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Research review paper

In-vitro in-vivo correlation (IVIVC) in nanomedicine: Is protein corona the missing link?

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ABSTRACT

One of the unmet challenges in nanotechnology is to understand and establish the relationship between physicochemical properties of nanoparticles (NPs) and its biological interactions (bio-nano interactions). However, we are still far from assessing the biofate of NPs in a clear and unquestionable manner. Recent developments in the area of bio-nano interface and the understanding of protein corona (PC) has brought new insight in predicting biological interactions of NPs. PC refers to the spontaneous formation of an adsorbed layer of biomolecules on the surface of NPs in a biological environment. PC formation involves the spatiotemporal interplay of an intricate network of biological, environmental and particle characteristics. NPs with its PC can be viewed as a biological entity, which interacts with cells and barriers in a biological system. Recent studies on the bio-nano interface have revealed biological signatures that participate in cellular and physiological bioprocesses and control the biofate and toxicity of NPs. The ability of in-vitro derived parameters to forecast in-vivo consequences by developing a mathematical model forms the basis of in-vitro in-vivo correlation (IVIVC). Understanding the effect of bio-nano interactions on the biological consequences of NPs at the cellular and physiological level can have a direct impact on the translation of future nanomedicines and can lead to the ultimate goal of developing a mathematical IVIVC model. The review summarizes the emerging paradigms in the field of bio-nano-interface which clearly suggests an urgent need to revisit existing protocols in nanotechnology for defining the physicochemical correlates of bio-nano interactions.

1. Introduction

The study on protein corona (PC) has led to a paradigm shift in the field of nanomedicine which has opened new avenues for the translation and safe usage of nanoparticles (NPs). The spontaneous formation of a PC on the NP surface in body fluids is now believed to be a critical determinant of the biofate of NPs (Lundqvist et al., 2008). Several years of research on NPs has failed to determine specific conclusions regarding the physicochemical correlates of their biological activity. Current in-vitro data of NPs poorly translate into in-vivo predictions (Shcharbin et al., 2014). Toxic effects of NPs have also raised some concerns and the cause for these effects is still largely unknown. The growing interest in the study of PC is due to its possible correlation with several important phenomena in biological systems (Albanese et al., 2014). Several challenges encountered in nanotechnology research are due to poorly designed experimental protocols. In order to obtain reliable and realistic data, existing protocols in nanotechnology must be carried under physiologically relevant in-vitro conditions taking into account the interactions occurring at the bio-nano interface. Therefore, gaps in knowledge still remain.

The basic aim of this review is to provide researchers working on basic and translational nanomedicine with a mechanistic understanding of the bio-nano interface and its role in developing an in-vitro in-vivo correlation (IVIVC) model. We also focus on the lacunae in the experimental designs, now used in nanotechnology which may be responsible for a poor IVIVC. The review is divided into two sections. The first part deals with the effect of physicochemical property of NPs on the PC formation and the second part deals with the impact of NP-PC complex on its biological activity. An in-depth analysis of the intermolecular forces between biomolecules and NPs at the bio-nano interface is beyond the scope of the current discussion. For a comprehensive review on the biophysical aspects of PC formation, readers may refer to

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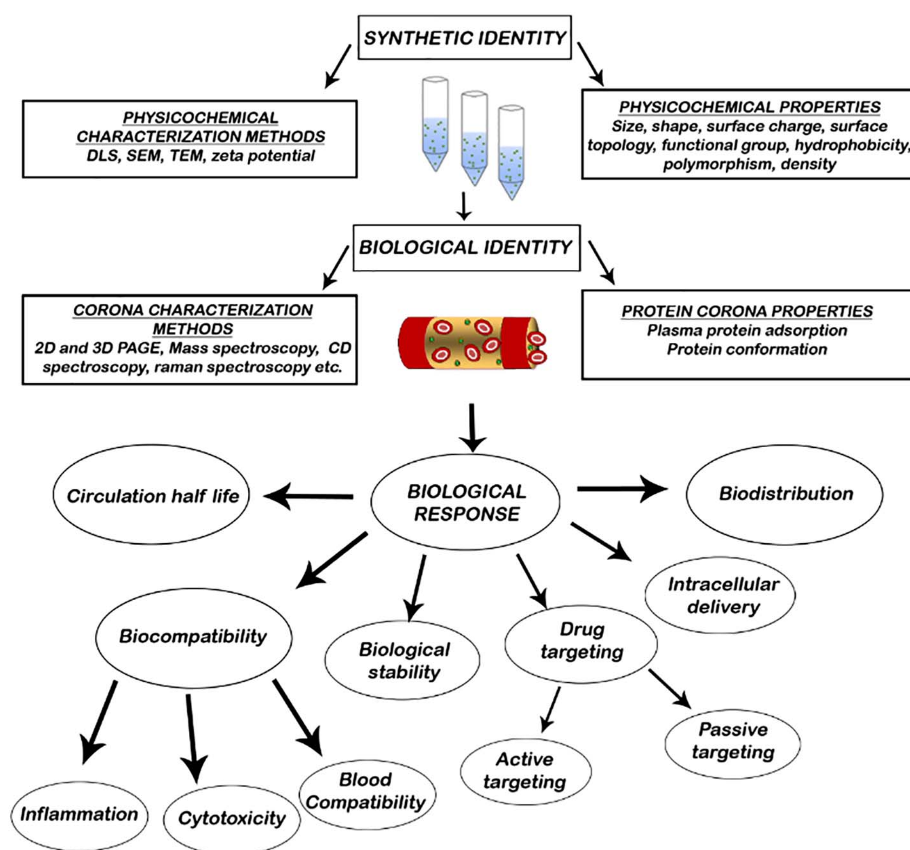

Fig. 1. Possible relationship between synthetic identity, biological identity and biological response of nanoparticles.

Nel et al., 2009. The main focus of the current discussion is on the biological consequences of bio-nano interactions and its implications on IVIVC.

2. Synthetic identity versus biological identity of NPs

The adsorbed layer of biomolecules defines the biological identity of NPs whereas physicochemical properties of NPs define its synthetic identity. The delineation of the synthetic identity and biological identity of NPs might seal the existing gap in knowledge and bring about predictability in nanomedicine research (Fadeel et al., 2013) (Fig. 1). The interest in the study of bio-nano interface gained significance by the seminal works initiated by Dawson and co-workers (Cedervall et al., 2007a,b; Lundqvist et al., 2008). Biological identity is a derived property of a NP and depends on the environment, which in the case of nanomedicines comprises of a myriad of physiological biomolecules. The PC formation shows a “memory” effect depending on the sequence of biomolecules, the NP encounters. The protein which interacts with the NP first has the largest abundance in the PC. It is hypothesized that the “memory” effect may be a consequence of initial NP-protein interaction (e.g. a conformational change in the adsorbed protein), which influences the subsequent protein-protein interaction during PC formation (Vilanova et al., 2016). The NPs with its PC can get access to otherwise inaccessible areas by biomimicking endogenous and exogenous substances and can have unanticipated consequences (Lara et al., 2017). As a matter of fact, the phenomenon of biomimicry has been used as a strategy for selective drug targeting by cloaking NPs with platelet membrane which provides a shielding effect against immune response (Hu et al., 2013b).

