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Recent Advances in Protein Corona Study and Compatibility of Employed Fluorescence Correlation Spectroscopy as a Method.

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ABSTRACT

Incorporation of engineered nano particles in biological system is very common. The study of biomolecular response triggered on exposure to nanoparticle is very important. Nano particles in the living organism encounter a complex environment such as interact with proteins and form complexes adsorbing on surface. The entity of adsorbed protein layer in the biological matrix confers a biological identity to the nano particles which is called protein corona. Composition of protein corona is dynamic, more abundant protein molecules first form the corona followed by replacement by less abundant proteins of higher binding affinity. Tightly bound structured first layer is called hard corona and loosely bound diffused layer is called soft corona. The interaction of nano particles with bio-organism is largely mediated by protein corona.A comprehensive study of nanoparticle protein interaction is necessary for designing nanoparticles and its safety on biological applications. Till now understanding of nano particle protein interaction is very shallow due to lack of sufficient sophisticated techniques. Our aim is to help nano-bio research community summarising the major developments in the study of protein corona in recent years and discussing compatibility of FCS technique which is directly employed for the study.

Keywords: Nano particles, protein, hard corona, soft corona, FCS.

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INTRODUCTIONS

With the radical advancement of nanotechnology, large number of new nano materials are synthesized intending applications in various fields e.g. electronics, medicines, catalysis, gas storage etc [1-5]. Nanomaterials has now great impact on human life and economy. Almost 3000 consumer products based on nano materials are in the markets [6-7]. It is predicted that nanomedicine market will grow up toUSD350.08 billion by 2025 [8]. The study on concomitant release of nano materials in environment, its biological fate and hazardous effect on living system is paramount of importance. Nano particles (NPs) can be incorporated to the living system by intake through the lungs, guts, skins or direct administrations through intravenous injection (e.g. Fe_3O_4NPs as contrasting agent in magnetic resonance imaging, Lipid NPs in drug delivery) [9-13]. The interactions between NPs and biomolecules can be the basis of medicines and toxicity. Incorporated NPs come in contact with biological fluids such as blood, lungs epithelial lining fluids, proteins and other biomolecules [14-17]. Protein molecules on the surface of NPs are adsorbed and within 30 seconds form corona which evolves with time and proceeds towards saturation [18,19]. Human blood contains almost 3500 proteins among them only few hundreds form corona [20-26]. The composition of protein corona (PC) is not static. As the protein coated NPs migrate through the various compartments, a new corona tends to form in each new environment depending on the abundant and binding affinity of the proteins present [27-29]. The physicochemical properties of NPs are largely masked hence interactions of bare NPs with biomolecules are largely changed on formation of corona around the NPs [30]. PC largely alter the physical properties of NPs like zeta potential, morphology, aggregations and modulates interactions of NPs with biomolecules, their intracellular translocations, kinetics and reactivity [31-35]. The dynamic protein exchange in corona suggests the binding of protein on the surface of NPs is not as much strong as covalent bonds [36]. The study of the protein corona formations with each of the sheer existing nano materials is not possible, hence a comprehensive understanding is necessary on (i) binding kinetics (ii) surface properties of the NPs (iii) nature of the protein molecules and their conformations. It is still unclear how corona first form upon contact with NPs and physicochemical properties of the NPs and proteome of the medium affect the corona formations and alter the compositions. The meticulous investigations suggest the corona formation has resilient effect on the circulation, biodistribution, pharmacokinetics and pharmacodynamics of the nanomedicines [37-39]. Thus, rigorous investigations of the PC formation are required while studying therapeutic activity, pharmacological and toxicological understanding of nano material-based agents [40]. The configurations of various proteins are found to vary among the individuals. Protein configurations are largely affected by diverse genetic makeup, epigenetic regulations, geographical inhabits, life styles and disease conditions—thus results the concept of personalized PC [41-44]. Nano particles of specific properties can be designed by characterising thePC, thus help in designing advance drug carriers [45]. Hazardous nanoparticles might be removed from the mammalian bodies by the modulation of PC causing less damage [46]. It is reported in literatures PC formation has profound impact on biological activities [47]. The protein concentrations and configurations in plasma proteome expressions were found to vary with diseases and conditions such as obesity, atherosclerosis, dyslipidaemia, diabetes, hypercholesterolemia, haemodialysis, pregnancy, rheumatism etc [48-54]. The characteristics of altered PC with disease conditions need to be considered and identification of altered PC might help in early diagnosis of many diseases. The modulation of PC might improve the efficacies of nano materials based therapeutic medicines and their clinical translations [55].The biomolecular coronas are of two types-hard corona and soft corona. The more abundant protein molecules in contact with bare nano particles form a tight binding layer (kinetical controlled binding) that are slowly replaced with less abundant high binding affinity protein molecules (thermodynamical controlled binding) and persists in medium for long time [56-57]. The exchange of the constituent protein molecules in the hard corona is very slow. A diffuse layer of the protein molecules is formed surrounding the hard corona. The protein molecules of soft corona are easily exchanged and remain in dynamic equilibrium with the vicinity. The study of hard corona is possible because of the structural stability in experimental time scale but for soft corona it is very difficult due to rapid molecular exchange [58-59].

