CHAPTER 1
Protein Nanoparticle Interaction in a Nutshell

Abstract: Nanomaterials have been used by mankind for centuries without knowing the dimensions of the materials they used. However, it is only the last ten years or so that the subject of nanoscience and technology has gained tremendous attention. From the biological and medical applications viewpoint, the primary interest in nanoparticles stems from the fact that they are small enough to interact with biomolecule (protein, DNA, carbohydrate). Interestingly, the interaction between nanomaterials and bio-molecules results in the formation of a biological corona on the NP’s surface that is quite dramatically different from that adsorbed on a flat surface of the same bulk material. In the present chapter a brief description has been made about nanoparticle, its characterization, and interaction with protein. This knowledge is important from the perspective of safe use of nano materials.

1.1 Introduction
Nano-world deals with tiny (extremely small) objects which have dimension in nanometer scale (at least in one dimension). The science of nonmaterial deals with their generation and properties and the phenomenon exhibited by them due to their small size. Nano object can be spherical, wiry, tubular or needle-like. Although nanoscience and nanotechnology have recently gained tremendous attention, they have been known for a long time. The subject has attracted interests because of the application in various fields, such as chemical and textile industries, material fabrication, electronics and nanomedicine.1

1.1.1. Nano Definition
Greek meaning of nano is “dwarf”, but by scientific definition “nano” is one-billionth of a meter (10⁻⁹ m) (Figure 1). Nanometer is the scale used to measure object in the nanoworld. Comparing an object with a diameter of a nanometer to that of meter is like comparing the size of a small marble to the size of the earth.¹

A nanometer object can be easily formed if one lines up 10 hydrogen atoms next to each other as the diameter of each hydrogen atom is ~0.1 nm. The bond distance between carbon atoms in diamond is 0.154 nm. It should now be absolutely clear that we are dealing with enormously small objects when we enter the nanoworld.
How Small is a nm?

1 μm = one millionth of a meter
1 nm = one billionth of a meter
≈ 1/50,000 thickness of a hair!
≈ a string of 3 atoms

If we shrink all distances by 110,000,000,000 X, the sun and earth would be separated by 1 m, a football field would be 1 nm.

Figure 1. A cartoon representation of “nanometer”.

1.1.2. Nanomaterials

Nanomaterials are of various types. Small nano-particles are known as quantum dots. These are the materials which posses nano-dimension in all three directions. Most of them are spherical and having diameter in 1-50 nm range. Nanoparticle of a variety of materials such as metal, metal oxide, semiconducting and magnetic materials have been prepared. Apart from quantum dots, nanomaterials can be further sub-divided into two major classes.

1.1.2.1. One Dimensional Nanomaterial. These materials are long, but their diameters are extremely small within a few nanometers. Nano-wires and nanotubues belong to this category and such structures have already being synthesized from metal oxide and other materials.

1.1.2.2. Two Dimensional Nanomaterial. In addition to quantum dots, nanowires and nanotubes, there are another class of nanomaterials which are two dimensional. Nanofilms, nanosheets or nanowalls belong to this category. The area of any two-dimensional nanomaterial can be large, (several mm²) but their thickness should be very small (within a few nanometers).
1.1.3. Properties of Nanomaterial

The main characteristic feature of nanomaterial is that their size determines their properties. In real world material, physical and chemical properties do not alter with the size, but in nano world property is largely dependent on particle size. In case of nanomaterial if one goes on reducing the size of a material to that of a very, very small particle, say, 1 nm, then all the atoms forming the particle will be on the surface; increasing the particle size will result in a decrease in the number of particles at surface. It is possible that magnetic material in real world lose its magnetic property when its size is reduced to nanometer range. A classic example of such is gold. Gold is non-reactive in nature and gives typical shines as a metal. However, if one can reduce the particle size to nanometer scale, it is no longer metallic, or shiney and becomes highly reactive in nature. The melting point of solid changes when we reduce its size to nanometer dimension. For example, small particles of gold melt at a much lower temperature than the bulk gold.

All the above phenomena are attributed to quantum confinement effect. What is quantum confinement effect? A particle having a size of 50 nm and above, behaves almost like a bulk material. However, if the size is reduced to 10-50 nm, the property of the material will vary linearly with its size. If one further reduces its size, then some unusual properties would be displayed due to quantum confinement effect. In this region, electrons confined in a very small volume or box exhibit energy, which depends on the length of the box.\footnote{1}

1.1.4. Characterization of Nano Object

It should now be abundantly clear that objects of nanometer size cannot be seen by naked eye. Neither can it be visualized using any ordinary microscope. To characterize nanoparticle we need some sophisticated microscope like TEM (Transmission Electron Microscope), SEM (Scanning Electron Microscope) or AFM (Atomic Force Microscope) which have very high resolution (Figure 2). TEM and SEM have been in existence for decades. TEM is like photography but done in vacuum with an electron beam of wavelength smaller than the size of an atom (~ 0.1Å).