The characterization of the PC and its correlation with observed biological effects of NPs is currently the biggest challenge in bionanoscience. The accessibility of NPs to cells and organs throughout the body and its environmental impact makes the study of PC relevant to biological systems. A lack of a rational understanding of the biological identity may well be the “missing-link” in the development of a predictive in-vitro model. The bio-nano interface has ushered a totally new field of study less known to the scientific community and we believe that this could be a possible turning point for the safe translation of NPs. However, understanding the bio-nano interface is extremely challenging, given the complexity to replicate a dynamic physiological environment and the difficulty to adapt currently available analytical methods to these situations. Here, we systematically analyze current gap in knowledge and provide a comprehensive overview of the possible future challenges in decoding the bio-nano interface.

3. Effect of physicochemical properties of NP on protein adsorption

The surface characteristic of engineered NPs is regarded as the principle determinant of bioactivity in a targeted drug delivery. A slight change in physicochemical properties alters the biological effect of NPs for unknown reasons. This brings unpredictability in terms of the toxicity and biofate of NPs and results in poor in-vitro in-vivo correlation. The study of PC may help us in underpinning the unpredictable behavior of NPs.

3.1. Size, shape, curvature and charge effect

In the early research, the principle determinant of NP delivery and toxicity was particle size (Park et al., 2013; Xiong et al., 2013). However, now it is realized that not only size but curvature, surface chemistry, hydrophobicity, charge etc. of NPs also appear to affect protein binding and it is quite clear that size alone cannot explain the unpredictable and heterogeneous outcomes of NPs. However, size is one of the most important determinants of PC composition and therefore its biological effect. The highly curved surface and small size of NPs

decrease protein-protein interactions and oligomerization of adsorbed proteins (Klein, 2007; Kurylowicz et al., 2014). Besides, a change in the size of particles results in a consequent change in surface area and hydrophobicity. Particle size not only influences the qualitative composition of PC but also affects the quantitative component of biomolecules within the PC (Hu et al., 2014; Schäffler et al., 2013).The size of NPs also plays an important role in the temporal pattern of PC formation demonstrated by a quick formation of PC on smaller sized NPs in comparison to larger particles (Piella et al., 2017). Therefore the formation of PC is a size driven kinetic process resulting in the formation of the multilayered PC.

There is a strong dependence of NP charge and protein identity on the formation of PC. Positively charged NPs shows affinity towards proteins with isoelectric points (pI) < 5.5 while the negatively charged NPs have an affinity towards proteins with pI > 5.5 (Gessner et al., 2002). Higher curvature interferes with binding of proteins on smaller size NPs. Depending on the experimental procedures, a difference in qualitative composition of PC on NPs can lead to significant differences in the estimation of dissociation coefficient (Cedervall et al., 2007a, 2007b). In a comparison of > 130 experiments for the determination of dissociation coefficients, it was found that different methods, NP systems, and proteins can lead to significant differences in the estimation of dissociation coefficient (Hühn et al., 2016). A critical observation in this regard is a clear tendency of positively charged NPs towards smaller dissociation coefficients. The surface charge of NPs could, therefore, be a key predictor of PC composition. A systematic evaluation of surface charge density of surface modified magnetite NPs in cell culture medium shows a fivefold increase in the particle size within few minutes of incubation (Calatayud et al., 2014). The final properties of the NPs (with the adsorbed PC) are in stark contrast to the actual particle size and charge, which highlights the need to characterize the final properties of the NPs in a physiologically relevant biofluid.

Very few studies have reported the effect of shape on the formation of PC (Jansch et al., 2013). Irregular shaped NPs have been shown to significantly lower the uptake in the human macrophage cell line. NP shape also influences the toxicity of NPs. The toxicity of rod-shaped silver NPs was shown to be toxic whereas rod-shaped NPs with the same mass concentration were found to be safe on the human lung epithelial cell (Stoehr et al., 2011).

3.2. Effect of protein corona on drug loading and colloidal stability

Depending upon the agglomeration kinetics and subsequent sedimentation, NPs may lose their inherent physical properties (Cho et al., 2011). NPs in physiological condition can lose its stability due to the prevalent ionic concentration and adsorption of biomolecules resulting in self-assembly of NPs on biomolecules acting as a template. Self-aggregation of metal NPs may complicate interpretation of NP-cell interaction in aqueous media. The aggregation of metal NPs can be prevented by pretreatment with serum (Wells et al., 2012) which may be due to charge based repulsion imparted by albumin present in serum (Rezwan et al., 2004). Changing the coating around NP has a profound effect on colloidal bio-stability of NPs. Preforming a bovine serum albumin (BSA) PC can enhance the NP stability and can help in long circulation (Peng et al., 2015). Protein adsorption also prevents nucleation and growth of NPs and improves biostability (Müller et al., 2011). Adsorption of β-LG, a globular protein improves stability, enhances cytosolic delivery and was found to improve the pharmacokinetics of paclitaxel (Li et al., 2015). Iron oxide NPs coated with citrate ions is de-stabilized in all fetal-calf-serum whereas an outstanding stability and a non-fouling surface were observed on polymer coated NPs (Safi et al., 2011). Citrate-stabilized gold NPs demonstrated remarkable stability under harsh hypertonic conditions only when bovine serum albumin was adsorbed on NP surface (Dominguez-Medina et al., 2013).

PC can also be exploited for improving the drug loading capacity much higher than conventional loading methods. PC of serum proteins formed on gold nanorods stabilized with cetyl trimethyl ammonium bromide (CTAB) has been used to load DNA oligonucleotide and an anticancer agent, doxorubicin at a much higher capacity (Kah et al., 2012). The rate of DNA release from NPs can be customized by manipulating the PC (Rius et al., 2013).

3.3. Surface chemistry effect

Protein adsorption can be tailored by varying the surface functional groups on NPs. The gold standard for controlling surface properties and maintaining corona free condition is with the use of polyethylene glycol (PEG) to obtain the so-called "stealth" behavior. Manipulating the surface functional group and functional group density on NPs can affect the pattern of protein adsorption. Metal NPs with similar surface charge bind to different plasma proteins (Deng et al., 2009). The surface functionality of NPs and the composition of amino acids on adsorbed proteins demonstrate a direct statistical correlation with protein adsorption on the surface of colloidal alumina NPs (Meder et al., 2013).

Studies have shown that surface chemistry of NPs is an important factor affecting the evolution and composition of PC on NPs. The PC on the surface of native SiO_2 NPs, amine ($-NH_2$) and carboxy ($-COO(-)$) modified SiO_2 NPs shows a unique composition due to a difference in surface functionality. The difference in composition of PC translated into different physicochemical and biological property of NPs. Carboxy-modified NPs demonstrates high stability in terms of agglomeration rate, the size of aggregates and lower toxicity on cells as compared to unmodified and amine modified counterparts (Mortensen et al., 2013).

The arrangement of lipid functional groups is a key regulator in liposome–protein interactions. A minor change in the composition of lipid (in liposomes) substantially affects the PC composition (Pozzi et al., 2015). Fibrinogen absorption on biomaterial is found to be very sensitive to small changes in chemical composition (Weber et al., 2004). The protein concentration has a direct effect on the hydrodynamic radius of NPs with different surface chemistry (carboxyl and amino group) which is independent of their zeta potential (Maffre et al., 2014). The surface functional groups have an influence on the average surface charge on NPs. The effect of zeta potential vis-a-vis the spatial arrangement and accessibility of the charged functional group show that the different relative spatial arrangement of the amino group on the surface of physicochemically similar mesoporous silica NP (in terms of size shape and ζ potential) was found to have a profound influence on its in-vitro and in-vivo interaction with endothelial cells and WBCs (Townson et al., 2013). Interaction with serum proteins in-vitro is significantly enhanced by the exposure to even small amount of surface amine group which results in a rapid in-vivo clearance from circulation. Manipulating the surface functional groups can therefore, change the biological outcomes of NPs.