The biomolecular corona study often needs separation of hard corona using the techniques such as centrifugation, size exclusion chromatography, magnetic separations followed by identification using mass spectrometry, liquid chromatography, gel electrophoresis and magnetic resonance [60-64]. The invitro study and analysis of nano particle protein binding interactions include analytical techniques like fluorescence correlation spectroscopy (FCS), diffused light scattering (DLS), circular dichroism (CD), atomic force microscopy (AFM), confocal laser scanning microscopy (CLSM), confocal Raman microscopy (CRM), surface plasmon resonance (SPR) etc [65-70]. For the ex-situ study, the used techniques are transmission electron microscopy (TEM), mass spectroscopy (MS), gel electrophoresis, criyo-TEM or

inductively coupled mass spectroscopy (ICPMS) [61, 63, 68, 71,72]. The major disadvantage for ex situ study is isolation of the protein coated NPs is required, thus the original environment is changed and during isolation using the analytical methods such as centrifugation, some of the compositions may be changed. Positron emission tomography (PET) or single photon computed tomography (SPECT) is exploited to in vivo estimation of radio labelled nano materials [73-74]. The ultimate fate of the incorporated NPs can be studied by estimating the NPs and PC. Total internal reflection microscopy (TIRF) is exploited to study the membrane translocations of the NPs [75]. Isothermal titration (ITC) measure enthalpy changes and stoichiometry of protein binding [76]. Each of the techniques has their own advantages and disadvantages. Choice of the methods depend on the properties of NPs and adsorbed proteins. Combination of many methods depending on the properties of NPs, proteins and medium provide more information about the insights.

A growing recent trend is the exploitation of FCS in delineating the binding interactions of protein molecules with NPs. FCS is an ultra-sensitive, non-invasive, single molecular spectroscopic technique [77]. It allows accurate measurement of kinetic and thermodynamic parameters in complex environment using femtoliter sized confocal volume [78-79]. So far FCS is exploited in studying protein conformations, immunoassay, single cell analysis, DNA hybridisations and so on [80-83].

A brief theoretical background of FCS

The statistical autocorrelation function is the basis of FCS [79]. The fluorescence intensity fluctuations arise due to diffusion of the probe molecules into and out of the very small (femtoliter) volume elements (prolate ellipsoidal confocal volume) [84]. The sample concentration used in FCS experiment is very small (nanomolar to picomolar order) so that at a time the number of molecules in the confocal volume become very less [85]. The auto correlation function is defined in the form of fluorescence intensity measured at time t and delayed by τ as,

$$G_D(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \quad \text{.....(1)}$$

A solution of the autocorrelation function for the single species diffusion is

$$G_D(\tau) = \frac{1}{N\left(1+\frac{\tau}{\tau_D}\right)\left(1+\frac{\omega^2\tau}{\tau_D}\right)^{\frac{1}{2}}} \quad \text{.....(2)}$$

