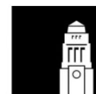

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Application of isothermal titration calorimetry in evaluation of protein–nanoparticle interactions

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Abstract Nanoparticles (NPs) offer a number of advantages over small organic molecules for controlling protein behaviour inside the cell. Protein binding to the surface of NPs depends on their surface characteristics, composition and method of preparation (Mandal et al. in J Hazard Mater 248–249:238–245, 2013). It is important to understand the binding affinities, stoichiometries and thermodynamical parameters of NP–protein interactions in order to see which interaction will have toxic and hazardous consequences and thus to prevent it. On the other side, because proteins are on the brink of stability, they may experience interactions with some types of NPs that are strong enough to cause denaturation or significantly change their conformations with concomitant loss of their biological function. Structural changes in the protein may cause exposure of new antigenic sites, "cryptic" peptide epitopes, potentially triggering an immune response which can promote autoimmune disease (Treuel et al. in ACS Nano 8(1):503–513, 2014). Mechanistic details of protein structural changes at NP surface have still remained elusive. Understanding the formation and persistence of the protein corona is critical issue; however, there are no many analytical methods which could provide detailed information about the NP–protein interaction characteristics and about protein structural changes caused by interactions with nanoparticles. The article reviews recent studies in NP–protein interactions research and application of isothermal titration calorimetry (ITC) in this research. The study of protein structural changes upon adsorption on nanoparticle surface and application of ITC in these studies is emphasized. The data illustrate that ITC is a versatile tool for evaluation of interactions between NPs and proteins. When coupled with other analytical methods, it is important analytical tool for monitoring conformational changes in proteins.

Keywords Isothermal titration calorimetry · Nanoparticles · Proteins · Interactions · Gold nanoparticles · Protein–nanoparticle interaction

Introduction

According to the National Nanotechnology Initiative, nanotechnology is the research and development of nanosystems (e.g. nanoparticles) on the scale of 1–100 nm [3]. Nanoparticles (NPs) are potentially useful tools for medicine and biology, as they are of similar size to important biological components (e.g. proteins, DNA, cell membranes) and thus able to interact in a sophisticated and controlled way at the cellular level. However, knowledge of the factor that govern the fate and effect of NPs administered as drugs is still limited [1, 2].

Nanobiotechnology is a scientific field combining nanotechnology and biology. It is one of the most critical research endeavours of the twenty-first century. The most promising applications of nanobiotechnology are in medicine (nanomedicine).

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When entering a biological fluid, NPs will be coated with proteins and other biological molecules and form protein corona. The composition of the corona around NPs will be determined by the concentration as well as on- and off-rate constants for each protein. The composition of corona is changeable until over time equilibrium is reached. Each time when the NPs enter a new environment, composition of corona will be changed until a new equilibrium is reached. The corona will create a new NP surface. Organization of a NP surface may be important to the fate of the NPs and its biosafety aspects [4]. NP corona, through interactions of NPs and proteins, could have impact on the structure and properties of adsorbed proteins.

A protein corona plays important role in further application of NPs in prevention and treatment of diseases. However, relatively little is known about potential biological risks from NP therapeutic applications. Clinical studies have indicated adverse health effects on NPs exposure. There is an essentially immediate increased risk of heart attack in the elderly that is associated with NP-induced changes in blood viscosity and blood clotting capacity from NPs exposure through respiration in some systems [5]. It is clear that NPs can act as a double-edged sword, either as a toxic agent or as a platform for therapy, depending on context. This has led to increased interest in obtaining and understanding of protein–NP interactions and the biological implications of these interactions to aid in the controlled synthesis of these promising materials. It is important to understand how NPs enter the body, tissues and cells, where they go, and what are the consequences of them being there. If we want to fully understand the biological impact of NPs use, we should address all the complicated molecular aspects of nano–bio interactions [6, 7].