1.1.4.1. Transmission Electron Microscope (TEM). The TEM works on the same basic principal as the light microscope. However, TEM uses electron beam instead of light (photon). As the TEM uses electron instead of light, the much lower wavelength of the
electron provides very high resolution and makes it possible to see the material at atomic and sub-atomic level. Also, TEM uses strong electromagnetic lenses to focus the electrons into a thin beam. This narrow beam of electrons is transmitted through an extremely thin specimen, an image of the specimen is formed and magnified, and the image appears on a screen. Sometimes it is also directed to appear on a layer of photographic film or detected by a sensor. In TEM, the electron beam pierces through the thin slice of matter before capturing the image. So specimen for TEM must be very thin and able to withstand the high vacuum present within the instrument. The thickness for biological specimen must not exceed 1 μM and the specimen should keep at liquid nitrogen before use to withstand the vacuum in the microscope. The resolution and other features of TEM can be improved by incorporating additional stages and detectors on the same microscope. A TEM can also be modified as a scanning Transmission Electron Microscope or STEM. With a good TEM, we can see objects at 2 or 3 Å resolution (1 Ångstrom is 0.1 nm).

This technique requires extremely thin and electron transparent sample. Sample preparation is so tedious, that there are always chances of artifact formation. There is also the possibility of sample bleaching (damaged) by the high energy electron beam.

1.1.4.2. Scanning Electron Microscope (SEM). In SEM, a mono-energetic electron beam is focused to a point on the sample surface through pairs of scanning coils in the objective lens. The beam is deflected horizontally and vertically so that it scans in a raster fashion over a selected rectangular area of the sample surface. Secondary electron emerges from the illuminated area and they carry the information regarding the topography of the surface. The brightness of signals depends on the number of secondary electrons reaching the detector. The difference in signal intensity from the different locations on the specimen allows an image to be formed. SEM has a large depth of field. This allows a large amount of the sample to be focused at a single shot. The samples need to be conductive. For non-conductive sample a coating of conductive material should be laid before the experiment. One can see an object ~50 nm or so in most of the SEM instruments.

Many specializations/modifications are possible with a working SEM column. X-Ray which is generated by the interaction of electrons with the sample can be detected
in a SEM equipped with energy dispersive X-ray spectroscopy (EDX). Elemental composition can be obtained using this method. EDX relies on the investigation of an interaction of some source of X-ray excitation and a sample. Its characterization capabilities are due in large part to the fundamental principle that each element has a unique atomic structure allowing unique set of peaks in its X-ray spectrum. To stimulate the emission of characteristic X-rays from a specimen, a high-energy beam of charged particles such as electrons is focused into the sample being studied. At rest, an atom within the sample possesses ground state (or unexcited) electrons in discrete energy levels or electron shells bound to the nucleus. The incident beam may excite an electron in an inner shell, ejecting it from the shell while creating an electron hole where the electron was. An electron from an outer, higher-energy shell then fills the hole, and the difference in energy between the higher-energy shell and the lower energy shell may be released in the form of an X-ray. The number and energy of the X-rays emitted from a specimen can be measured by an energy-dispersive spectrometer. As the energy of the X-rays is characteristic of the difference in energies between the two shells, and of the atomic structure of the element from which they were emitted, this allows the elemental composition of the specimen to be measured.

1.1.4.3. Atomic Force Microscopy (AFM). The basic working principle of AFM is based on measuring the force between atoms. A typical AFM consists of microfabricated cantilever with a sharp tip. The tip is brought close enough to the surface under examination, to start feeling the local attractive and repulsive force. The tips deflection rate is varied depending on the surface nature. A laser beam reflects the deflection off the backside of the cantilever into a quadrant of photo detectors. Using these data, deflection can be monitored and an image of surface can be generated. The tip of AFM is generally made of silicon nitride and normally mounted as an inverted pyramid on a cantilever arm from the probe. The radius of curvature of the tip is only a few nanometers. This allows one to precision control the tip motion to approach a surface and scan a region of interest. The sharp tip scans the samples point by point. To avoid air borne or mechanical disturbance in this sophisticated instrument, the microscope as a whole is mounted in a vibration free platform. Apart from these, there are certain other techniques which are generally used for NP characterization; these are mentioned in Figure 2.
1.1.4.4. Fourier Transform Infrared Spectroscopy (FTIR). Fourier transform infrared (FTIR) spectroscopy is a useful method for getting information about the surface properties of NP. FTIR is an effective analytical tool for the identification of surface functionalization (capping) present within nanoparticle. The principle of FTIR is that the gas/solid/liquid to be analysed is led through a cuvette with an IR light source at one end that is sending out scattered IR light, and a modulator that "cuts" the infra red light into different wave lengths. At the other end of the cuvette a detector is measuring the amount of IR light to pass through the cuvette. By data processing, Fourier Transformation mathematics is used to turn the measured absorption values into concentrations for the analysed samples. Because chemical bonds absorb infrared energy at specific frequencies (or wavelengths), the basic structure of compounds can be determined by the spectral locations of their IR absorptions. The plot of a compound's IR transmission vs. frequency is its "fingerprint", which when compared to reference spectra identifies the material. FTIR spectrometers offer speed and sensitivity impossible to achieve with earlier wavelength-dispersive instruments. The FTIR spectroscope accessory allows spectra from a few nanograms of material to be obtained quickly. It relies on the fact that most molecules absorb light in the infra-red region of the electromagnetic spectrum; this absorption corresponds specifically to the bonds present in the molecule. The sample is irradiated by a broad spectrum of infra-red light and the level of absorbance at a particular frequency is plotted after Fourier transforming the data. The resulting spectrum is characteristic of the organic molecules present in the sample. This instrument covered the wavelength range from 2.5 μm to 15 μm (wavenumber range 4000 cm⁻¹ to 660 cm⁻¹) and used for the identification and quantification of organic species. The lower wavelength limit was chosen to encompass the highest known vibration frequency.