3.4. Effect of hydrophobicity

NPs can be engineered to prevent the exposed surface from adsorbed protein by modifying surface hydrophobicity such that the biological property of NPs is governed by its physicochemical characteristics rather than its adsorbed protein layer. Zwitterionic NPs of variable hydrophobicity were fabricated by Moyano et al., which do not form hard coronas at physiological serum concentrations (Moyano et al., 2014). Hydrophobic NPs shows high adsorption of albumin than hydrophilic NPs, even though the affinity of albumin was comparable towards both types of NPs (Lindman et al., 2007). The surface curvature, surface topography and hydrophobicity affect the denaturation state of the adsorbed proteins and this effect has been found to be compounded by the surface chemistry of NPs (Roach et al., 2006). Fibrinogen was found to be more sensitive to curvature induced denaturation than albumin. The small size of silver NPs (20 nm) results in PC of more hydrophobic proteins compared to larger sized NPs (100 nm) (Shannahan et al., 2013). These hydrophobic proteins show curvature

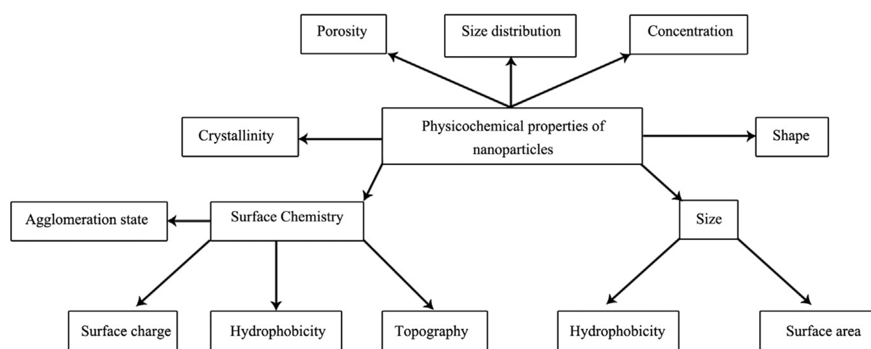

Fig. 2. Physicochemical characteristics of nanoparticles and their interdependence.

induced folding thereby shielding the NPs in the aqueous environment. Electrostatic and hydrophobic interactions (depending on the surface chemistry) play a significant role in the qualitative and quantitative composition of PC which can have broad nanotoxicological consequences.

The physical property of NPs should not be viewed in isolation as it is the interplay between different physicochemical properties which dictates the adsorption of biomolecules. The adsorption of proteins on NPs should be seen as an overall effect of the physicochemical properties (Fig. 2). A complete physicochemical characterization of NPs applying a broad range of methods is of utmost importance. A careful consideration of surface structure may allow us to tailor the materials/surface coatings with customized bioactivity.

4. Protein corona and its biological implication

4.1. Plasma protein binding

The NPs injected into blood is bound by plasma proteins instantaneously forming a PC. A "hard corona" formed on NP surface is attributed to the formation of irreversible protein binding whereas a quick reversible fast exchange of biomolecules forms the "soft corona" (Cedervall et al., 2007a, 2007b). The identification and stoichiometric studies of PC could be technically challenging due to the dynamic nature of PC (Cedervall et al., 2007a, 2007b). A qualitative difference in the number of plasma proteins forming the PC has been observed using different tools to analyze the full repertoire of NP-binding blood proteins (Docter et al., 2014b; Simberg et al., 2009; Pozzi et al., 2014b). The effect of protein adsorption on the different biological activity of NPs and their role in the development of an IVIVC model are highlighted below.

4.2. Effect on coagulation cascade

PC has been linked to the procoagulant activity of materials. The amine modification of silica NPs prevents abnormal activation of the coagulation cascade as observed in the unmodified silica NPs after systemic exposure in mice (Yoshida et al., 2013). The interaction between unmodified silica NPs and intrinsic coagulation factors such as factor XII have been found to be deeply related to its harmful effects (Nabeshi et al., 2012). Intranasal exposure of these unmodified NPs also activates the intrinsic coagulation cascade resulting in induction of an abnormal activation of the coagulation system (Yoshida et al., 2015). Surface modification with amino group inhibits the adsorption of factor XII. The amount of serum proteins (albumin, transferrin, and IgG) bound to amino group functionalized NP has been found to be lower than that with unmodified NPs (Hata et al., 2014).

The in-situ generation of thrombin in the PC and zeolite structure of Ca-zeolite is found to be critical for its high procoagulant activity, which is not achieved by naked zeolites (Li et al., 2014). The clotting time of the Ca-zeolite in plasma (to form a hard PC) has been found to be shortened by a factor of 9 in comparison to the Ca-zeolite without a hard corona. The abundance of thrombin in the PC of Ca-zeolite was found to be about 650 times higher than that in the Na-zeolite composite, which shows that the proteins forming the PC is a major determinant of biological activity.

4.3. Effect on protein conformation

Changes in protein conformation caused due to exposure of cryptic peptide epitopes after its interaction with NPs can have unanticipated effects, with potential for immunotoxicity and inflammation in cells (Shemetov et al., 2012). The protein crowding on NP surface results in the formation of oligomers which may provoke avidity effect. NPs are known to promote or inhibit protein fibrillation of adsorbed proteins. Fibril formation occurs by nucleation which is a key determinant of fibrillation. The enormous surface area presented by NPs decreases the lag time for the formation of a protein nuclei and therefore, enhances the rate of protein fibrillation (Linse et al., 2007). The conformation of the protein at the bio-nano interface depends on properties of biomolecules, the microenvironment and the NP in question.

Protein conformation of adsorbed proteins in situ also correlates with the biological functionality of NPs. The conformational change in BSA, adsorbed from cell culture media reduces the membrane adhesion of NPs and thereby reduces internalization efficiency in human monocytic cells, THP-1, compared with the bare NPs. On the contrary, the unfolded BSA on the surface of nanoporous polymer NPs triggers receptor-mediated uptake in macrophage-like cells (dTHP-1) without a significant impact on the overall internalization efficiency (Yan et al., 2013). Similarly, the conformational change of adsorbed proteins causes receptor-mediated endocytosis of modified quantum dots (Prapainop et al., 2012). Change in the conformation of PC can, therefore, be used for cell-specific targeting.

4.4. Protein corona and its effect on biocompatibility

Biocompatibility is the property of being nontoxic i.e. free from inflammatory response and cell injury (Baguneid et al., 2006). Studies on PC can give us important information on the biocompatibility of NPs.

4.4.1. Effect on cell uptake

The study on PC has led to a paradigm shift in the way we look at how NPs are taken up by cells. NPs encounter cells with a dynamic PC which can significantly influence the ensuing sequence of events including cellular uptake, internalization, and pathway activation. This knowledge can be used for customizing the selectivity of NPs to avoid adverse health effects by enhancing or suppressing cellular uptake.