Proteins binding to NPs undergo conformational changes. This further complicates the structure of the protein corona. A structural change in the protein may cause loss or a gain of function. It could also lead to the exposure of amino acid residues, which are normally buried in the core [4].

Isothermal titration calorimetry (ITC) is a quantitative physical technique used to determine reaction thermodynamics and kinetics. It can be used to study solutions, solids and heterogeneous mixtures because it is invariant to sample physical form [8]. This label-free technique is based on heat measurement absorbed or generated during binding event in a sequential (titrating) manner. It allows simultaneous determination of binding constants (Ka), reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS) during single experiment, providing a complete thermodynamic profile of investigated interaction [9]. ITC is becoming the method of choice for characterization of intermolecular interactions and recognizing reactions with exquisite sensitivity, since both low- and high-affinity interactions can be quickly and accurately characterized [10]. ITC is an attractive approach for the study of biomolecular interactions such as the interactions of proteins with NPs. The combination of thermodynamic data obtained with ITC and structural data for proteins has significantly enhanced our understanding of macromolecular interactions in solutions allowing predictions of the size and thermodynamic properties of the binding interface, and the characterization of contributions from protein conformational changes and solvation in the extended binding interface [11]. Different studies indicated the complexity of protein–NP interactions [12].

The main aim of this review is to summarize current knowledge in the study of NP–protein interactions and on the application of isothermal titration calorimetry in evaluation of these interactions with focus on structural changes of proteins upon adsorption on nanoparticle surface.

Influence of nanoparticle–protein interactions on protein adsorption on nanoparticle surface

Nanoparticle–protein corona

Proteins are large biomolecules consisting of one or more long chains of amino acid residues. Amino acids in almost all proteins are charged, and they are responsible for electrostatically interactions of proteins with substrates, transition states and products [13].

Most proteins fold into unique three-dimensional structures and carry net surface charge depending on the pH of the surrounding medium. At a specific pH (isoelectric point), the positive and negative charges will balance and the net charge will be zero. It occurs in the pH range of 5.5–8 for most of the proteins. A protein will have the lowest solubility at its isoelectric point. If there is a charge at the protein surface, the protein will prefer to interact with water, rather than with other protein molecules. The charge makes protein more soluble. Without a net charge at the protein surface, protein–protein interactions and precipitation are more likely.

Adsorption of proteins at the NP surface is aided by several forces (hydrogen bonds, Van der Waals interactions, electrostatic interactions, etc.) (Fig. 1). The main driving forces for protein folding are hydrophobic interactions. Van der Waals interactions provide selectivity to noncovalent interactions. Hydrogen bonds are a particular type of electrostatic interactions between a weak acid donor group and an acceptor group with a lone pair of electrons. They stabilize the secondary structure of proteins [14].

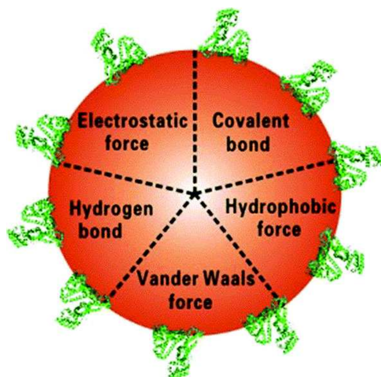

Fig. 1 Intermolecular forces which can occur between NPs and proteins

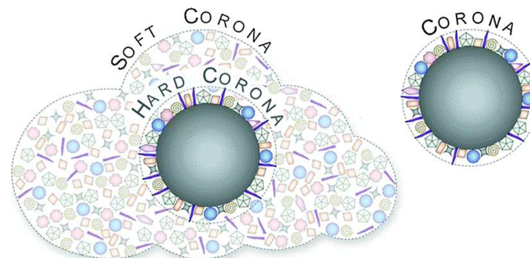

Fig. 2 Hard and soft corona

The formation of NP–protein corona is a multifactorial process dependent on the NPs, interacting proteins and medium properties. The medium where NP–protein corona is formed can cause the NP to undergo a complex sequence of modifications that are far from being fully understood. Even within one environment the NP–protein corona and interactions between NP and proteins in the corona are constantly changing [15].