1.1.4.5. X-Ray Diffraction (XRD). X-ray powder diffraction (XRD) is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The analyzed material should be finely ground and homogenized as the pre-condition of measurement. X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate, and directed toward the sample. The interaction of the incident
rays with the sample produces constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law \((n\lambda=2d \sin \theta)\). This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a crystalline sample. These diffracted X-rays are then detected, processed and counted. By scanning the sample through a range of 20 angles, all possible diffraction directions of the lattice should be attained due to the random orientation of the powdered material. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each mineral has a set of unique d-spacing. Typically, this is achieved by comparison of d-spacings with standard reference patterns. Copper is the most common target material for diffraction, with CuKa radiation = 1.5418 Å. These X-rays are collimated and directed onto the sample. As the sample and detector are rotated, the intensity of the reflected X-rays is recorded. When the geometry of the incident X-rays impinging the sample satisfies the Bragg Equation, constructive interference occurs and a peak in intensity occurs. A detector records and processes this X-ray signal and converts the signal to a count rate which is then output to a device such as a printer or computer monitor.

Common Methods for Nanoparticle Characterization

![Diagram](image_url)

**Figure 2.** A schematic diagram representing methods to characterize nanoparticle.
1.2. Nanotechnology

Nanotechnology refers to a field of applied science and technology whose basis is nanoparticles. The nanotechnology stretches across the whole spectrum of science, touching medicine, physics, engineering and chemistry (Figure 3).

![Figure 3. Application of nanomaterial in different domains of science and technology.](image)

The rapid development of nanotechnology in the past decades offers wide prospects in using micro- and nanoscale materials in different areas of industry, technology, and medicine. This is why understanding the mechanisms of interactions between nanoparticles (NPs) and living matter (Figure 4) is crucial for the safe implementation of nanotechnologies in various fields.

**What Happens to NP in Biological System?**

![Figure 4. Possible consequences when nanomaterial is exposed to biological system.](image)

Therefore, we need to understand how NPs enter the body, tissues, and cells, where they go when they get there, and what are the consequences of them being there. Furthermore,
if we want to fully understand the biological impact of NPs, we should address all the complicated molecular aspects of nano-bio interactions.  

### 1.3. Mechanisms of Protein Adsorption

When a nanomaterial enters a physiological environment, it is initially encapsulated by high concentrations of free protein. Proteins migrate to the nanomaterial surface either by diffusion, or by traveling down a potential energy gradient. Once in the vicinity of the surface, protein adsorption occurs spontaneously only if it is thermodynamically suitable; in other words, if:

\[
\Delta G_{\text{ads}} = \Delta H_{\text{ads}} - T\Delta S_{\text{ads}} < 0
\]

where \(\Delta G_{\text{ads}}\), \(\Delta H_{\text{ads}}\), and \(\Delta S_{\text{ads}}\) are the changes in Gibbs free energy, enthalpy, and entropy, respectively, during the time of adsorption, and \(T\) is the temperature. There are a number of transient interactions that actually contribute to favorable changes in enthalpy \((\Delta H_{\text{ads}} < 0)\), or entropy \((\Delta S_{\text{ads}} > 0)\), including the formation of covalent and noncovalent bonds, rearrangement of interfacial water molecules, or conformational changes in either the protein or near the nanomaterial surface. The precise mechanisms involved during adsorption and their relative contributions depend on the nature of protein and the physicochemical properties of the nanomaterial.

