]. According to Cedervall et al. size-exclusion chromatography is potentially less perturbing of protein–particle complexes than centrifugation, because the outcome of a centrifugation assay may be affected by the duration of washing steps and the solution volumes used in these steps [13]. As a consequence Lynch et al. underline that highly abundant proteins may be retrieved either due to insufficient washings or because they are truly associated with the NP. Sedimentation of large proteins, protein aggregates, and co-precipitation have also to be taken into account. Nevertheless, centrifugation assays conducted with care and associated with other methods still allow to retrieve enough proteins for safe identification of proteins by mass spectrometry [13].

Aggarwal et al. report that [167] a combination of SDS-PAGE and Western blotting may provide general information concerning the proteins adsorbed onto NPs, although quantitative results are difficult to obtain and these methods may be used mainly for comparison purposes. SDS performed on fluorescent dye-labeled protein separates the proteins based on molecular weight [168] and allows measuring protein concentration based on fluorescence measurements. The same authors remind that another possibility for spotting the corona’s proteins, once they are separated from NPs is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). This can be used in association with N-terminal sequencing and/or mass spectrometry, applied on individual excised protein spots from the 2D gel and compared to a known database of proteins. Immune-blotting and Western blotting may also help in identifying specific proteins [167].

Stoichiometry, affinity and enthalpy of protein–NP interaction may be assessed by isothermal titration calorimetry (ITC) [13,118].

Kinetics of adsorption may be studied by means of surface plasmon resonance (SPR) studies which may provide information on protein–protein interaction kinetics as well as on protein association to and dissociation from NPs [13].

Dynamic light scattering (DLS) has been used to determine the thickness of the protein layer on NPs [169]. A study by Casals et al. [125] provides an example of how DLS may be used to follow the evolution of NPs coating in metallic Au NPs exposed to protein-rich media.

The ζ-potential and the isoelectric point correlate with the degree of coverage of the particle surface and provide indirect information on the changes in surface net charge of the NP after protein adsorption [36]. This technique may be also exploited to obtain information on the degree of reversibility of the adsorption process by measuring the variation of ζ-potential after applying negative gradients at the surface of the NPs–protein conjugate [36].

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) may help in grasping information on the morphological changes occurring at the NP’s surface after protein adsorption. These techniques have also been employed to highlight association of NPs, NP-induced protein fibrillation, etc. [136]. aberration corrected.

### Table 1

<table>
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<td>Identity of the proteins on nanoparticles</td>
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<tr>
<td>(gel filtration)</td>
<td></td>
<td>Rates of exchange with proteins in the medium</td>
<td></td>
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<tr>
<td>Centrifugation associated with MS</td>
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<tr>
<td>Isothermal titration calorimetry (ITC)</td>
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<td>[13,118]</td>
</tr>
<tr>
<td>Surface plasmon resonance (SPR)</td>
<td>Kinetic evolution of the protein corona</td>
<td>Protein–protein interaction kinetics of protein association to and dissociation from nanoparticles</td>
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<tr>
<td>Dynamic light scattering</td>
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<td>Chemical composition of the protein corona</td>
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<td>[167]</td>
</tr>
<tr>
<td>TEM</td>
<td>Modifications of the solid surface</td>
<td>Provides information on the morphology of the NP–protein surface</td>
<td>[1]</td>
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<tr>
<td>SEM</td>
<td>Modifications of the solid surface</td>
<td>Provides information on the morphology of the NP–protein surface</td>
<td>[1,135]</td>
</tr>
<tr>
<td>Total internal reflection fluorescence microscopy (TIRFM)</td>
<td>Biodistribution</td>
<td>Allows to observe single molecule fluorescence at surfaces</td>
<td>[172]</td>
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<tr>
<td>Live cell confocal microscopy</td>
<td>Biodistribution</td>
<td>Allow to follow high resolution imaging of movement through intracellular environments</td>
<td>[173]</td>
</tr>
<tr>
<td>Surface-enhanced Raman scattering (SERS)</td>
<td>Biodistribution</td>
<td>Measures the enhanced Raman scattering of molecules adsorbed on metal surfaces</td>
<td>[174]</td>
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that measures the enhanced Raman scattering of molecules adsorbed and live-cell confocal microscopy [173].