Specific association and dissociation rates for each protein decide about the fate of their interactions with the NP surface. Long-term or irreversible binding of proteins on the NP surface leads to the formation of a "hard corona". Quick, reversible binding of proteins on the NPs binds a "soft corona" (Fig. 2).

NPs that have entered human body have to be considered as evolving systems that are shaped by exposure to different protein-rich environments. Kinetics of protein adsorption on the NPs surface is influenced by several factors. One of the factors is the amount of proteins to interact with the NP. For example, when NPs are present in human plasma, the composition of proteins in NP corona varied with plasma concentration where plasma proteins were applied at concentration between 3 % and 80 % of plasma. On the other side, relative amounts of abundant proteins adsorbed on silica or polystyrene NPs increased with increasing plasma concentrations [16].

A typical NP–protein corona in human plasma consists of proteins such as serum albumin, immunoglobulins, fibrinogen and apolipoproteins [15]. Many scientific studies focused on the interactions of plasma proteins and different NPs. De Paoli Lacerda et al. performed an array of photophysical measurements to quantify the interaction of model gold NPs having a wide range of NP diameters with common blood proteins. The results provide main core of the physical basis of the biological activity of NPs in nanomedicine applications and under conditions of environmental exposure. Further studies are necessary to understand how the nature of the protein adsorption process changes the protein conformation and the tendency of the "dressed" particles to aggregate [6]. A detailed study on the interaction of carbon NPs with bovine serum albumin and human serum albumin using three analytical techniques (fluorescence spectroscopy, circular dichroism spectroscopy and ITC) demonstrated strong interactions between carbon NPs and human serum proteins [1]. A high-density lipoprotein was found in the protein corona on polystyrene NPs [17]. Many review articles have been written to summarize all existing research about plasma proteins and NPs. One of these reviews gives insight into existing research of NPs interactions with plasma proteins as related to NP biodistribution, biocompatibility and therapeutic efficacy [3].

Adsorption of a protein on the NPs surface also depends on the affinity of the protein towards the NP surface and its ability to completely occupy the surface. The way in which protein molecules arrange themselves on the NP surface may affect the biological reactivity at the cellular level [18].

Implication of NP uptake by cells

Cytotoxicity and immune modulation are the most important repercussions of NP uptake by cells. This is important when considering that NPs have a property to dissolve after reaching the acidic lysosomal compartments of the cell and thus contributing to cellular toxicity. To understand the fate of NPs in the biological content, it is important to study factors involved in uptake of NPs. Those factors are protein adsorption, physical characteristics of the NPs and the properties of interacting cells [15].

It was shown that kinetics of uptake of the same NPs differs with different cell types [19, 20]. Physicochemical properties of protein-coated gold nanoparticles in biological fluids and cells before and after proteolytic digestion are presented by Chanana et al. [21].

Apolipoproteins are a class of proteins that are often found in NP–protein corona. They have ability to aid in the uptake of NPs by binding to specific receptors on cells [22, 23].

Influence of NPs surface on protein adsorption and cellular interactions

Different NP properties influence protein adsorption on the surface on NPs (e.g. NPs composition, functional groups, pH, temperature and hydrophobicity). NP surfaces need to be carefully designed to elicit the desired effect for biomedical applications since NPs come into contact with the biological environment through their surfaces. To endow NPs with desirable surfaces, modification of their surfaces is needed. The most common method for NP surface modifications is PEGylation. PEGylations present NPs surface modification with polyethylene glycol (PEG) [24].