During adsorption, interactions between the protein and nanomaterial typically occur through a portion of the protein known as a ‘domain’. For example, adsorption of high molecular weight kininogen (popularly known as Fitzgerald factor involved in blood coagulation) to iron oxide nanoparticles occurs through ‘domain 5 (D5)’ of HMWK. D5 is histidine-rich and interacts with iron oxide via its imidazole sidechain. Adsorption does not necessarily happen through a single domain, but may instead involve the simultaneous interaction of multiple domains of the same protein with the nanomaterial surface. The net binding energy of an adsorption event \((\Delta G_{\text{ads}})\) determines the stability of the protein-nanomaterial complex. Proteins that adsorb with a large \(\Delta G_{\text{ads}}\) have a low probability of desorption, and tend to stay associated with the nanomaterial, whereas proteins that adsorb with a relatively low \(\Delta G_{\text{ads}}\) easily desorb and return to solution. Nanomaterials that are charged or hydrophobic tend to form strong interactions with proteins than those that are hydrophilic (Figure 5).
1.4. Factors that Influence Protein-Nanoparticle Interaction

There are various factors that affect the protein-nanoparticle complex formation, such as the physiochemical properties of the NPs themselves, the composition of the biological fluid, and the resulting protein corona with possible reversible/irreversible conformational changes of the proteins after interaction with NPs. In this section, the most important physical-chemical characteristics of the NPs influencing the nature of NP-protein coronas are discussed.

<table>
<thead>
<tr>
<th>Force</th>
<th>Origin and nature</th>
<th>Range (nm)</th>
<th>Possible impact on the interface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodynamic interactions</td>
<td>Convective drag, shear, lift and Brownian diffusion are often hindered or enhanced at nanoscale separations between interacting interfaces</td>
<td>$10^2$ to $10^5$</td>
<td>Increase the frequency of collisions between nanoparticles and other surfaces responsible for transport</td>
</tr>
<tr>
<td>Electrodynamic interactions</td>
<td>VDW interactions arising from each of the interacting materials and the intervening media</td>
<td>1 to 100</td>
<td>Universally attractive in aqueous media; substantially smaller for biological media and cells owing to high water content</td>
</tr>
<tr>
<td>Electrostatic interactions</td>
<td>Charge-charge interactions between proteins or repel other interfaces</td>
<td>1 to 10</td>
<td>Overlapping double layers are generally repulsive as most materials acquire negative charge in aqueous media, but can be attractive for oppositely charged materials</td>
</tr>
<tr>
<td>Solvent interactions</td>
<td>Lyophilic materials interact favourably with solvent molecules</td>
<td>1 to 10</td>
<td>Lyophilic materials are thermodynamically stable in the solvent and do not aggregate. Lyophobic materials are spontaneously expelled from the bulk of the solvent and forced to aggregate or accumulate at an interface</td>
</tr>
<tr>
<td>Steric interactions</td>
<td>Polymers adsorbed to inorganic particles or biopolymers expressed at the surfaces of cells give rise to spring-like repulsive interactions with other interfaces</td>
<td>1 to $10^5$</td>
<td>Generally increase stability of individual particles but can interfere in cellular uptake, especially when surface polymers are highly water-soluble</td>
</tr>
<tr>
<td>Polymer bridging interactions</td>
<td>Polymers adsorbed to inorganic particles or biopolymers expressed at the surfaces of cells containing charged functional groups can be attracted by oppositely charged moieties on a substrate surface</td>
<td>1 to 100</td>
<td>Generally promote aggregation or deposition, particularly when charge functionality is carboxylic acid and dispersed in aqueous media containing calcium ions</td>
</tr>
</tbody>
</table>

Table 1. Physicochemical forces involved in protein-nanoparticle interaction.

1.4.1. Effect of NP Size

The curvature of the NP surface, which is dependent on the size of the NPs, can influence on the adsorption of biomolecules, which may undergo different conformational changes from their native structure relative to those observed for the proteins adsorbed.
onto flat surfaces of the same material. This was demonstrated when gold NPs of varying sizes (i.e., 5, 10, 20, 30, 60, 80 and 100 nm) were incubated sequentially with common blood proteins, including albumin, fibrinogen, γ-globulin, histone, and insulin.\textsuperscript{11} The gold NPs had significant interaction with each of the proteins; the binding constant and the degree of cooperativity of protein-NP binding (i.e., the Hill constant, n), depended mostly on the NP size. Determination of the binding association constant, k, for all protein-NP pairs should give an idea of the exchange rates of the proteins over nanoparticle surface. For instance, the k values were determined for the binding of common blood proteins to gold NPs with a size range between 5 and 100 nm. According to the results, the k values increased gradually with increasing NP size between 5 and 60 nm.\textsuperscript{11} In contrast, the k values showed an irregular trend for gold NPs in the size range of 60-100 nm; the authors claimed that the conformational state of the adsorbed proteins was responsible for such irregular changes. Furthermore, the k values for the binding of chymotrypsin to amino acid capped gold NPs and binding of citrate-coated gold NPs to bovine serum albumin were also determined and were found to be dependent on NP sizes.\textsuperscript{12,13} Cedervall et al. used NPs with different hydrophobicities and probed the protein adsorption on their surfaces. According to their results, there was a considerable difference in the degree of protein surface coverage of the NPs depending on their size, with a larger degree of protein coverage on the larger particles.\textsuperscript{14} By decreasing the NP size from 200 to 70 nm, a curvature-induced suppression of the protein adsorption was observed. It has been also confirmed by fluorescence quenching of histone by gold NPs that the thickness of the protein corona gradually increased with increasing size of the NPs.\textsuperscript{9,11}