The detection volume assumed to have three dimensional gaussian shapewith axial size r_0 and z_0. $\omega = \frac{r_0}{z_0}$, i.e. axial size ratio. N is the average number of diffusing species. $N=CV_{eff}$.C is the concentration of diffusing fluorescent species and $V_{eff} = \pi r_0^2 z_0$. τ_D is the correlation time of translational diffusion. τ_D is related with diffusion coefficient as follows,

$$\tau_D = \frac{r_0^2}{4D} \quad \text{.....(3)}$$

The diffusion coefficient of the species is related with the size of the diffusing species by Stokes-Einstein equation,

$$D = \frac{KT}{6\pi\eta r_H} \quad \text{.....(4)}$$

Measuring τ_D and with the help of equation (3) and (4), size of the diffusing species (hydrodynamic radius, r_H) can be determined.

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Ignoring the chemical kinetics, this idea can be extended for more than one diffusing species and considering linear combinations, equation (1) can be written as,

$$G_D(\tau) = \frac{\sum_i^m \bar{F}_i^2 G_i(\tau)}{\sum_i^m \bar{F}_i^2} \quad \text{.....(5)}$$

The data fitting for FCS can be done either by conventional methods or by maximum entropy method analysis (MEMFCS) developed by Maiti et al [86-89].

While exploiting FCS as analytical technique in studying the nano particle protein interactions, either fluorescent nano particles or fluorophore tagged proteins (if proteinis non fluorescent) are required.

Nano particle protein binding

Binding of NPs with protein molecules can be described by Hill equation.

$$N + P \rightleftarrows NP$$

Inequilibrium the fraction of bound NPs,

$$Y = \frac{[NP]}{[N]_{tot}} = \frac{[NP]}{[N]+[NP]}$$

Equilibrium binding constant, $K = \frac{[NP]}{[N][P]}$

$$[P]_{tot} = [P] + [NP] \text{ or, } [P] = [P]_{tot} - [NP]$$

$$\text{and } [N]_{tot} = [N] + [NP] \text{ or, } [N]_{tot} = \frac{[NP]}{K[P]} + [NP]$$

$$\text{or, } [N]_{tot} = \frac{[NP]}{K\left([P]_{tot} - [NP]\right)} + [NP]$$

solving the resulting quadratic equation,

$$Y = \frac{\left([N]_{tot} + [P]_{tot} + K^{-1}\right) - \sqrt{\left([N]_{tot} + [P]_{tot} + K^{-1}\right)^2 - 4[N]_{tot}[P]_{tot}}}{2[N]_{tot}}$$

In the limit, $[P]_{tot} >> [N]_{tot}$,$[P]_{tot} \approx [P]$, the simplified form of the above equation is

$$Y = \frac{[NP]}{[N]_{tot}} = \frac{[NP]}{[N]+[NP]} = \frac{K[P]_{tot}}{1+K[P]_{tot}}$$

This equation is known as the limit of so-called Hill equation, when n=1

The generalised Hill equation takes the form, $Y_{Hill} = \frac{K[P]_{tot}^n}{1+K[P]_{tot}^n}$

Hill himself has not given any physical interpretation ofn. In the Hill equation n is the measurement of co-operativity. Whenbinding of two proteins are not influenced by each other, then n=1.If binding of one

protein increase the binding affinity of nanoparticles for other protein then n>1 (positive cooperativity) and vice versa. If n=1, binding of two proteins is independent of each other. The major limitations in Hill binding equation are the binding was assumed to be reversible and occurs without any intermediate steps, that may not always be true. Only for the cases of strong positive cooperativity, n provides a lower limit of the number of binding sites and control the steepness of the binding curve [90-94].

From Hill binding model, $$R_H = R_0\left(1+\frac{cZ_{\max}}{1+\left(\frac{K_D^{'}}{[P]}\right)^n}\right)^{1/3}$$

Here R_0 is the radius of bare nano particles, $c=V_p/V_0$ is the volume ratio of protein and nano particle, Z_{max} is the maximum number of adsorbed protein molecules. [P] is the concentration of free protein in solution, K_D' is the concentration in the midpoint of binding curve (obtained by plotting Y_{Hill} against [P]). $K_D^{'} = \sqrt[n]{K_D}$ wheren number of protein molecules bind with one nano particle, K_D = equilibrium dissociation constant [95].