The interactions of NPs with cells and tissues are an important factor for any potential translation into biomedical applications that require high specificity and a rapid internalization of the NPs into the target cells. Surface properties of most NPs are essential to ensure colloidal stability, and they play a role in determining the kind of NP–cell interactions. It has only recently been found that the proteins from biological environments can drastically modify the surface of NPs, therefore deterring the intended therapeutic action. The surface charge of NPs is expected to influence the uptake pathway as well as their effective performance [25].

An important issue that requires further attention is the indirect influence of the NP physicochemical properties on cytotoxicity, cell signalling, etc. [15].

Structural changes of proteins induced by nanoparticles

General principles of protein behaviour on nanoparticle surfaces

The main consequence of the interaction of proteins with NPs is the alteration of the biomolecule structure. This effect strongly depends on the NP surface characteristics and protein stability. Structural changes may affect further the protein functions.

Protein–NP interactions have to be separated in the interactions of proteins and peptides with nanosized particles and interaction of proteins and peptides with the surface as a macrostructure. This separation is necessary in terms of the differences between the forces that act from an NP surface and an NP as a whole on a protein [7].

Each protein has an intrinsic stability. It is, in thermodynamic terms, the most favourable state of the polypeptide chain with the minimal entropy. Disturbance of the protein structure should be accompanied by an adding of additional energy to the system to shift it from the "potential hole". If the entropy of the "alternative state" is lower than the entropy of the "native" state, these transitions are reversible. If the entropy reaches the critical maximum value at which the protein is denatured and may form aggregates or fibrils, transitions are irreversible. This phenomenon is the basis for the classification of all proteins into "soft" and "hard" molecules [26]. From the point of the protein structure, this classification is mediated by the interactions underlying the structural stabilization of the protein. The main forces that can stabilize the protein structure are covalent and hydrogen bonds, hydrophobic and electrostatic interactions and Van der Waals interactions. The secondary structures of proteins are stabilized by hydrogen bonds alone and hydrogen bonds combined with hydrophobic interactions. The stabilization of tertiary structure involves practically all types of interactions. When the folding of the molecule has been completed, its stabilization is strengthened by Van der Waals interactions. Conformational stability varies in different protein domains.

One of the most important properties of the surface is its hydrophobicity or hydrophilicity. If the surface is hydrophobic (nonpolar), the force of interaction of the protein and the surface is only determined by the degree of the hydrophobicity of the surface. Hydrophilic surfaces (polar) will interact with proteins through hydrogen bonds. The degree of disruption of the protein structure depends on the balance between the formation and breakage of hydrogen bonds. A nonpolar structure is unable to interact through hydrogen bonds.

Another important property of a surface interacting with biomolecules is its charge. The charge on the surface of a protein molecule is a complicated function of the distribution of amino acids residues in the protein molecule and is unevenly distributed over its surface. This charge depends on the medium surrounding the protein molecule. The interaction of proteins with charged surface may lead to new "induced" charged regions on proteins.

The NP–protein interactions may cause different structural rearrangements of the protein molecule. One of the possible effects is exposure of the so-called hidden epitopes on the surface of the protein [7].

Conformational epitopes are a result of specific folding of the protein polypeptide chain. Continuous epitopes are regions on the protein primary structure which consist of 10–12 amino acids which are capable of eliciting an immune response. The NP surface may induce abnormal folding of bounded proteins. This type of folding will form novel conformational epitopes or may induce unfolding of the native structure to expose hidden epitopes [15].

Protein structural changes are undesirable in the case of biological recognition. Changed receptor or antibody structure can cause the loss of cell signal transduction or

immune response. Interaction of a protein or peptide with an NP and resulting structural perturbation may lead to the loss of the ability to interact with other proteins. Thus, NPs may disturb cell homoeostasis. However, it is important to remember that NP-induced structural changes of proteins have a positive effect too. One of the examples of positive effects of protein structural changes after interaction with NPs is inhibition of fibrillation of peptides and proteins involved in the pathogenesis of neurodegenerative. But, NPs may conversely promote this pathological process [7].