1.4.2. Effect of NP Shape
The shape of NP has a great impact not only on its physiochemical characteristics but also on the way proteins adsorb onto its surface and consequently on the way that cells interact with it. For instance, the shape of gold NPs has a considerable influence on their interactions with cell layers; more specifically, a peak in cell association for 50 nm spherical gold NPs is reduced drastically by changing the shape of the NPs to the rod geometry.\textsuperscript{15} It had been suggested that rodshaped and cylindrical nanoparticles experience longer association times than spherical nanoparticles, as greater
thermodynamic forces were involved in the interaction. Particle shapes and aspect ratios are also play an important role in determining nanomaterial safety.

1.4.3. Effect of NP Crystallinity

NP crystallinity also has some role in protein nanoparticle interaction process. Earlier Mahmoudi et al. have shown that a high amount of poly (vinyl alcohol) (PVA) as a coating agent, usually with a polymer/iron mass ratio greater than 3, reduces the crystallinity of supermagnetic iron oxide nanoparticle (SPION) and affects the cytotoxicity profile of the obtained materials due to the effect of the crystallinity on the protein adsorption profile on the particle surfaces.16

1.4.4. Effect of Surface Defects

Literature evidence suggests that surface defects present on nanoparticle surface have the ability to modulate protein nanoparticle adsorption process.17 For example, Nareoja et al. investigated the possible effects of surface defects on the denaturation of antibodies in sandwich immunoassay. Their first aim was to find out if surface defects can be attributed as an origin of nonspecific binding of the bio-conjugated nanoparticle label on the binding surface.18 Therefore, microtiter well surfaces and their patterns were studied with scanning probe microscopy (atomic force microscopy, AFM) and fluorescence microscopy. The second line of approach was to determine to what extent denaturing of surface-bound and soluble antibodies caused unwanted nonspecific binding. According to their observation neither surface defects nor denaturation of capture protein caused any increase in the nonspecific signal in a sandwich immunoassay utilizing bioconjugated nanoparticle labels.

1.5. Effects of NP Surface Properties

1.5.1. Effect of Charge. The surface charge of NPs is very crucial for defining the composition of the protein corona and also has an impact on their subsequent biodistribution. For example, some positively charged NPs are rapidly identified by opsonins,19 leading to the removal of the particles by the reticuloendothelial system (RES) and mononuclear phagocytic systems, with their final destination being the liver and spleen, resulting in a significant decrease in the application yield of these NPs. Many NPs are stabilized in physiological conditions by functionalization with negatively charged groups (carboxyl, sulfate, phosphate, etc.), resulting in a negative ζ potential of
about 30-50 mV in physiological buffer. Despite their negative surface charge, these NPs are immediately covered by plasma proteins when they are in contact with biological fluids, resulting in a significant reduction of their ζ potential to about 5-10 mV upon formation of the NP-corona complexes. Thus, the colloidal stability of these complexes cannot be solely due to electrostatic effect, but it is closely connected to the complex nature of the protein corona.20

1.5.2. Effects of Smoothness/Roughness. Surface effects can also be changed by nanoscale surface roughness (that is, local protrusions or depressions with radii smaller than that of the particle).21 Indeed, small-surface radius dictates the strength of nanoparticle-cell interactions. Simulations of NPs interacting with synthetic membranes suggest that nanoscale surface roughness greatly minimizes repulsive interactions (for example, electrostatic, hydrophilic), thereby promoting adhesion, which might translate into easier engulfment by cells.6

1.5.3. Effects of Hydrophobicity/Hydrophilicity. A clear correlation between the affinities of biomolecules (e.g., proteins) for the surface of NPs and the extent of structural changes has been observed.22 Due to their high affinities for hydrophobic surfaces, proteins adsorbed on such surfaces may have a less native-like structure (than when adsorbed on hydrophilic surfaces) leading to severe protein denaturation.23 In addition, NP surfaces may capture proteins depending on their isoelectric points in a rather narrow pH range. It has also been demonstrated that an increase in electrostatic interaction is generally accompanied by a reduction in the modification of the native structure (Figure 6).24

Figure 6. Impact of electrostatic and hydrophobic interaction on protein structure.
1.5.4. Effect of Functional Groups and Targeting Moieties. To investigate the role of functional group on protein-nanoparticle interaction, Ehrenberg and co-workers used cultured endothelium cells as a model for vascular transport of polystyrene NPs decorated with various functional groups (i.e., carboxyl, amidine, amine, lysine, cysteine, methyl and PEG). According to their observation the capacity of the various NP surfaces to adsorb proteins was indicative of their tendency to associate with cells. They concluded that NP-cell association was not dependent on the identity of the adsorbed proteins. Furthermore, quantification of the adsorbed proteins showed that high-binding NPs were maximally coated within seconds to minutes, indicating that proteins on the surface of NPs could mediate cell association over much longer time scales.