The FCS measurement is carried out in presence of large number of protein molecules so that the number of unbound protein molecules remain almost unchanged. The average hydrodynamic radius of nanoparticle protein complex (R_H) and bare nano particle (R_0) is determined, hence the corona thickness $\Delta R_H = R_H - R_0$ is found.

Studies of Protein corona using FCS

It is difficult to study the protein corona formations with large number of nano particles that are synthesized on daily basis but a generalised comprehensible understanding is necessary. One of the interesting in situ applications of FCS is the study of protein corona. In biological matrix the incorporated nanoparticles absorb various molecules like proteins, lipids, sugars resulting the increase of the size of NPs, hence diffusion time also increases. The change in diffusion time can be easily measured in situ. Albumin is the most abundant blood protein. Rocker et al studied the adsorption of HSA onto the amphiphilic small polymer coated FePt NPs. From their study it was found HSA molecules bind in the negatively charged surface of NPs with micromolar affinity and form protein corona with 3.3 nm thickness. The monolayer of proteins around NPs was found to form up to 800 µm concentrations of HSA and the diffusion time increases until saturation. From the time resolve fluorescence study the measured residence time of protein in the surface of NPs is ~100s. The protein monolayer formation was also observed with the same amphiphilic polymer coated CdSe/ZnS NPs and HSA but the binding was comparatively weaker. The kinetic parameters such as Hill's constant n, dissociation coefficient $K_D^{'}$ are determined. Jiang et al also conducted a similar study of human transferrin (Tf) adsorption onto the same amphiphilic polymer coated FePt NPs and observed the monolayer thickness 7 nm with binding affinity ~26 µm and larger $K_D^{'}$ compared to the case in HSA. The lesser affinity of Tf compared to HSA for FePt is due to the presence of charged residue in Tf.An interesting fact from the study of Hühn et al is that both positively and negatively charged Au NPs has the same affinity for HSA. This is very contradictory to the expectation due to the fact that composition of PC mainly depends on the surface charge of NPs. The possible explanations of this anomaly may be due to the rapid exchange between proteins in serum or the negatively charged polymer causes reduction in the adsorption of protein. A commonly used surface coating polymer used for reduction of protein adsorption is polyethylene glycol (PEG). Molecular weight and surface density of PEG largely affect the stealth abilities. PEG of 5 K MW and 5% wt surface density has been found to be best efficient for reduction of protein adsorption. The protein corona thickness for non-PEGylated NPs was found to be 3 nm. On PEGylation, corona thickness become 1.5 nm i.e a large reduction of protein adsorption occurred. The formation of protein monolayer on PEGylated NPs occurs due to partial penetration of HSA in the PEG layer around NPs. Vilanova et al elucidated the dynamic nature of the Protein corona. The kinetics (10^{-3}-10^{3}s) of the protein corona formation with model plasma made of three blood proteins (Human serum albumin, Transferrin and Fibrinogen) with silica NPs was studied through the experiment, simulation and theory. A memory effect in the adsorption of protein was observed on changing the order of administrations of these three

proteins. The experimental results show that Fibronectin (FIB) adsorption on NPs surface depends on the concentration of HSA. For the highest HSA concentrations (10 mg/ml) the diffusion time as well as the time to reach the FIB in saturation increases. Another experiment with FIB shows the similar results in presence of Tf. HSA binds with NPs surface through the triangular faces containing positive charge patches. Changing of the polarity in the residues of HSA causes the change in its secondary structure consequently the binding interactions with NPs changes. An interesting experiment was carried out to study the charge modified HSA absorption onto the surface of dihydrolypoic acid (DHLA) coated CdSe/ZnS QDs (QDs-COOH).HSA was modified by ethylenediamine (HSA-am) and succinic anhydride (HSA-su) to impart the positive and negative charge. While studying the corona formation, the diffusion time for HSA-am was found to be higher compared to HSA-su because of the higher affinity of the latter for QDs-COOH compared to the former one.