Curved NP surfaces compared to planar surfaces provide extra flexibility and enhanced surface area to the adsorbed protein molecules. These NP surfaces can affect the secondary structures of proteins and in some cases cause irreversible changes [27, 28].

NPs can induce conformational changes in proteins that can lead to fibril formation [29, 30].

Structural changes in proteins bounded to nanoparticles are very important research topic in protein science and nanobiotechnology nowadays. Cukalevski et al. reported structural changes in apolipoproteins upon adsorption on different NPs [4]. It was found that simple chemical modifications of the surface charge distribution of human serum albumin change the nature of protein adsorption onto DHLA-QDs [2]. The effect of gold nanoparticles ligand charge on cytochrome c complexes was investigated [31]. Chakraborty et al. investigated effect of gold nanoparticles and nanorods on the structure and activity of bovine serum albumin [12].

Analytical methods for studying NP–protein interactions

Several analytical methods are developed for characterization of NP–biomolecule interactions (fluorescence spectroscopy, circular dichroism, mass spectrometry, etc.). To study NP–protein interactions, mass spectrometry-based proteomics method is the most widely used. The advantages of the method include possibility of application over a range of NPs and use of stable isotope labelling by amino acids in cell culture (SILAC) [32]. Fluorescence spectroscopy provides measurements of the intrinsic fluorescence of the proteins. Rocker et al. had quantitatively analysed the adsorption of human serum albumin onto polymer-coated FePt and CdSe/ZnS nanoparticles (10–20 nm in diameter) using fluorescence correlation spectroscopy [33]. Circular dichroism spectroscopy uses changes in chiral properties of purified proteins to predict changes in secondary structure as a result of NP–protein interactions [34]. Nuclear magnetic resonance can provide high-resolution information on protein structure. Other techniques for characterization interactions between NPs and proteins are: isothermal titration calorimetry (ITC), quartz crystal microbalance (QCM) and fluorescent labelling [15].

In order to understand and study different aspects of NP–protein (biomolecule) interactions, various approaches and mathematical models are developed. Xia *et a.l* introduced the biological surface adsorption index approach (BSAI) to predict influence of hydrophobicity, hydrogen bonding and polarizability and ion-pair electrons of NP surface on the protein adsorption at nano–bio interface [35, 36]. A mathematical model for kinetic prediction of protein binding to NP surfaces was presented by Dell'Orco et al. [37]. This model can be used for prediction of any proteome–NP combinations [37].

With scientific progress in understanding processes taking place on NPs surfaces, it becomes clear that coupling of the available analytical techniques is necessary for further progress in the study of different aspects of NP–protein interactions. Chakraborty et al. presented the differential response of a protein adsorption on NP surface depending on the nature of the nanostructure and its surface chemistry [12]. These diverse responses need to be modulated in order to control the biological responses of nanostructures when the latter intend to be used for biomedical applications. Authors of this work performed experiments combining the following methods: fluorescence spectroscopy, CD, ITC and Fourier transform infrared (FTIR) analysis.

Isothermal titration calorimetry in NP–protein interaction characterization

ITC is a physical technique used to determine the thermodynamic parameters of interactions in solution. The method is suited for diverse applications because it is label-free and relies only on measuring the heat evolved during the molecular reaction. It is most often used to study the binding of small molecules to larger macromolecules (proteins, DNA), but it could be also used for studying interactions of drugs with target biomolecules, polymer interactions in nanotechnology, micelle formation by surfactants and enzymatic activity and inhibition, among many others [38].

Nanoisothermal titration calorimetry (nITC) is a new generation of isothermal calorimetry allowing measurement of heat effects as small as 100 *nJ* using one nanomole or less of biomolecule. The main advantages of nITC, when compared to standard ITC, are reduced titration time and higher sensitivity. Up to now, there is no research studies based on application of nITC although it has a great prospective for nanobiotechnology applications.