Kinetics of PC formation in a highly dynamic and complex biological environment is still poorly understood, however, efforts have been made in this direction (Casals et al., 2010; Ehrenberg et al., 2009). In order to bridge the gap between in-vitro studies carried out in a

controlled cell culture plate and a dynamic in-vivo environment with a multitude of competing proteins for adsorption on NPs would require novel simulation approaches. It has been demonstrated that shear stress plays a critical role in NP uptake by endothelial cells (Samuel et al., 2012). Physiologically relevant in-vitro experimental models are required to simulate in-vivo conditions. The central nervous system in this context represents a special challenge due to the co-existence of multiple cell types. The dominant cells in the immediate neural environment show rapid uptake due to which uptake by other cell types are suppressed (Pinkernelle et al., 2012). A dramatic difference in NP uptake between cell subtypes has been observed, with astrocytes showing maximum uptake (Fernandes and Chari, 2014). Isolated monoculture system often shows over-estimates of cell uptake as compared to multicellular environments and has limited biorelevance.

The choice of a cell growth media and anticoagulant for the collection of plasma can also affect the cellular uptake and the PC formed on NPs (Schöttler et al., 2016). The uptake of NPs is enhanced in macrophage cells whereas it is suppressed in HeLa cells due to the presence of heparin in media. The qualitative and quantitative aspect of PC depends not only on the physicochemical properties of NPs but also on the concentration of serum/plasma used. The serum concentration determines the internalization of amino-modified polystyrene NPs which correlates with its toxicity on cells (Kim et al., 2014). The in-vivo concentration of plasma proteins (i.e. 100%) is essential for a strong binding of fibrinogen to zeolite NPs. Importantly, the number of proteins bound to NP surface was found to be much less at 100% plasma exposure than with 10% plasma (Rahimi et al., 2015). The protein concentration in the immediate microenvironment of NPs can affect their colloidal stability, the conformation of the adsorbed proteins and cellular uptake of NPs. Pre-incubation of cationic gold NPs in 1% w/v BSA was found to increase the cellular uptake by three folds in MCF-7 cell lines as compared to NPs in 10% FBS (Dominguez-Medina et al., 2016). The concentration of protein and protein-to-NP ratio determines the physical chemistry of the PC. At a low protein to NP ratio, a non-equilibrium mechanism is found to exist resulting in irreversible protein adsorption on NPs. This is followed by BSA unfolding and the interaction between unfolded BSA molecules drives the process of NP aggregation. Even small amount of plasma proteins can result in a change in the secondary structure of bound proteins and may influence the biological fate of NPs (Dominguez-Medina et al., 2016).

Physical chemistry of PC on NPs can also be influenced by heat inactivation of serum. Cellular uptake of NPs of the same size was found to be significantly different when tested in a medium where the serum was heat inactivated (Lesniak et al., 2010). Hence, standardization of PC formed on NPs in a defined media is a critical part of NP testing which would otherwise result in variation among different experiments (Table 1). The interaction and response of NP towards cells depend on the cell-type, their origin and their evolutionary mechanisms of detoxifying foreign particles. This concept has been termed as the cell "vision" (Mahmoudi et al., 2012). The types of cell that a NP encounters determine the intracellular events and subsequent fate of NPs including its toxicity. This property of cells makes toxicity prediction of NPs complicated as NPs which are nontoxic to certain cell types may be toxic to other cells of a different origin. The implication of this concept has broader consequences on the environmental impact of NPs as cells of different origin are continuously exposed to a host of synthetically generated NPs.

Pristine NPs can strongly adhere to the cells and form a PC consisting of mainly proteins normally associated with the cell membrane and cytoskeleton as a result of cell damage which was found to be independent of their composition or surface modification (Lesniak et al., 2012). Interestingly, none of these biomolecules were identified on NPs when cellular interaction studies were performed in a media containing serum. Computer simulation study shows that PC enhances the interaction mode of NPs based upon their charge and hydrophobicity resulting in loss of target specificity of charged NPs towards macrophage cell membrane (Ding and Ma, 2014). Therefore, care should be taken while using in-vitro data to foresee in-vivo consequences due to the fact that the multiple factors affecting cellular uptake as discussed above can significantly affect the composition of PC resulting in different patterns of cellular uptake.

The different charge on NPs results in different cell uptake pathways. Whereas, cationic NP complex binds to the scavenger receptor, its anionic counterpart binds to the native protein receptor. The uptake of FePt NPs by HeLa cells at a physiological concentration of Tf and HSA is suppressed by the formation of a protein monolayer on NPs in comparison to the bare NPs. Both the proteins individually suppress the NP uptake by different mechanisms (Jiang et al., 2010a). The identity of proteins adsorbed onto TiO_2 NPs influences its cellular uptake by fibroblast cells (Allouni et al., 2015). The highest amount of NP uptake was found to be in serum-free conditions and in the presence of human serum albumin (HSA) and lowest for culture medium containing globulins or fibrinogen. Chirality also plays a fundamental role in synthetic materials and also affects cell adhesion. Self-assembled monolayers based on enantiopure polymer shows a significant change in BSA adsorption and cell adhesion than racemic polymer covered surfaces (Li et al., 2013).

A classic example of the relevance of in-vitro methods to the in-vivo behavior of NPs was reported by Lunov et al. (2011). The study compares the cellular uptake of carboxy and amino-functionalized polystyrene NPs in two different cell culture in a buffer or in serum. Macrophage cells internalized carboxy modified NPs almost four times more

Table 1
Factors to be considered during cell culture studies to reduce variation among experimental protocols.

Factors to be considered in cell culture studies	Ref.
Physiologically relevant protein concentration should be present in the media during cell uptake studies	Dakwar et al. (2014, 2015), Kim et al. (2014), Dominguez-Medina et al. (2016)
Using mixed cell culture should be considered to mimic multicellular environments, such as the intact CNS	Fernandes and Chari (2014)
Phase of cell cycle should be assessed, which can affect the outcomes during cell uptake were internalized nanoparticle concentration is split among cells	Kim et al. (2011)
Dynamic model of cell culture should be used to mimic physiological conditions	Freese et al. (2014)
Concentration of nanoparticles used should be above a critical threshold density	Chithrani and Chan (2007), Jiang et al. (2010b), Rahimi et al. (2015)
Colloidal stability of nanoparticle in cell culture media should be taken into account	Mohr et al. (2014)
Slight change in temperature of the cell culture media can change the composition of PC resulting in a consequent change in the cellular uptake of NPs	Mahmoudi et al. (2013, 2014)
Presence and absence of drugs or anticoagulant in plasma can affect cell uptake kinetics	Schöttler et al. (2016)
Cell uptake should not be considered as a predictor of transfection efficiency	Fernandes and Chari (2014)
pH and temperature of intracellular compartments vary from each other and will affect the protein corona formation and subsequent trafficking of particles	Donner et al. (2012), Yang et al., 2011
Different cell types have different uptake, processing and detoxifying mechanism for exactly same NPs. This is known as the "cell-vision"	Mahmoudi et al. (2012), Azhdarzadeh et al. (2015)

than THP-1 cells in the presence of serum. On the contrary, a rapid internalization of amine modified NPs in THP-1 cells in comparison to macrophages were observed in either medium. When these NPs were intravenously administered, carboxy modified NPs were removed from circulation by the macrophages of liver whereas amino modified NPs were preferentially targeted to tumor xenografts. The different behavior of NPs can be associated with the differential adsorption of biomolecules due to a change in surface functionality.