FCS study can be directly performed in complex environment such as in living cells. An in vitro study of fluorescent glucose coated Au NPs was done by Murray et al. The diffusion time of the NPs was found to increase inside the cell due to the increase of hydrodynamic radius. The incorporated NPs in HepG2 cells aggregate and diffusion time exceeds the technique limit. Silvestri et al investigated the impact of surface chemistry of Au NPs onits state of aggregation, interactions with biomolecules such as proteins and ultimate biological fate. They functionalised the surface of Au NPs with mercaptosuccinic acid (Au-MSA), N-4-thiobutyroil glucosamine (Au-glucosamine), HS-PEG5000 and HS-alkyl-PEG600 and studied the protein corona formation in cell media and internalisation in A549 cells. In a culture cell media and in live cells protein corona formation, aggregations, intracellular behaviour of these AuNPs are investigated. The PEGylated Au NPs was found to absorb less amount of proteins and has low tendency of agglomeration both in living cells and in media. The HS-alkyl-PEG600 coated Au NPs absorb protein four times less compared to MSA coated Au NPs and is very less prone to intracellular aggregation. From the practical point of view, the engineering of the NPs with molecules like PEG will avoid or decrease the interactions with protein as well as decrease the aggregation of NPs in the cell, hence there will be an impact on NPs translocation that will affect their applications in targeted drug delivery.

Driving force for protein adsorption on nano particle surface

The electrostatic interaction between NPs and protein molecules profoundly affects the protein corona formation. The negatively charged NPs surface electrostatically interact with the positively charged moiety of proteins. Due to very high ionic strength of the biological medium (150mM), Debye screening length is <1, hence local charge distribution (not overall charge)determines the electrostatic attachment. The protein molecule like HSA is overall negatively charged and in its conformational structures triangular faces are present containing positive charge patches. The correlation of corona thickness with the positive charge density of proteins and negative charge of NPs clearly indicates the electrostatic interactions. Therefore, protein molecules (like ApoE4) having large positive charge patches bind strongly in the NP surface. The evidence of electrostatic interaction can be established by charge modification of proteins. The Dihydrolypoic acid capped QDs bind much more strongly with succinylated HSA compared to the aminated one. For negatively charged QDsand charge modified HSA, binding affinity follows the order: HSA-su<native HSA<HSA-am.

Thermodynamics and binding reversibility

The inevitable interaction of NPs with the biomolecules is the precursor of NPs-protein corona formation. Some of the NPs bind weakly and reversibly some bind very strongly and almost irreversibly. The binding of protein molecules with NPs is enthalpy driven. For the binding of Carboxyl and PEG capped ZnS/CdSe NPs with the model protein BSA, Gibb's free energy, enthalpy and entropy was found to be negative. The reversibility of the binding can easily be checked by dilution method. FCS allows in situ measurement therefore the decrease in the size of protein coated NPs on dilution allows to understand the desorption of proteins, hence dissociation of PC. Corona formation for the DHLA-QDS with HSA was found to be fully reversible. NPs coated with Zewtter ions, D pennicilamine and nearly neautral polyethylene glycol were found to form irreversible corona. In mixtures, proteins having less affinity for solvents bind strongly with NPs. For various types of NP se.g Au, Ag, Fe_3O_4, CoO, CeO_2 in serum it was found to occur the formation of soft corona followed by dissociation and formation of hard corona and desorption of proteins was not found on dilution. The adsorption of proteins on NP surface was found to be highly dependent on physicochemical properties (e.g size, shape, morphology, charge, surface coating, hydrophobicity) of the NPs, the conformations of the proteins and composition of the biological medium. The affinity of the

proteins for the NPs determine the residence time of proteins on the NP surface, as proposed by the Vroman effect and largely dictate the formation hard and soft corona.

Many researches have been conducted to study the protein NP interaction including Au, FePt and polymer nanoparticles and plasma proteins including serum albumin, Gama globulin, transferrin, fibrinogen and apolipoproteins. Many factors such as temperature, pH, surface properties of the NPs and its morphology, composition of the biological medium and even protein-protein interactions likely to affect the protein corona compositions.