1.6. Structure of ZnO Nanoparticle
Zinc oxide is an inorganic compound with the formula ZnO. It usually appears as a white powder, nearly insoluble in water. In materials science, ZnO is often called a II-VI semiconductor because zinc and oxygen belong to 2 and 6 groups in the periodic table, respectively. This semiconductor has several favorable properties: good transparency, high electron mobility, wide bandgap, strong room temperature luminescence, etc. In nature ZnO can exists in three types crystal structures: wurtzite, zinc blende and rocksalt (Figure 7).

Figure 7. The three different structural forms of ZnO that exists in nature.
At ambient conditions, the thermodynamically stable form of ZnO is the wurtzite structure, in which every zinc atom is tetrahedrally coordinated with four oxygen atoms. The zinc blende form can be stabilized by growing ZnO on substrates with cubic lattice structure. The rocksalt NaCl-type structure is only observed at relatively high pressures.
~10 GPa. The hexagonal and zinc blende ZnO lattices have no inversion symmetry (reflection of a crystal relative to any given point does not transform it into itself). This and other lattice symmetry properties result in piezoelectricity of the hexagonal and zinc blende ZnO, and in pyro-electricity of hexagonal ZnO. The hexagonal structure has a point group 6 mm (Hermann-Mauguin notation) or $C_{6v}$ (Schoenflies notation), and the space group is $P6_3mc$ or $C_{6v}$. The lattice constants are $a = 3.25 \, \text{Å}$ and $c = 5.2 \, \text{Å}$; their ratio $c/a \sim 1.60$ is close to the ideal value for hexagonal cell $c/a = 1.633$. As in most II-VI materials, the bonding in ZnO is largely ionic, which explains its strong piezoelectricity (Figure 8). Due to this ionicity, zinc and oxygen planes bear electric charge (positive and negative, respectively). Therefore, to maintain electrical neutrality, those planes reconstruct at atomic level in most related materials, but not in ZnO - its surfaces are atomically flat, stable and exhibit no reconstruction. This anomaly of ZnO is not fully explained yet.

ZnO has a relatively large direct band gap of ~3.3 eV at room temperature; therefore, pure ZnO is colorless and transparent. Advantages associated with a large band gap include higher breakdown voltages, ability to sustain large electric fields, lower electronic noise and high temperature and high power operation. The bandgap of ZnO can further be tuned in the range of ~3-4 eV its alloying with magnesium oxide or cadmium oxide (Figure 8).

**ZnO: A Versatile Material**

- II-VI compound semiconductor:
  - Direct bandgap, with $E_g = 3.32 \, \text{eV}$.
  - Bandgap engineering: 3.3 eV to 3.9 eV.
- Multi-functional:
  - Hexagonal wurtzite class crystal $\Rightarrow$ piezoelectricity with large coupling coefficient.
  - Large and fast photoconductivity $\Rightarrow$ optical sensing.
  - Al or Ga doping $\Rightarrow$ transparent conductive oxide.
  - Li & Mg doping $\Rightarrow$ ferroelectric.
  - Alloyed with Mn $\Rightarrow$ magnetic oxide semiconductor.
- Integrate electrical, optical and piezoelectrical properties $\Rightarrow$ Efficient for sensor technology.
- Biocompatible and highly stable.

**Figure 8.** Summary of different properties of ZnO nanoparticle.
Most ZnO has $n$-type character, even in the absence of intentional doping. Inherent defect centres, such as oxygen vacancies and zinc interstitials, are believed to be responsible for visible photoluminescence in ZnO. Defects in ZnO strongly depend on the preparation and annealing conditions which in turn affect the photoconduction properties. With a wide band gap of 3.4 eV and a large exciton binding energy of 60 meV at room temperature, ZnO holds excellent promise for bioapplication because of its versatility and stability. ZnO is not a newly discovered material with reports of its physical characterization going back to 1935.\textsuperscript{27} ZnO is present in the Earth crust as a mineral zincite; however most ZnO used commercially is produced synthetically.