4.4.2. Effect on inflammation

Polymers that render surfaces bioincompatible, i.e. inflammatory, are claimed to do so by the adsorption of specific proteins and, perhaps by influencing the conformation of those proteins once they are bound. There is a compelling need to standardize existing toxicity-assessment protocols of NPs in view of the knowledge available regarding PC. Of those proteins believed to mediate the tissue response to polymer coated surfaces, fibrinogen (Fib) seems pre-eminent (Retzinger, 1995). The effect of surface adsorbed Fib largely remain unknown as it is absent in culture media used in in-vitro toxicological tests (Marucco et al., 2016). The inflammatory functions of NPs have been attributed to the conformational change associated with the adsorption of Fib and exposure of the specific epitopes. The exposed epitopes of Fib promote interaction with the integrin receptor, Mac-1 and the activation of Mac-1 in turn increases the NF-κB signaling pathway, resulting in the release of inflammatory cytokines (Deng et al., 2011). However, not all fibrinogen binding NPs demonstrate this effect. The adsorption-induced unfolding of Fib molecules has been found to be strongly dependent on platelet adhesion onto biomaterials (Sivaraman and Latour, 2010). After binding, Fib remains susceptible to enzymes relevant both to the formation and dissolution of clots and to inflammation (Retzinger and McGinnis, 1990). On the contrary, the mechanism of hemocompatibility of thermally modified titanium dioxide NPs has been attributed to the αC domains of Fib which electrostatically interacts with the heated NPs resulting in a shielding effect of the platelet binding sites (Zhao et al., 2014). The size of TiO_2 primary NPs and particle aggregation modulate the affinity of Fib towards the TiO_2 surfaces (Marucco et al., 2013). The Fib molecule associated with SiO_2, carbon and TiO_2 NPs induces a significant inflammatory response by enhancing the cytotoxicity and the mediators of inflammation in alveolar macrophages (Marucco et al., 2016). It is, therefore, critical to the understanding of the role played by Fib in the toxic response to NPs.

The shear rate of blood also affects the adsorption of both fibrinogen (Fib) and von Willebrand factor (vWf) on biomaterials (Zhang et al., 2008). The platelet adhesion under shear flow conditions can be induced by a very low concentration of Fib. The vWf was found to be a critical factor which significantly affects platelet adhesion under high shear. Therefore manipulating surface properties to reduce adsorption of vWf can be used as an approach to improve hemocompatibility of materials (Kwak et al., 2005; Wu et al., 2008).

The concentration of NPs also affects the type of cytokine secretion by macrophages (Borgognoni et al., 2015). Proinflammatory triblock copolymers adsorb Fib on their surface, which was found to correlate with the inflammatory cytokines quantitatively (O'connor et al., 2000). The manipulation of surfaces with nonionic surfactants to reduce adsorption of albumin has been used as a strategy to inhibit the anti-inflammatory properties of the NPs. Therefore surface property of NPs can be manipulated to render it biocompatible by preventing the adsorption of selective plasma proteins (Dutta et al., 2007).

4.4.3. Effect on complement activation

The binding and activation of complement by the three general pathways is a part of the innate immune system to recognize and eliminate foreign particles including NPs. The physicochemical property determines the extent and pathway chosen by a specific NP during complement activation (Lundqvist et al., 2008). The complement system also acts as a recognition mechanism for NP clearance by phagocytosis (Owens and Peppas, 2006; Rybak-Smith and Sim, 2011).

Surface characteristic of the NPs is a major determinant of the composition of PC which acts as a recognition system for the activation and amplification of the complement system (Yu et al., 2014). The complement activity of glycopolymer-grafted NPs is attributed to the elevated levels of two pro-complement proteins (in the PC), factors B and C3, which depends on a threshold grafting density. Engineering of NP surface chemistry and topology has been used to optimize NP for immunotherapy. The surface modification correlates with in-vivo antigen-specific immune responses upon antigen re-stimulation. Therefore, NP vaccines can also be surface engineered for the activation of the complement cascade, generating a danger signal to the immune system (Reddy et al., 2007). The surface topography of NPs critically affects the activation of the complement system (Hulander et al., 2011). Coating of NPs with polysaccharides (dextran and chitosan) and albumin was found to reduce complement activation (Bertholon et al., 2006) and increase circulation time of NPs (Peng et al., 2013). Complement activation by NPs can be controlled by manipulation of surface functional groups. Surface modification of NPs with methoxy, carboxy, and amine groups has been used to control complement activation by the alternative pathway (Morales et al., 2009). Negatively charged liposomes have been shown to activate complement system via the classical pathway (Bradley et al., 1999) whereas positively charged liposomes activate the alternative pathway (Devine et al., 1994). The presence of cholesterol, encapsulated drugs and the size of liposomes contributed to complement activation by liposomes (Kuznetsova and Vodovozova, 2014). The protein binding profiles of liposomes has been found to be in agreement with the data on complement activation in invitro tests.

4.4.4. Effect on hemocompatibility

Hemocompatibility of NPs is critical for intravenously delivered NPs. Hemolysis by NPs can cause endothelial cell dysfunction, vascular thrombosis and a host of clinical manifestations related to hemoglobin release (Rother et al., 2005). Coating NPs with biocompatible polymers can decrease hemolytic toxicity. Hemolytic potential of a dendrimer formulation was found to be reduced by derivatizing with chitosan which can be safely used for intravenous applications (Zhou et al., 2015). Surface functional groups play an important role in hemocompatibility. Optimized surface functionality has been shown to reduce or even completely prevent the hemolytic activity of NPs (Yildirim et al., 2013).

The formation of a PC on the surface of NPs dramatically attenuates the hemolytic activity of hydrophobic and hydrophilic NPs (Saha et al., 2014). The conventional hemolytic assay done in the absence of plasma may provide misleading results as NPs show disparate hemolytic activity in the presence and absence of plasma proteins (Moyano et al., 2014) (Fig. 3). Porous silica NPs demonstrated negligible cytotoxic effect on RBCs when the hemolytic assay was conducted in the presence of blood plasma, regardless of the surface charge of the silica NP, which otherwise is known to induce hemolysis when cytotoxicity assays are performed in a phosphate buffered solution. The absence of hemolysis is due to the formation of PC on NPs thereby shielding the original microenvironment of bare NPs (Paula et al., 2012). A modified hemolytic assay procedure (Dobrovolskaia et al., 2008) to account for the false-positive or false-negative results due to the presence of NPs shows that the interference caused by the presence of NPs is due to adsorption of released hemoglobin on NPs during hemolysis. The adsorbed hemoglobin precipitates are removed from the supernatant by centrifugation which yields a false negative result. The inherent absorbance of some NPs at or close to the assay wavelength is an important source of interference in the hemolytic assay. However, a contrary report showing no correlation between PC and hemocompatibility has also been reported (Dobrovolskaia et al., 2014).