Effect of temperature and pH on protein nano particle interactions

Normally mammalian body temperature is 37°C. In fever condition body temperature may raise up to 40°C. Cell temperature in certain cases may be up to 50°C. For the safety study of administered NPs, study of the effect of temperature on nanoparticle protein binding is important. With the increase of temperature, the dissociation of protein corona complex increases, therefore lowering of temperature should favour binding. Mehmudi et al has experimentally found that most favourable temperature for binding of HSA and Tf is 43°C. With increase of temperature translational entropy increases and protein molecules undergo conformational modifications, hence change of potential energy landscape and optimum temperature of binding arise. The thinnest corona was foundat 43°C.

Morphological effect of Nano particles on protein corona formation

The nature of protein corona and quantity of its formation largely depend on the physicochemical properties e.g. shape, size, surface charge, zeta potential and hydrophobicity of the relevant NPs. The smaller size NPs tend to adsorb less amount of protein due to higher degree of surface curvature and form thick and tight corona whereas larger size NPs tend to adsorb more proteins and the protein molecules are more evenly layered. From the study of polystyrene NPs, it was found both size and surface charge affect the corona formation. Nanoparticles of rod-shaped structure are more prone to adsorb proteins compared to the spherical one. The surface charge or zeta potential of NPs plays a vital role in binding proteins having positive or negatively charged residues. It was found in literature report that positively charged NPs efficiently adsorb proteins of PI<5.5 and negatively charged NPs are more prone to adsorb proteins of PI>5.5. Generally, Protein molecules are more strongly adsorbed on the surface of hydrophobic NPs as compared to the hydrophilic NPs. Proteins like serum albumin and haemoglobin fetal subunit beta strongly prefer binding on the surface of hydrophobic NPs although some of the proteins like vitronectin and antithrombin III has more affinity for hydrophilic NPs, talin 1 and prothrombin are more prone to bind with medium hydrophobic NPs and apolipoprotein has been found to bind with all kind of NPs. The attachment of proteins on the surface of hydrophobic NPs lead to the formation of new surface modified NPs resulting the disruption of old stabilizing interactions such as hydrogen bonding, Vander Waals force of attractions, hence a loss of protein conformation occurs. The porosity of NPs decreases protein adsorption due to size exclusion. As discussed earlier, Coating of NPs with (polyethylene glycol (PEG) or polysaccharides minimize the protein adsorption.

Success of FCS

FCS is mathematical descendants of Quasi-elastic light scattering spectroscopy (QUELS). In both QUELS and FCS very small probe concentration is used. FCS is highly sensitive in single molecular level detection, characterisation, studying mechanical properties of biomolecules such as folding and unfolding of proteins and binding of biomolecules and relevant kinetics. The fluctuations of fluorescence intensity are fitted into the correlation function which give the instantaneous number of molecules passing the confocal volume and the size of the complex. Thus, directly measuring the size of bare NPs and protein adsorbed NPs, the corona thickness is measured from the differences in their size. However, in case of NP-Protein interaction study using FCS, either NPs or proteins must be fluorescent. FCS technique exploit incoherent emission while the similar technique DLS exploit incoherent scattering of light. The major disadvantage of the DLS technique is all the components in the sample scatter the light resulting the disturbance in measuring the diffusion coefficients. FCS and DLS techniques have their own advantages and disadvantages. FCS technique is suitable for fluorescent NP or protein and DLS for non-fluorescent molecules.

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CONCLUSIONS

The complete understanding of the triggered physiological response on NPs exposure is required to understand the bio-compatibility of NPs. Protein corona formation has large impact on designing future generation nanomedicines since it affects the blood circulation time, bio distribution, targeted drug delivery and biodegradations. The interaction of NPs with protein molecules may divert the protein folding pathways and diseases like Alzheimer, Parkinson etc. that occurred due to protein misfolding could be successfully averted, hence the rational idea of NPs based drug designing for neurovegetative diseases is emerged. The major progress in the study of protein adsorption on NP surface is very recent and all it is due to the availability of many sophisticated techniques including FCS. Still there is a large lacuna in complete understanding. We are optimistic to become able to understand sufficiently through the multidisciplinary approach, elegant combinations of powerful analytical techniques with theoretical modelling, high throughput technologies, data mining and bioinformatics. We speculate the delineation of complete nano-bio interactions will be the major breakthrough in designing future generation bio compatible nano materials.

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