1.7. Technique to Identify Protein-Nanoparticle Interaction

When in contact with biological fluids (serum, plasma, lung lining fluids, etc.), NPs interact spontaneously with proteins and other biomolecules, which dramatically change their surface properties. Thus, the NP's surface acquires a new biological identity which most likely would influence its stability and interaction with living matter, thereby affecting the nanoparticle biodistribution in (in vivo) experiments. For example, the adsorption of opsonins, such as immunoglobulins, complement proteins, fibrinogen, etc., can enhance the phagocytosis rate with immediate removal of the particles from the blood stream, while the adsorption of proteins, such as serum albumin and apolipoproteins, and other protein carriers of the blood stream, can prolong NP circulation. When nanomaterials are meant for biological applications, it is necessary to investigate their physicochemical properties in the biological milieu. There are several techniques available\textsuperscript{29} to characterize protein nanoparticle interaction some of which are discussed below.

1.7.1. Spectroscopy Methods

1.7.1.1. UV/Vis. Protein binding to NPs induces changes in the absorption spectra of the NP or proteins, and these changes can be used to investigate the binding.\textsuperscript{30,31} The shift and broadening of the absorption spectra of the NP-protein complex depend on the NP size and aggregation state and the local dielectric environments. For example, it was shown that the red shift and the widening of the peak in the absorption spectra of azurin (Az)-gold NP solutions depend on the Az concentration.\textsuperscript{9} UV/vis spectroscopy can thus be used to analyze NP-protein binding, although quantitative and conclusive results are
difficult to achieve. In fact, protein-nanoparticle complexes are characterized by a different size distribution compared to the bare nanoparticles, with possible formation of NP-dimer and NP-trimer conjugates, which will give a different scattering contribution to the overall absorption spectra. Compared with other methods, UV/vis is faster, more flexible, and less complicated, but the method is not conclusive and needs to be performed in association with other complementary spectroscopic and structural techniques.

1.7.1.2. Fluorescence. Labeling of proteins with fluorescent probes allows one to use of fluorescence spectroscopy to investigate their structural and dynamic properties. The fluorescent labeling has to be thoroughly designed to avoid introducing major conformational changes to the native structure of the protein. In addition, one can also argue that the addition of a dye can affect the interaction of the protein with the NP, if the dye has higher affinity for the NP surface compared to protein’s functional groups. Fluorescence spectroscopy is highly sensitive to protein dynamics because the excited fluorescent state persists for nanoseconds, which is the time scale of many important biological processes, such as the rotational motion of protein side chains, molecular binding, and conformational changes. NP-protein binding can be further measured by the steady-state or time-resolved fluorescence spectroscopy and fluorescence resonance energy transfer (FRET). Intrinsic protein fluorescence from Trp groups can also be used to monitor changes in the protein microenvironment upon binding to NPs.

1.7.1.3. Circular Dichroism. Different protein secondary structures (α-helix, β-sheet, etc.) have their own characteristic CD spectra in the UV region. This method has been widely used for monitoring conformational changes induced by protein-NP interactions. NPs are not usually chiral in nature and thus do not have any affect on the protein CD spectra, although, if their size is large, they can give a scattering contribution which can affect the CD signal. The approximate fraction of a specific secondary structure present in the protein can thus be determined by analyzing its far-UV CD spectrum as a sum of fractional multiples of reference spectra typical of the secondary structural type. Like all spectroscopic techniques, the CD signal reflects an average of the entire molecular population. Thus, while CD can determine if a protein contains about 40% α-helix, it cannot determine which specific residues are involved in
the α-helical formation. The CD spectrum of a protein in the “near-UV” spectral region (250-350 nm) can be very sensitive to certain aspects of tertiary structure. At these wavelengths the active chromophores are the aromatic amino acids and disulfide bonds, whose CD signals are sensitive to the overall tertiary structure of the protein. In particular, signals in the 250-270 nm regions are attributable to phenylalanine residues, in the 270-290 nm regions to tyrosine, and in the 280-300 nm regions to tryptophan. Disulfide bonds give rise to broad weak signals throughout the near-UV spectrum. The near-UV CD spectrum can be efficiently used to detect small changes in tertiary structure due to protein-nanoparticle interactions and/or changes in solvent conditions. Although CD cannot be applied on complex protein mixtures, it can provide useful insight on protein structure changes resulting from adsorption to a NP surface.

1.7.1.4. Isothermal Titration Calorimetry (ITC). ITC is a powerful technique that can measure the binding affinity constant, enthalpy changes, and binding stoichiometry between NPs and proteins in solution. On the basis of the measurement of small changes of temperature, Gibbs energy and entropy changes can be easily calculated. To quantify protein binding as a function of the NP characteristics, typically a protein is titrated into the NP solution and the heat response is recorded. The heat changes are then fitted to the isothermal function, and thermodynamic parameters are obtained. In a study of quantum dot (QD) HSA interaction, the thermodynamic parameters of the system were calculated at different temperatures. Results indicated that electrostatic interactions played a major role in the binding reaction, as negative enthalpy and positive entropy values were obtained. In a separate study Cedervall et al. showed that ITC is suitable for studying the affinity and stoichiometry of protein binding to NPs. Lindman et al., also showed that ITC is a straightforward method for measuring protein adsorption to nanoparticles in a quantitative manner. In particular it can be used to derive specific effects driving adsorption and give accurate degrees of adsorption and provide good complement to more qualitative or structural methods.