Trp fluorescence by the adsorbed proteins may rely on several techniques. Using both transmission and scanning transmission modes [170,171], TEM instruments with improved resolution are now available: they allow imaging the volumes and surface edge atomic structures of NPs [172] and unfolding processes, based on the change of fluorescence emission of Trp residues and unfolding processes at the surface –[177].

Finally total internal reflection fluorescence microscopy (TIRFM) – which allows to observe single molecule fluorescence at surfaces [172] and live-cell confocal microscopy [173] – which allows high resolution imaging of movement through intracellular environments – are helpful to follow the particle biodistribution. The same objective may be achieved by using surface-enhanced Raman scattering (SERS), that measures the enhanced Raman scattering of molecules adsorbed on metal surfaces [1]. The same techniques also provide information on the state of aggregation of NPs [174].

An accurate monitoring of the conformational changes undergone by the adsorbed proteins may rely on several techniques.

- Far UV-circular dichroism (CD) is useful for monitoring the secondary structure changes of proteins and peptides; near UV-CD provides information on the tertiary structure. Both are used to monitor unfolding processes.
- Trp fluorescence is employed for monitoring conformational changes and unfolding processes, based on the change of fluorescence emission of Trp residues, that is strongly influenced by the hydrophobicity of its chemical environment [175]. In particular, the changes in tertiary conformation may be correlated to the extent of blue shift of the emission spectrum.
- ANS binding allows to detect molten globule states or to highlight the exposure of hydrophobic patches to solvent. These techniques are often used together, in order to get an exhaustive picture of the modifications ongoing in the protein structure during unfolding [164].
- Fourier transform infrared spectroscopy (FTIR) [150,159,176] and Raman spectroscopy are well established methods for the analysis of protein secondary structure, in solution and when adsorbed to surfaces. The frequency shift of mode of vibration of some peculiar bonds, such as the amide I [177] may provide information on the degree of conformational integrity of the adsorbed protein. In fact the presence of a number of Amide I band frequencies has been correlated with the presence of α-helical, antiparallel and parallel β-sheets and random coil structures. FTIR can be a very powerful tool when used in combination with other techniques, like CD.
- Nuclear magnetic resonance (NMR) [178,179] is useful to probe conformational changes through modification of dipolar interactions between distinct parts of the protein. Localized conformational information has been obtained for some adsorbed peptides by using solid-state NMR [180]. A recent study by Calzolai et al. [181] reports that NMR measurements, chemical shift perturbation analysis and dynamic light scattering, allowed to identify the interaction site of ubiquitin with gold NPs at aminoacid scale, in solution.
- AFM has the ability to image adsorbed proteins near native conditions. Silva reports that [182] high resolution topographs of ribonuclease A adsorbed on mica surface have shown the presence of well-defined monolayer two-dimensional structural arrays of oligomers. Determination of the force vs. distance curves by AFM provides a direct understanding of the strength of the molecular interactions between the protein and the surface [183].
- SDSL coupled to ESR spectroscopy (either CW or pulsed) may be successfully employed to get information on the folding state of the protein and on conformational transitions. It allows measuring spin–spin distances and represents a powerful tool for following the mechanism of conformational perturbation at a level of aminoacid residues detail. In addition, it has been employed to grasp information on the orientation of proteins on the surface [36,165].
- A study by Yu et al. [184] on cytochrome c adsorbed on chemically modified gold nanohole arrays demonstrates that SERS may be successfully employed for studying the orientation of proteins adsorbed on nano-patterned metal surfaces.
- Analytical ultracentrifugation and gel permeation chromatography may provide information on the rate of conformational transition occurring in proteins upon adsorption on solid surfaces. In a study on the adsorption of human carbonic anhydrase to silica surfaces [46], analytical ultracentrifugation and gel permeation chromatography data allow to conclude that conversion of the adsorbed protein from the native to non native conformation is slow or readily reversible.
- Activity assays are a useful tool for grasping indirect information on the folding state of a protein and, in particular, of the active site of