NPs are in a dynamic state in blood and so are the blood cells. NPs have never been subjected to hemolytic studies in a dynamic flow

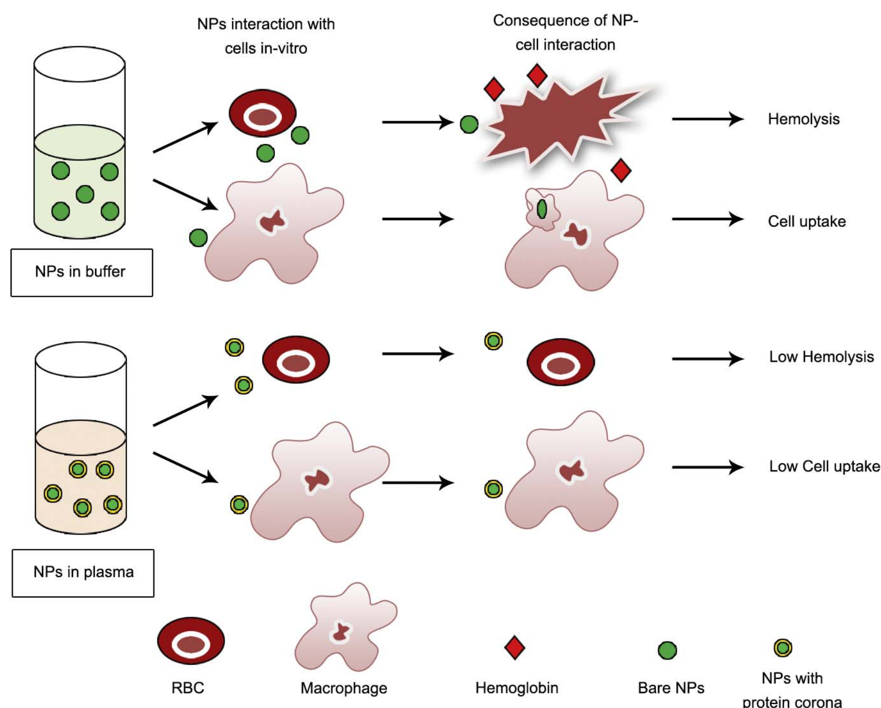

Fig. 3. Effect of nanoparticles on RBCs and macrophages in the presence and absence of protein corona.

condition. Hemolytic studies on intravenous solutions done by a dynamic in-vitro method have shown differences in hemolysis as compared to the conventional static method (Obeng and Cadwallader, 1989). A meaningful extrapolation of in-vitro data for in-vivo predictions can only be realized by using physiologically relevant and facile experimental approaches.

4.4.5. Effect on platelet aggregation

Platelets play a pivotal role in blood homeostasis. The platelet activation is very sensitive to the blood microenvironment and the properties of the NPs (Simak et al., 2009). In-vitro platelet aggregation is used to assess the thrombogenic potentials of NPs and hence is an important part of hemocompatibility testing. Previously, platelet aggregation was recognized as a process of noncovalent bridging of integrin $\alpha_{IIb}\beta_3$ or GPIIb-IIIa receptors on the platelet surface by fibrinogen. Now it is recognized that platelet aggregation is a multi-step procedure involving a host of ligand and receptors dependent on the prevailing blood flow conditions (Jackson, 2007).The shear-dependent mechanisms of platelet aggregation have ramifications on the NP-induced platelet aggregation process. Fibronectin is now regarded as an important component in promoting thrombus growth and stability under elevated shear (Matuskova et al., 2006) which was earlier thought to play a minor role in platelet aggregation. Moreover, the rheological conditions in an animal microcirculation are likely to significantly differ from those operating in human arteries. It is, therefore, unclear whether observations from in-vivo animal models can be extrapolated to platelet aggregation processes relevant to humans with a different blood flow and blood proteome. The blood proteome of an individual dictates the composition of PCs and the formation of this personalized PC can have implications for the safe usage and diagnostic applications of NPs (Hajipour et al., 2015). Platelet aggregation test on NPs has been conducted on a conventional aggregometer (Laloy et al., 2014) and is a static method which lacks the dynamic environment in which blood exists in the body.

Mechanism of platelet aggregation by NPs also remains largely unknown which may also vary among different classes of NPs involving different pathways. Only large cationic dendrimers showed aggregation of human platelets induced by the disruption of membrane integrity which depends on the number of surface amine groups (Dobrovolskaia et al., 2012). Activation of glycoprotein integrin receptor GPIIb/IIIa has been shown to be the mechanism of platelet aggregation by carbon-based materials, depending on the size of the NPs (Radomski et al., 2005). Depending on the type of NPs, platelet activation can be attenuated by inhibitors known to block extracellular calcium influx (Semberova et al., 2009). Pristine multi-walled carbon nanotubes (MWCNT) penetrate platelet plasma membrane causing depletion of platelet intracellular Ca(2 +) stores (Lacerda et al., 2011).

Type of plasma proteins adsorbed onto NPs greatly affects the platelet aggregation potentials of carboxylated multiwalled carbon nanotubes (CNTCOOH) (Ge et al., 2011). While pristine NPs induced platelet aggregation, HSA corona attenuated the effect. Adsorption of different proteins has an effect on bio-functionality of carbon nanotubes like platelet fragmentation due to IgG and platelet aggregation due to histone protein adsorption. The adsorption behavior of carbon nanotubes is critically determined by the π-π stacking interactions between carbon nanotubes and aromatic residues (De Paoli et al., 2014). Preconsideration of the interactions at the nano-bio interface may help us to design safe and biocompatible carbon nanotube NPs.

4.4.6. Effect on cytotoxicity

A factor often neglects in nanotoxicity studies is the effect of biomolecular components present in the medium. The study of the interplay between materials and local biological environment should be taken into consideration during toxicity profiling. A novel screening method for NP toxicity based on whole human blood model has been tested for TiO_2 NPs. The TiO_2 NPs at very low concentrations induces thrombo-inflammation (Ekstrand-Hammarström et al., 2015). The cytotoxic and pro-inflammatory responses are influenced by the composition of PC and the type of cell used in the study (Brown et al., 2014). Cytotoxicity of NPs depends on specific proteins adsorbed on the NP surface. A slight change in surface chemistry can modulate cytotoxicity due to an associated change in PC composition (Kennedy et al., 2014). In-vitro toxicity assessments can only be conducted accurately when NPs display enhanced dispersion stability without sedimentation in the cell culture media. The cells may be either in the form of suspension culture (which can interact with dispersed NPs) or may be in the form of adherent cancer cell lines (which interacts with sedimented NPs). The availability of NPs to cells will, therefore, depend on the dispersion

stability of NPs and the type of cell culture used (Anders et al., 2015). The media in which NPs are dispersed dictates the composition of PC. The presence of divalent ions and the composition of macromolecules in the media have a significant influence on the formation of PCs both qualitatively and quantitatively (Strojan et al., 2017).

PC, in general, reduces the cytotoxic effects of NPs. Preformed BSA corona demonstrated a substantially lower cytotoxicity in comparison with NPs in three different cell lines (A549, HepG2, and L929 cells). The adverse effects triggered by amorphous silica NPs (ASP) is significantly ameliorated due to the formation of a PC (Docter et al., 2014a). PC also prevents the TiO_2-associated photo-generated radical production, due to the radical trapping ability of the serum proteins (Garvas et al., 2015).