1.7.1.5. Electrophoresis. Electrophoresis is the most widely used method for the separation and analysis of complex protein mixtures, where charged molecules dispersed in a fluid migrate under an electric field. Electrophoresis can be broadly classified into two types, capillary electrophoresis (CE) and gel electrophoreses (one-dimensional gel...
electrophoresis (1-DE) and two-dimensional gel electrophoresis (2-DE). Among different
types of electrophoresis technique one-dimensional gel electrophoresis is the most
popular for analyzing protein-nanoparticle complex.

1.7.1.6. One-Dimensional Gel Electrophoresis. Running SDS-PAGE is probably the
most common practice of separation protein mixtures by molecular weight under an
electric field. The proteins travel through a vertical gel of acrylamide cross-linked
with bisacrylamide and are separated according to their size due to their different
electrophoretic mobilities. The proteins must be fully denatured and negatively charged,
which can be achieved simply by boiling them with a reducing agent such as
dithiothreitol (DTT) or β-mercaptoethanol and an anionic detergent (SDS). While the
reducing agent will only reduce the disulfide bonds within cystine residues, SDS
denatures the proteins by binding to the amino acids, causing electrostatic repulsion and
thus inducing protein unfolding. The same method has been successfully applied to
denature and detach proteins adsorbed onto NP surfaces. The negative charge of the
proteins given by SDS causes protein repulsion and thus detachment from the NP surface.
The proteins resolved in the gel can be stained with one of several available commercial
stains like (coomassie brilliant blue, silver nitrate staining, deep purple, etc.) and
densitometry analysis is routinely used to quantify protein abundance. SDS-PAGE is an
extremely quick, cheap, and reliable technique that allows up to 14 protein coronas from
different samples to be resolved simultaneously within the same gel.

1.8. Outcome of Protein-NP Interactions
Although NPs have significant potential for use in many bio-medical applications, there
are also potential “toxic” phenomena including the production of reactive oxygen
species, uncontrolled aggregation or amyloidosis diseases, and whose biological and
physiological implications must be understood. NPs possess large surface/volume ratios,
and as a possible outcome in a physiological medium, NPs are always surrounded by
proteins whose conformation may be disrupted or induced to aggregate, which can trigger
uncontrolled cellular responses. The adsorbed proteins are almost comparable to the
native protein that the cells do not recognize them as denatured, but may be sufficiently
different from the native form that they trigger an inappropriate cellular process. Earlier
Linse et al. have reported that NPs accelerate the probability of appearance of a critical
nucleus for nucleation of protein fibrils from human β2-microglobulin in solution.\textsuperscript{44} These phenomena increase the risk of toxic clusters formation and amyloid generation. A recent study using iron oxide NPs modified with chlorotoxin to image brain tumors showed NP transport across the blood–brain barrier for excellent tumor imaging.\textsuperscript{45} However, the mechanism of transport is unknown. It is possible that circulating apolipoproteins may have contributed to the uptake of these NPs, indicating the beneficial role of serum protein binding. However, the protein corona surrounding the NP is often uncontrolled and thus not beneficial for targeting because it can lead to accumulation in the reticuloendothelial system.\textsuperscript{46}

1.9. Future Prospective

The developments in nanotechnology are leading to potential applications in multiple areas, such as in cosmetics, coatings, electronics, information technology, and more recently health care and the life sciences. Due to its rapid propagation into all branches of science and technology, the current century may have reason to be named the “Nanotechnology Age”. The number of publications in the field of nanotechnology has been escalating enormously in recent few years; however, the majority of these investigations are focused on the synthesis, characterization, and surface properties of NPs, whereas the biological issues have been neglected. Due to the importance of the potential toxicity of NPs and the need to ensure the safe implementation of nanotechnologies, the biological issues have to be addressed with utmost care.

Revolutionary advances in science and technology enable the solution-oriented design of nanomaterials. In-depth understanding of physics and chemistry fundamentals at the nanoscale, combined with modern, robust, computational capability across length scales, would enable the directed design and synthesis of libraries of high-quality nanomaterial building blocks. Understanding of the fundamentals will assist in the development of models and tools and help to verify the accuracy of that understanding. Knowledge of the relationships among structure, properties, functions, and processing methods would provide the basis for application-based nanoparticle design.
1.10. References

1. Rao, C.N.R. Nanoworld, an introduction to nanoscience and technology.
2. Wikipedia (http://en.wikipedia.org)