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<td>Circular dichroism (CD)</td>
<td>Conformational state of proteins</td>
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<td>[146]</td>
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<td>Trp fluorescence</td>
<td>Conformational state of proteins</td>
<td>Used to monitor conformational changes and unfolding processes, based on the change of fluorescence emission of Trp residues</td>
<td>[1,117,136,167]</td>
</tr>
<tr>
<td>ANS binding</td>
<td>Conformational state of proteins</td>
<td>Allows to detect molten globule states or to highlight the exposure of hydrophobic patches to solvent</td>
<td>[146]</td>
</tr>
<tr>
<td>Infrared (IR) and Raman spectroscopy</td>
<td>Conformational state of proteins</td>
<td>Analysis of protein secondary structure, in combination with other techniques</td>
<td>[150,159,176,177]</td>
</tr>
<tr>
<td>Nuclear magnetic resonance (NMR)</td>
<td>Conformational state of proteins</td>
<td>Used to probe conformational changes in proteins and peptides adsorbed onto solid surfaces at a level of molecular details and to identify protein–NP sites of interaction</td>
<td>[178,181]</td>
</tr>
<tr>
<td>Neutron reflectivity (NR)</td>
<td>Conformational state of proteins</td>
<td>Used to determine conformations of peptide and protein layers adsorbed onto solid surfaces at a level of molecular details</td>
<td>[179]</td>
</tr>
<tr>
<td>Atomic force microscopy</td>
<td>Topography of protein adsorption</td>
<td>Imaging of proteins adsorbed onto solid surfaces; determination of the strength of the molecular interactions between the protein and the surface</td>
<td>[182,183]</td>
</tr>
<tr>
<td>ESR-SDSL</td>
<td>Topology and orientation</td>
<td>Monitoring secondary and tertiary structure changes of proteins and peptides</td>
<td>[36,165]</td>
</tr>
<tr>
<td>SERS</td>
<td>Protein orientation</td>
<td>Molecular recognition processes; unfolding processes at the surface</td>
<td>[184,249]</td>
</tr>
<tr>
<td>Analytical ultracentrifugation and gel permeation chromatography</td>
<td>Kinetic of conformational changes</td>
<td>May provide information on the rate of protein conformational transition occurring upon adsorption</td>
<td>[46]</td>
</tr>
<tr>
<td>Activity assays</td>
<td>Functional state of the adsorbed protein</td>
<td>Allows to correlate the conformational state of the adsorbed protein to activity changes</td>
<td>[135,143,148]</td>
</tr>
</tbody>
</table>
adsorbed proteins, as the enzyme activity is strictly dependent on protein conformation and changes in protein folding may result in loss of activity.

4. Adsorption of nucleic acids

Similar to proteins, nucleic acids exhibit a high tendency to adsorb onto surfaces through electrostatic interactions and coordinative bonds, hydrogen bonds or hydrophobic interactions. The presence of binding sites at well-defined distances at the surface of crystalline solids may lead to specific adsorption nucleic acids and, in some cases, to a molecular recognition. Like for proteins, adsorption may lead to modification of the activity of nucleic acid. However, in the case of DNA and RNA, more severe adverse effects on cell functions are expected if irreversible adsorption or even unfolding occurs.

Direct interaction with DNA (primary direct genotoxicity) is one of the possible modes of action of genotoxic molecular substances as well as particulates [18,185–187].

To get in contact with DNA, particles need to enter cells and cross the nuclear membrane. However, DNA aberrations may also arise from the contact of particles with DNA during mitosis or, alternatively, as a result of interferences with mitotic spindle.

A growing number of studies devoted to the evaluation of genotoxicity of engineered NPs are currently published [18,187,188] since the evaluation of carcinogenic and mutagenic potential is a key area in the risk assessment of new materials.

Not many examples of genotoxicity triggered by the direct interaction of inorganic NPs with nucleic acids are available (Table 3). However, reports of specific interaction may be found in the literature from other scientific area.