ITC and nITC use stepwise injections of one reagent into a calorimetric cell containing another reagent to measure the heat of the reaction. The suspension of NPs is

injected in aliquots into the sample solution (proteins) when measuring binding of proteins to the NPs surface. The role of reference cell is to follow heat changes produced by solvent (buffer). The scheme of the standard ITC instrument is presented in Fig. 3. The observed heat is either released (an exothermic reaction) or taken up (an endothermic reaction). The heat is measured by determining the power required to maintain a constant temperature with respect to reference solution. The reaction heats tend to zero as the titration progresses, and the binding sites become saturated. Thermodynamic parameters which can be obtained with ITC or nITC are stoichiometry of the interaction (n), association constant (K_a)/dissociation constant (K_d), free energy (ΔG_b), enthalpy (ΔH_b), entropy (ΔS_b) and heat capacity of binding (ΔC_p) (Fig. 4). Average enthalpy and entropy of the NP–protein reactions can give indication about the nature and type of the interaction. From mentioned parameters, the Gibbs free energy of binding, ΔG, and the entropic contribution to the binding event (-TΔS) can be calculated [38–40].

Problem which occurs in practice with application of ITC and nITC method is that derived thermodynamic values are apparent values. This means that ITC and nITC measurement values include contributions not only from the binding itself but also from other coupled equilibrium.

The main practical considerations concerning ITC and nITC applications are: (a) buffer—the choice of buffer is not usually critical for an accurate measurement, but it is important to find the best conditions under which sample is most stable; (b) performing blank runs where ligands are injected into buffer for ensuring small background heats of dilution that would affect binding measurements; and (c) stoichiometry.

The question that might arise is why ITC and nITC methods are essential in characterization of (bio)interactions? The answer can be found in the following explanation. Almost any chemical reaction or physical change is followed by a change in heat or enthalpy. A measure of heat for an endothermic or an exothermic process is equal to the amount of the product(s) that has (have) occurred, n