4.5. Effect of protein corona on drug targeting and transfection efficiency

4.5.1. Effect on passive drug targeting

Formation of a PC on intravenously injected NPs is known to alter their target specificity, distribution, and clearance from the body (Owens and Peppas, 2006). The formation of the PC is found to be instantaneous comprising of almost 300 different proteins (Tenzer et al., 2013). A soft corona with rapid exchange rate has been proposed on the surface of the hard corona. Jansch et al. proposed that the long-lived corona may have relevance for the in-vivo fate and circulation half-life of NPs (Jansch et al., 2012).

Intravenously injected NPs bind to serum opsonin which subsequently results in internalization by phagocytosis and is regarded as a limiting step to long-circulating NPs-based targeting (Kumari et al., 2010; Owens and Peppas, 2006). Manipulating the surface architecture for controlling the environmental interactions has been used as a strategy for optimizing the in-vivo functionality of NPs. Steric hindrance of PEG chains has been used as the most successful anti-opsonization strategy for long circulating NPs. Excessive PEGylation, however, can reduce the targeting potential of carriers because of strong inhibition of their uptake by the target cells. PC composition of PEGylated liposomes has been analyzed for optimizing the amount of PEGylation to strike a balance between anti-opsonization strategies and target specificity (Pozzi et al., 2014a).

The concept of “stealth effect” is thought to be a property of non-fouling surfaces. On the contrary, studies on PC show that protein adsorption on NPs can provide a “stealth like” effect (Fig. 3). Despite the fact that the proteins which are responsible for phagocytic clearance of NPs were present in the PC (immunoglobulin, coagulation, and complement proteins) the uptake by macrophages was much less than the pristine NPs. It is hypothesized that this may be due to a structural change in the functional motifs of these proteins or the shielding of the functional motifs by the presence of other proteins in the PC (Caracciolo et al., 2015). Therefore, a change in accessibility and orientation of proteins in the PC may be responsible for the stealth property acquired by NPs in blood. An important effect of the PC is, therefore, a reduction in the purely nonspecific interactions between the pristine NPs and the cell membrane which results in a decrease in cell uptake (Lesniak et al., 2013). As a matter of fact, the PEGylated liposomes also show the adsorption of complement proteins (C3 and C4) under dynamic flow conditions (Palchetti et al., 2016). This also indicates that the MPS escape of NP's is a complex process which requires further investigations on the events at the bio-nano interface. A change in body distribution of NPs was observed due to a change in properties of NPs caused by the presence of the cargo which influences the formation of PC (Fedeli et al., 2015). Intravenous administration of NPs should maintain colloidal stability in the blood plasma. A significant difference in colloidal stability of NPs due to the adsorbed proteins in media composed of bovine serum albumin (BSA) solution, mouse, and human plasma was observed. Therefore translation of iv administered NPs from mouse models to humans would be irrelevant due to the interspecies difference in the composition of blood proteome. This could be one of the reasons for the clinical failure of tumor targeted NPs by the EPR effect (Park, 2014). Recently it has been shown that rat and pig model is better than mouse model for extrapolating animal to human pharmacokinetic data for gold NPs (Lin et al., 2016). Contrarily, the use of a pig model is questionable due to the pulmonary intravascular macrophages (PIM) present on the lungs (not present in rats and humans) which clear the NPs within minutes of administration (Moghimi, 2016). Therefore the choice of the appropriate animal model is another important aspect of nanomedicine research.

For the clinical translation of NPs, protocol related interferences caused by the biological media in which NPs are studied should be considered (Pisani et al., 2017). Protein interaction during the evolution of PC on NPs has been studied by a system biology approach which can be generalized to NPs with different properties. This approach can be applied for the adsorption of specific protein onto NPs which show minimal interaction with opsonins to create stealth properties (Pochert et al., 2017). Shielding the NPs from cells of the immune system is required for long circulation. The PC enriched with histidine-rich glycoprotein has been found to be responsible for the antiopsonin characteristics of SiO_2-NPs in enriched human plasma (Oliveira et al., 2017).The route of entry and the consequent biofluid encountered by NPs en route provides a unique PC composition resulting in change in bioactivity depending on route of administration. The translocation of NPs across pulmonary surfactant film depends on the hydrophilicity of NPs and the hydrophobic NPs are trapped in the surfactant film. The formation of a pulmonary surfactant lipoprotein corona on the NP surface has been reported which can be used to design inhalable NPs with desired functionality and safety (Hu et al., 2013a).

The choice of protein by a particular NP (for the formation of hard corona) is largely unknown. The in-vitro protein binding studies carried on NPs with different surface modification and size has shown to have a strong correlation with the observed changes in biokinetics of NPs (Konduru et al., 2015; Stolnik et al., 2001). The hard corona can evolve significantly as the concentration of protein in the testing environment changes from in-vitro studies to in-vivo studies, which may have deeper implications for the extrapolation of in-vitro data to in-vivo predictions (Monopoli et al., 2011).

4.5.2. Effect on active targeting

Optimized drug therapy requires the accumulation of drug-NP construct at the desired target. However, target specificity of NPs has been problematic even in the presence of cell-specific targeting ligands such as monoclonal antibodies. Dawson and colleagues have provided an explanation for the lack of targeting ability of functionalized NPs (Salvati et al., 2013). They showed that the formation of PC can shield transferrin appended NPs from their intended target. However, the PC formed in-vivo was found to be different from the PCs formed in-vitro qualitatively and the binding and internalization of antibody labeled liposomes were partially retained after in-vivo PC formation (Hadjidemetriou et al., 2015). This discrepancy in results among in-vitro and in-vivo studies can be attributed to the design of in-vitro and in-vivo experimental models. Firstly, the PC composition on NPs may differ under different conditions i.e. in-vitro static condition vs. in-vivo dynamic condition. PC formed on circulating liposomes has been found to be more negatively charged as compared to their static counterpart (Palchetti et al., 2016). Secondly, the hemo-rheology, presence of RBCs and the margination of NPs may influence the PC formation and can differ among different animal models depending on blood flow conditions and the blood proteome of the animal used in the study (see Section 7.1). Thirdly, the type of cells selected for the study has an influence on the cellular uptake and subsequent processing of NPs according to the cell “vision” concept. Fourthly, the type and composition of media in which the study is performed in-vitro and the proteome of the animal model for in-vivo studies determine the PC composition on NPs and consequently their cellular uptake. Therefore, a cell “sees” the NP with a PC formed under dynamic shear flow and not under static in-

Table 2
Some notable feature of protein corona studies in relation to drug delivery.