The interaction of RNA with solid surfaces is relevant to studies concerning the origin of life. In fact, one of the leading theories is based on the assumption that life started in an RNA-based world. The interaction of RNA with minerals, as catalytic surfaces, might have played a role in the resurgence of the first self-organized systems capable of self-replication. The idea of an origin of life mediated by the surface of minerals was first proposed by Bernal in 1949 [189] and supported by several authors. Studies hunting for a specific interaction between RNA and widespread minerals have been published. Clays were reported to catalyze the formation of RNA oligomers in a regioselective mode [190,191] and to confine and conformationally restrict RNA, similarly to what was observed within the ribosome of living cells [191]. RNA adsorbed on clays was also reported to be less susceptible of degradation by RNase but still able to act as template for molecular replication [192]. Both clays, which are aluminosilicates, and RNA strands are negatively charged and therefore is unlikely that electrostatic interactions could be the driving forces for adsorption. It has been proposed that soluble cations as Ca$^{2+}$ than Na$^+$ could potentially mediate such interaction by screening the negative charge with monovalent cations, or by making divalent cation bridges between the two layers of negative charge formed by the nucleic acid and the mineral surface [191]. Theoretical calculations strengthen the role of Na$^+$ ions in the interaction of DNA and RNA nucleobases with the clay surface [193]. Adsorption of DNA against electrostatic repulsion was also observed on silica. In this case the process was suggested to be driven by dehydration of both the DNA molecules and the silica surface as well as by the formation of intramolecular hydrogen bonds [194]. The possible interaction of clays with DNA is relevant in nanotoxicology. Nanometric clays (nanoclays) are currently used as filler to improve mechanical and thermal resistance of polymers. Exposure of humans to these nanomaterials is therefore likely to occur. Only few toxicity studies have been published on this kind of materials [195–198]. Two of them explore the possible genotoxicity of different type of nanoclays and both report no evidence of adverse effects [196,197].

Stabilization of nucleic acids does not occur at the surface of all minerals. Specificity was not observed on pyrite, a very common mineral composed by iron sulfide (FeS$_2$). In this case DNA-like polymers were shown to adsorb onto pyrite surface through N–Fe interaction involving nitrogen groups of the nucleobases. However, the adsorption was aspecific and inhibited the hybridization process with complementary nucleic acid [199].

DNA molecules are chiral and may interact stereospecifically with chiral surfaces. Tang and co-workers nicely demonstrated a stereoselective interaction between DNA and a chiral surface possibly

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**Table 3**

Summary of studies reporting a primary direct genotoxicity of inorganic particles.

<table>
<thead>
<tr>
<th>NP type</th>
<th>Cell type/model</th>
<th>Localization of particles</th>
<th>Genotoxic effects observed</th>
<th>Mechanism of interaction proposed</th>
<th>Techniques used to evaluate particle-DNA interaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO$_2$ anatase (5 nm)</td>
<td>ICR-mice</td>
<td>Accumulation in liver DNA</td>
<td>Alteration of the secondary structure of DNA, cleavage at the higher dose</td>
<td>Insertion into DNA base pairs and binding to DNA nucleotide through Ti–O or Ti–N bonds</td>
<td>UV/Vis, ICP-MS, EXAFS, CD, agarose gel electrophoresis</td>
<td>[250]</td>
</tr>
<tr>
<td>Au nanoparticles (30 nm), nuclear targeting</td>
<td>Cancer cells</td>
<td>Nucleus</td>
<td>Cytokinesis arrest, failure cell division, apoptosis</td>
<td>Van der Waals forces</td>
<td>AFM</td>
<td>[251]</td>
</tr>
<tr>
<td>Carbon nanoparticles</td>
<td><em>Escherichia coli</em></td>
<td>Extracted DNA</td>
<td>Induction of DNA aggregation</td>
<td>Hydrogen bonding between surface silanol groups and the phosphate-sugar backbone of DNA</td>
<td>Fourier transform infrared spectroscopy</td>
<td>[252]</td>
</tr>
<tr>
<td>Quartz particles</td>
<td>Alveolar epithelial cells</td>
<td>Localization of quartz particles in the nuclei and mitotic spindles</td>
<td>Induction of DNA strand breaks and induction of (8-OHdG)</td>
<td>Direct oxidative damage to DNA</td>
<td>Agarose gel electrophoresis of plasmid DNA after incubation with fibers</td>
<td>[107]</td>
</tr>
<tr>
<td>Asbestos fibers</td>
<td>Different cell lines</td>
<td>Nucleus</td>
<td>Induction of DNA strand breaks and induction of (8-OHdG)</td>
<td>Indirect, formation of intranuclear protein aggregates of topoisomerases</td>
<td></td>
<td>[254]</td>
</tr>
<tr>
<td>SiO$_2$ NPs (50–100 nm)</td>
<td>HEP-2 and RPMI 2650 cells</td>
<td>Nucleus</td>
<td>Inhibition of replication, transcription, and cell proliferation but not cell viability</td>
<td>Direct (Ag$^{+}$ ions) or indirect (oxidative stress via disruption of the mitochondrial respiratory chain)</td>
<td></td>
<td>[255]</td>
</tr>
<tr>
<td>Ag NPs (6–20 nm)</td>
<td>Human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251)</td>
<td>Nucleus and mitochondria</td>
<td>DNA damage, cell cycle arrest in G2/M phase</td>
<td></td>
<td></td>
<td>[255]</td>
</tr>
</tbody>
</table>
through hydrogen bonds [200] giving insights into the stereospecific interaction of cells with surfaces [201].