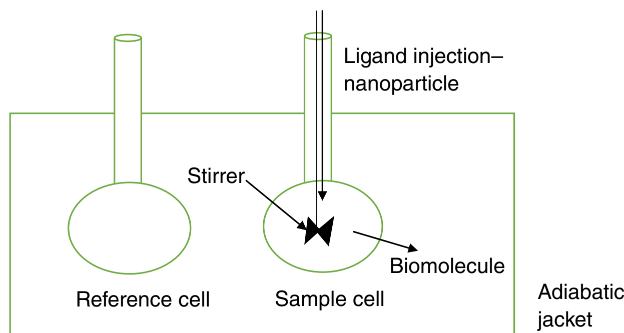

Fig. 3 Sample and reference cell of ITC instrument

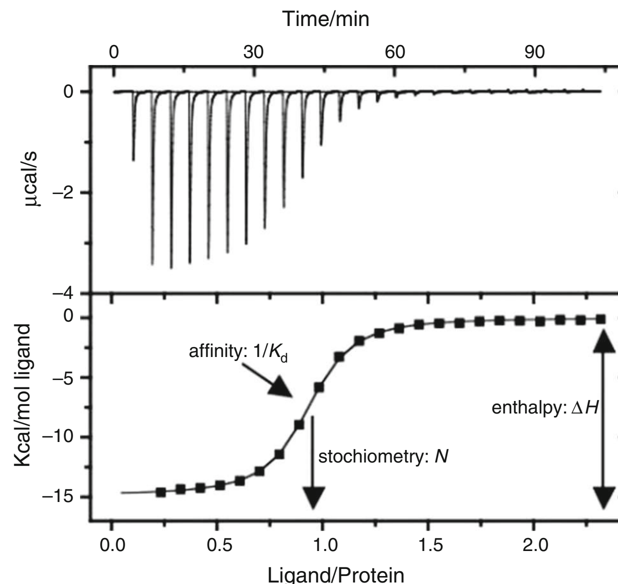

Fig. 4 Raw data (upper panel) generated by an ITC experiment represent the heat released (or adsorbed) during titration (μcal/s). These raw data are converted into the binding isotherm (lower panel) by integration of each injection producing the thermal energy (Δ*H*) of each titration step. The signal is reduced until only the background heat of dilution remains. Corrections of such heat are necessary and usually performed by control experiments. The change in enthalpy (Δ*H*), the stoichiometry (*n*), the dissociation constant (K_d) and affinity ($1/K_d$) can be derived from the binding isotherm. The change in enthalpy is presented by the distance of the two asymptotic lines corresponding to the minimal and maximal heat formation. Stoichiometry is molar ratio at the inflection point of the sigmoidal curve. The slope at the inflection point present the association constant (affinity)

(expressed in *moles*, *mmoles*, *μmoles*, *nmoles*, etc.), and the enthalpy change for the reaction, ΔH (typically in *kcal/mol* or *kJ/mol*). A measure of the rate at which heat is exchanged with surroundings is equal to the rate of the reaction and again the enthalpy change. A calorimeter is therefore an ideal instrument to measure how a reaction proceed or what is the rate at which a reaction occured. Calorimetric measurements can be performed with reactants that are spectroscopically silent and can be run across a range of biologically relevant conditions (pH, temperature, etc.) [41].

ITC has been applied in studying biomolecular interactions (protein–protein interactions, protein–ligand interactions, lipid/membrane interactions, polysaccharide interactions, etc.). Today, one of the most important applications of ITC is in the study of protein/peptide interactions with nanoparticles. X. Zhou et al. described a method for identifying unknown target proteins from a mixture of biomolecules for a given drug or a lead compound [42]. The method is based on combination of chromatography and ITC, where the ITC is a tracking tool. Welsch et al. reviewed recent work on the adsorption of

proteins on two types of colloidal particles, where thermodynamics of adsorption was measured with ITC [43]. The potential of ITC for characterization of different functionalities carried by nanoparticles as well as their interaction with living systems was investigated by Bouchemal [10]. Cedervall et al. applied ITC in studying the affinity and stoichiometry of protein binding to nanoparticles [44]. ITC was used in the study of membrane proteins [9, 45]. Pilloni et al. investigated PEGylated magnetite–silica nanocomposites in biocompatibility study [46].

Since ITC has been developed, many studies based on empirical thermodynamical rules have been performed in order to evaluate titration heat and thermodynamic framework underlying isothermal titration calorimetry [11, 47–49].

Application of ITC in evaluation of structural changes of proteins upon adsorption on NPs surface

In recent years, ITC has been used in the studies of protein structural changes upon adsorption on NPs surface. It is very important since protein structural changes can affect the overall bio-reactivity of NPs and proteins. Thus, contribution of these studies to the overall understanding of NP–protein interactions is enormous.

ITC cannot give direct information about structural changes. Thermodynamical data obtained from ITC experiment provide insight into favourable interactions between NPs and proteins which leads to the prediction of structural changes as it is described in Chapter 3.1. ITC is usually coupled with other analytical methods to give more detailed information about NP–protein interactions and protein structural changes in NP–protein interactions. Fluorescence spectroscopy and circular dichroism are methods of choice to combine with ITC in characterization of NP–protein interactions.

Fleischer and Payne investigated effect of protein corona on cellular-level events, including NP binding, internalization and transport. A combination of technics (microscopy, fluorescence spectroscopy, ITC and circular dichroism) was necessary to use for completely probing of protein–NP–cell interactions. A model system composed of polystyrene NPs functionalized with amine or carboxylate groups to provide a cationic or anionic surface. NPs were tested with bovine serum albumin (BSA). The results showed that BSA adsorbed onto the surface of both cationic and anionic NPs, forming a net anionic protein complex. These protein–NP complexes have similar diameters and effective surface charges, but they show the exact opposite behaviour in terms of cellular binding [50].