The immobilization of DNA on different solid supports is an important issue in different fields ranging from medicine to analytical chemistry and molecular electronics. Immobilization may be performed by simple adsorption or by covalent bonding. In such applications DNA strands need to preserve their conformational structure for hybridization with complementary chains and therefore organic linkers are often used to minimize the interaction with the surface.

Au/DNA conjugates find application in biosensing and nanobiotechnological applications [202]. DNA thiolated strands are generally used to synthesize Au/DNA NPs since thiol groups are known to form stable bonds with gold surfaces. DNA unfolding was not observed on Au NPs at low coverage suggesting that intramolecular hydrogen bonds and base stacking which stabilize the double DNA strand are stronger than nonspecific interactions between the bases and the gold surface [203].

Recently a large interest has been focused on DNA/carbon nanotubes conjugates. The remarkable sensitivity of CNT conductivity to the surface adsorbrates allows using CNTs as highly sensitive nanoscale sensors [204]. CNTs are also of large interest in medicine as gene nanocarriers [205,206]. Interaction of DNA with non-functionalized single-walled CNTs (with diameter ranging from 1.2 to 1.6 nm) resulted in DNA wrapping around the tube and structural changes [207,208] similar to those observed in chromosomes during histones-mediated DNA assembly. The same authors studied the interaction of SWCNT with poly-A fragment and found that the interaction occurs through adenine and phosphate groups [209]. Interestingly, some authors recently report that single-walled CNT may recognize particular sequences of single stranded DNA depending upon the chirality of their graphenic structure [210].

Adsorption of nucleic acid on inorganic particles may occur only when the size of particles is larger than the nucleic acid molecule's size. When particles become very small (few nanometers), the interaction with DNA helixes resembles that reported for soluble metals (such as Cr(VI) or Cd) as they fit into the DNA grooves through very size-dependent specific interactions, as demonstrated for Au NPs [211] and for C60 fullerol [212].

Finally, when an inorganic particle approaching nucleic acids exhibits reactive sites at the surface which may generate radical species, oxidative damage of nucleic acid may occur. This type of interaction will be discussed in the next session.

5. Surface driven oxidative damage to biomolecules

Reactive oxygen species (ROS•) is a term that includes oxygen-based free radicals such as superoxide anion (O2•−), hydroxyl (HO•) and biomolecules-derived species as alkylperoxyl (RO2•) and alkoxyl (RO•) radicals. Reactive non-radical species such as hydrogen peroxide (H2O2) and singlet oxygen (1O2) are also considered ROS [213]. These reactive species are physiologically produced in cells from several different sources and cover many important physiological functions [214].

ROS have been demonstrated to be involved in many signal transduction pathways. However, increased ROS may also be detrimental leading to cell death or to acceleration in aging and age-related diseases. Increased ROS levels cause random damage to proteins, lipids and DNA. In addition to these effects, a rise in ROS levels may also represent a stress signal that activates specific redox-sensitive signaling pathways [214].

Oxidative stress, resulting from an imbalance between production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and antioxidant defenses, is considered an important mechanism of particle-induced health effects [215,216].

Particles may generate reactive oxygen species by a direct mechanism (surface-derived ROS), or by an indirect one relying on the alteration of mitochondrial functions or the activation of cells of the immune systems (cell-derived ROS) [54,112,217]. Oxidative stress may also be a consequence of Leahing of redox-active ions like iron [218] or of the impairment of ROS homeostasis by depletion of endogenous antioxidants [89,110,112,219,220].

Finally, damage may follow the direct reaction of biomolecules with the surface. In Fig. 4 the different paths which may lead to particle-induced oxidative damage are summarized.

Internalization and localization of NPs inside cells play a key role in the particle-derived oxidative stress since free radicals and singlet oxygen are extremely unstable. Therefore in order to trigger oxidative damage they need to be generated close to the target molecule.

Both generation of ROS and direct damage to biomolecules are related to the existence of sites accessible to the fluid and able to undergo redox cycling. The chemical nature of these reactive sites depends upon the type of solid (see Section 2.5) [9,20,54,221].

Covalent solids as silica or carbon nanotubes may exhibit radical defects (dangling bonds) generated by the homolytic rupture of the covalent bonds when ground. However, while in crystalline silica forms, e.g. quartz, such radical species are known to promote the generation of free radicals in biological fluids [54] and to cause oxidative damage to biomolecules [222], in carbon nanotubes they have been related to a scavenging activity toward free radicals [68,69]. Radical defects are not present on amorphous silica forms which are therefore essentially unable to cause direct oxidative damage.

Direct oxidative damage may also derive from redox reactions involving redox-active metal ions exposed at the surface. Such ions may be present as contaminants [64,217], may derive by adsorption of ions from fluids, or may be present in the structure of the solids, e.g. in asbestos, pyrite or iron oxides [57,61,65,221,222,223]. As discussed in Section 2.5, the oxidative potential depends upon the coordinative and oxidative states of exposed ions. In some cases a strong reactivity has been observed. For example asbestos fibers were shown to induce strand breaks in supercoiled plasmid DNA [88,107,224–226] and lipid peroxidation [227]. Similar reactivity toward DNA was observed in pyrite [223]. Alternatively oxidative stress may derive from metals leached from the surface by endogenous chelators [228].

Semiconducting materials are intrinsically able to exchange electrons with molecular entities which come into contact with the surface, if activated by light or heat.

One of the most common semiconductors is TiO2. As described in the Section 2.5 under sunlight illumination TiO2, when in contact with oxygen or water, generates large amounts of radical species such as O2•−, HO• and 1O2. As these species are highly aggressive, they may efficiently degrade organic molecules or act as bactericidal agents. Therefore, TiO2 has been proposed as catalyst for the degradation of pollutants in waste waters [229,230] or as antibacterial and self-cleaning components in coatings and textiles [231,232]. At the same time, direct DNA and RNA damage [233,234] and lipid peroxidation [16,17,109] caused by UV-irradiated TiO2 have been reported.

Depending upon the method of synthesis TiO2 particles may expose at the surface few persistent active centers which are still reactive in the dark. Recently, some of us reported the ability of TiO2 powders to generate free radicals form organic molecules in the absence of illumination [73]. This reactivity was related to the presence of persistent Ti3+ species and electron holes.

The capacity of CdSe quantum dots to induce oxidative damage to DNA was also reported under photoactivation or in the dark [76].

Conductive indium-doped tin oxide (ITO) powders was also reported to generate free radicals [111]. However the nature of the reactive sites has not been clarified.

Carbon-based NPs and multilwalled carbon nanotubes are intrinsically unable to generate free radicals. At the opposite they may act as antioxidant by scavenging free radicals [67–69,235] and therefore may potentially protect biomolecules from oxidative damage.

C60 are known to quench various free radicals, behaving as a “free radical sponge” C60 [77,236]. Exploiting this properties, fullerences have been proposed in antioxidant therapy as neuroprotective agents [237]. However, if excited with UV light they may be promoted to a to
triplet state which may transfer energy to oxygen to generate singlet oxygen \( (^{1}\text{O}_2) \) [77] and for this reason they have been proposed in photodynamic therapy of tumors.