Li et al. reviewed diverse analytical techniques to determine protein–NP interactions such as UV–Vis spectroscopy, fluorescence spectroscopy for monitoring binding affinity and binding ratio, circular dichroism and Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy for monitoring conformational changes of NP-bound proteins, ITC and fluorescence spectroscopy for monitoring of mechanism of NP–protein interactions [51].

Eren et al. investigated lysozyme–silica interactions and the resulting complexation through adsorption isotherms, dynamic and electrophoretic light scattering, circular dichroism and ITC [52].

There are no many publications based on the investigation of protein structural changes by ITC since this is rather new analytical field. Among the existing one, there is a study of Mandal et al. where authors investigated interactions of carbon NPs with serum albumin with ITC, circular dichroism and fluorescence spectroscopy [1]. Binding studies of Cucurbit [7] uril with gold nanoparticles bearing different surface functionalities were investigated [48]. The differential response of a protein depending on the nature of the nanostructure and its surface chemistry for controlling the biological response of nanostructures for their potential biomedical applications was presented by Chakraborty et al. [12].

The role of ITC in the creation of protocols for the preparation of chitosan-based nanoparticles and the role of calorimetry to find chitosan-coating conditions to offer to nanoparticles the desired proprieties for the delivery of drugs, biologics and vaccines were investigated in the work of Fotticchia et al. [53].

ITC was used to detect phytate binding to the protein lysozyme in the study of Darby et al. [54].

Conclusions

Adsorption of proteins at the NP surface is aided by several interactions (hydrogen bonds, Van der Waals interactions, electrostatic interactions, etc.). Specific association and dissociation rates for each protein decide about the fate of their interactions with the NP surface Long-term or irreversible binding of proteins on the NP surface leads to the formation of a “hard corona”. Quick, reversible binding of proteins on the NPs binds a “soft corona”.

The formation of NP–protein corona is a multifactorial process dependent on the NPs, interacting proteins and medium properties. The medium where NP–protein corona is formed can cause the NP to undergo a complex sequence of modifications that are far from being fully understood. Even within one environment the NP–protein corona and interactions between NP and proteins in the corona are constantly changing [15].

One of the main consequences of the interaction of proteins with NPs is the alteration of the biomolecule structure. This effect strongly depends on the NP surface

characteristics and protein stability. Structural changes may affect the protein functions. The main interactions that can stabilize the protein structure are covalent and hydrogen bonds, hydrophobic and electrostatic interactions and Van der Waals interactions. The secondary structures of proteins are stabilized by hydrogen bonds alone and hydrogen bonds combined with hydrophobic interactions. The stabilization of tertiary structure involves practically all types of forces. When the folding of the molecule has been completed, its stabilization is strengthened by Van der Waals interactions. Conformational stability varies in different protein domains.

Several analytical methods are developed for characterization of NP–biomolecule interactions (fluorescence spectroscopy, circular dichroism, mass spectrometry, etc.).

Over the years, isothermal titration calorimetry (ITC) and later nanoisothermal titration calorimetry (nITC) have been adopted for measuring NP–protein interactions. Both methods provide information regarding the molecular mechanism of an interaction and, hence, can serve as a platform to better understand (bio)chemistry and (bio)-physics of molecular interaction. ITC and nITC provide a correlation between structural and thermodynamic contributions towards examined interactions.

When coupled with other methods (fluorescence spectroscopy, circular dichroism, etc.), ITC provides more detailed information about interaction mechanism, reaction products, changes on nanoparticle surface and what are the possible consequences of interaction on the functions of interacting biomolecules and on living organisms.

In recent years, ITC has been used in the studies of protein structural changes upon adsorption on NPs surface. Protein structural changes can affect the overall bio-reactivity of NPs and proteins. Thus, contribution of the ITC studies based on protein structural changes to the overall understanding of NP–protein interactions is enormous.

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