6. Role of the interactions with biomacromolecules in the in vivo response to nanoparticles

The complexity of the processes occurring at the solid/biofluid interface is particularly high in the case of nanoparticles. NPs are in fact potentially able to distribute through the organs and in the various cellular and extracellular compartments, thus implying that they may experience different conditions in terms of pH, ionic strength and fluid composition. At the same time, NPs may come into contact with macromolecules as nucleic acids unaccessible to bulk inorganic materials and even micrometric particles.

The fate of a NP in the body and the adverse/beneficial responses to them is largely related to the events occurring at the interfaces [136]. In particular the following events need to be considered: i. modification of the surface properties affecting biodistribution; ii. perturbation of the composition of biological fluids; iii. perturbation of the biomacromolecule physiological functions; iv. generation of bioactive protein/NP adducts.

Physico-chemical properties as size, charge and hydrophilic degree have been reported to modulate biodistribution, bioaccumulation and biocompatibility of NPs [22,238–240]. According to Aggarwal et al. [167] and Lynch et al. [136], it is the NP–protein corona, whose composition is largely determined by the physico-chemical properties of NPs, that actually determines both biodistribution and the final subcellular location of a specific NP.

An aspect that deserves attention, as far as protein adsorption on NPs is concerned, is the effect of protein depletion in fluids following adsorption. Several in vitro studies report changes in the biological activity as a consequence of the modified composition of cellular media interacting with NPs. Data are available for metal oxide, polystyrene NPs and carbon nanotubes [35,131,241–244]. Similarly, depletion of proteins may occur in biofluid. For examples, selective binding of supernatant proteins from bronchoalveolar lavage fluid by double-walled nanotubes has been reported by Salvador-Morales et al. [245]. The authors show that a chronic level of exposure to carbon nanotubes may result in sequestration of surfactant proteins A and D, with consequently higher probability for the insurgence of pathological states.

In the previous chapters the occurrence of modification of the enzymatic activity of enzymes or perturbation of the activity of nucleic acids following adsorption (Sections 3.3 and 4) and of oxidative damage (Section 5) has been discussed. How such modifications overcome the capacity of cells to re-establish their physiological (or pathological) state needs to be clarified.

Finally, as far as the adverse effects that may arise from exposure to NPs are concerned, it is worth to remember that the response of the immune system to NPs has been related to the generation of bioactive proteins/NP adducts. Protein adsorption may in fact lead to the exposure of receptors involved in the various paths driving to inflammation [246]. At the same time NPs may improve antigenicity of conjugated weak antigens thus serving as adjuvants [247].

7. Conclusions

A deep understanding of the mechanisms driving the interaction between biological fluids or cells constituents and surfaces is instrumental for designing strategies apt to prevent the toxicity and premature clearance of NPs used in diagnosis and therapy, and for avoiding adverse reactions to materials used as implants or toxic effects that may follow the accidental exposure of organisms to nanomaterials.
Conclusive perspective of the effects induced on macrobiomolecules by the most relevant physico-chemical surface features.

<table>
<thead>
<tr>
<th>Physico-chemical surface feature</th>
<th>Type of interaction force</th>
<th>Effect on macrobiomolecules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Affinity changes?</td>
</tr>
<tr>
<td>Hydrophobic interaction</td>
<td>Increase with protein softness</td>
<td>Correlate with protein softness</td>
</tr>
<tr>
<td>Hydrogen bonding</td>
<td>Secondary structure changes may occur</td>
<td></td>
</tr>
<tr>
<td>Surface charge</td>
<td>Driven by coulombic interactions</td>
<td>Correlate with protein charge distribution</td>
</tr>
<tr>
<td>Coordinative bonds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reactive sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size/curvature</td>
<td>Higher curvatures may prevent efficient close packing of the proteins</td>
<td>Generally induced by lower curvature. Some protein–dependent exceptions reported</td>
</tr>
<tr>
<td>Chirality</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stereoselective molecular recognition of protein and nucleic acids</td>
<td></td>
</tr>
</tbody>
</table>

The physico-chemical properties of the surface definitely play a pivotal role in modulating the various possible processes at the interface between biological fluids and solid surfaces; the peculiar features and behavior of biomacromolecules further complicate the model systems; it has to be born in mind that adsorbed onto solids. These evidences come mainly from studies on both real and model systems (where well characterized surfaces interact with proteins with well-defined properties) with the contribution of theoretical calculations.

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