Protein corona effect	Remarks	Ref.
Formation of biomolecular corona block the functionalized NPs resulting in loss of target specificity	Targeting can be accomplished by dealing with the corona effect	Salvati et al. (2013), Varnamkhasti et al. (2015)
Protein markers adsorbed from plasma on NPs can act as a targeting ligand towards cognate receptors on target cells and acquire targeting capabilities	Research on the formation and evolution of PC on NPs as a function of time in the blood can be used for targeting drugs to specific locations. e.g. apolipoprotein on NP surface has been exploited to target prostate carcinoma cell line via scavenger receptor	Pozzi et al. (2014a), Barrán-Berdón et al. (2013)
Protein corona formed on the NPs depends on the route of entry of NPs and on the relevant bio-fluid which the particle encounters which affects the subsequent biokinetics.	Biological behavior of NPs was found to be distinctly different for particles entering via lung than for those injected directly into the bloodstream due to a difference in PC on NPs	Kreyling et al. (2014), Ruge et al. (2011)
Formation of a protein corona can alter the loading of drugs and release from nanoformulations	Drug release study should be carried in the relevant biofluid	Kah et al. (2012), Rius et al. (2013)
Protein corona can alter the pathway of cellular uptake and tissue distribution of NPs	New strategies for targeting cells can be developed	Fleischer and Payne (2012), Schäffler et al. (2014)
Protein corona can mediate the uptake of compounds which are otherwise impermeable to cells by drug encapsulation	Intracellular targeting of impermeable drugs can be accomplished by tailoring the particle surface and protein corona	Paula et al. (2013)
Protein corona can be tailored to reach at a compromise between an antiopsonization strategy and cellular targeting	Concentration of surfactant and size of PEG molecules can be tailored for optimum drug targeting	Sánchez-Moreno et al. (2015), Pozzi et al. (2014a)
Albumin coated NPs resulted in a massive shift from the liver to lungs and a significant accumulation in the brain	Finding the right surface property and composition of adsorbed protein effects may allow us to custom design NPs for specific applications such as brain targeting.	Mahmoudi et al. (2015), Schäffler et al. (2014)
Specific proteins adsorbed on SPIONs (depending on the size and surface charge) results in their deposition to non-target sites like its entry into the brain endothelial barrier cells	A precise in-vitro-screening can be useful in predicting the final in-vivo behavior of NPs	Sakulkhu et al. (2014)
Protein corona formed around NPs can provide stealth effect by preventing uptake by macrophage	Accessibility and orientation of proteins in corona may be responsible for the stealth property acquired by nanoparticles in blood	Caracciolo et al. (2015), Mirshafiee et al. (2016)
Preferential adsorption of ApoE has been found to be a major determinant for targeting of NPs to the brain	NPs adsorbing ApoE mimics the ApoE coated LDL-particles and can cross the blood brain barrier by endocytic mechanism	Gessner et al. (2001), Shamenkov et al. (2006), Müller and Keck (2004)
Aggregation of nanoparticles in serum and protein corona fingerprinting studies has been successfully used for predicting in-vivo behavior	Non-aggregating particle shows the most promising body distribution.	Mohr et al. (2014)

vitro conditions. Moreover, the cell with which the NP interacts may itself be under flow conditions (RBCs and WBCs) depending on the cell-type being considered. The in-vitro set-up which encompasses all these aspects of a realistic in-vivo condition may need improvement in existing experimental design. This shows that we are still far from simulating in-vivo conditions in the laboratory for testing NPs.

Reducing the PC effect for active targeting can be accomplished by chemical modification of NPs. Gold nanostars functionalized with a ligand and surface treated with a blocking agent, demonstrated active targeting towards HER2-positive tumors, both in-vitro and in-vivo (D'Hollander et al., 2017). Some of the striking observations on the effect of PC on drug targeting are highlighted in Table 2.

4.5.3. Effect on transfection efficiency

Mapping the NP-cell interactome for cellular uptake of NPs may provide interesting clues for the efficient delivery of nucleic acids and other bioactives to the desired compartment in the cell. In a recent report, a molecular level description of the PC on silica-coated magnetite NPs shows that during the internalization and trafficking of NPs, extracellularly formed serum derived PC is preserved on NPs (Wang et al., 2013). The PC also protects the cell from damage caused by pristine NPs until degraded by hydrolytic enzymes present in the lysozymes. The serum biomolecules associated with NPs has a different intracellular processing mechanism than when the same biomolecules are freely transported from extracellular fluid (Bertoli et al., 2014). NPs can be customized to modulate intracellular events for different applications. We have just begun to understand the events during intracellular trafficking of NPs from the perspective of PC which can impact several key cellular bioprocesses. Intracellular formation of PC can also modulate the process of mRNA translation. In a cell-free system using HeLa cell lysate, it was demonstrated that a PC formed by adsorption of ribosomes and translation factors on NPs resulted in enhanced translation of the mRNA by reducing the recycling time of ribosomes (Chan et al., 2017).

A dramatic change in NP characteristics has been observed for lipid-based vectors exposed to serum resulting in aggregation. Prolonged incubation causes vector disintegration (Li et al., 1999). Structural integrity and size in serum were found to be the major determinants regulating the efficiency of lipofection. A change in intracellular trafficking mechanism (from clathrin-dependent to caveolae-mediated pathway) due to protein adsorption is thought to be responsible for a 2-fold enhancement in transfection efficiency of lipoplexes in serum (Caracciolo et al., 2010). In the absence of serum, the nano size and the capacity of lipoplexes to bind DNA determines the transfection efficiency whereas, in the presence of serum, the transfection efficiency increases substantially (Marchini et al., 2009). The lipid composition of lipoplexes has a significant effect on both the qualitative and quantitative aspect of protein adsorbed from fetal calf serum. Contrary to the common view, PEGylation has been shown to increase protein adsorption on lipoplex and the effects are dependent on the type of PEG conjugate employed (Betker et al., 2013).

A study on the compositional evolution of PC shows that the hard corona on lipoplexes is quite stable whereas PC of low-affinity biomolecules is formed on the surface of cationic liposomes, whose relative abundance changes with the plasma concentration (Caracciolo et al., 2011). An exquisite control over the spatiotemporal behavior of NPs by PC fingerprinting could be a possible turning point in targeted drug delivery. A classic example is the selective targeting of lipid-based NPs made of 1, 2-dioleoyl-3-trimethylammonium propane (DOTAP) tovitronectin $\alpha\nu\beta3$ integrin receptor expressed on transformed cells (Caracciolo et al., 2013).

For an optimal selection of the bio-vector, it is essential to select a biological fluid in which the NP is exposed in-vivo. A remarkable difference in transfection efficiency of NPs has been observed when studies were carried out in two different media viz. a synthetic media used in in-vitro screening and a physiologically relevant media to mimic in-

vivo situation (Dakwar et al., 2015). A noteworthy observation in this regard is the absence of statistical correlation between transfection efficiency and physicochemical properties of the NPs (Dakwar et al., 2014). A deeper understanding of the role played by specific proteins adsorbed on transfection vectors and its effect on intracellular trafficking might help in the rational designing of safe and efficient vectors for nucleic acid delivery.

5. Physiologically based pharmacokinetic models (PBPK)

The use of data on absorption, distribution, metabolism and elimination to predict the in-vivo kinetics of a molecule or its metabolites in biological model forms the scientific basis of physiologically-based pharmacokinetic (PBPK) modeling (Lipscomb et al., 2012). Recently, the physiologically based pharmacokinetic model has been used to generate mathematical rate equation which defines the kinetics of NPs in tissues and organs which are treated as individual compartments.

Models developed previously have used the parameters such as size, surface charge, dose etc. as the factors which determine the biodistribution of NPs (Alexis et al., 2008; Avgoustakis et al., 2003; Li et al., 2010). Only a few studies have demonstrated a quantitative relationship between NP property and tissue kinetics (Lankveld et al., 2010; Li et al., 2012). The IVIVC concept has been successfully used previously in the development of PBPK models for chemical molecules (Yoon et al., 2015). It is now explicitly clear that PC formed on NPs influence NP uptake, distribution, metabolism, elimination, and toxicity. A major limitation is the lack of appropriate data concerning the effect of PC formation on biokinetics. Without a clear understanding of these properties, effects of any change in NP characteristic on its biofate can only be tested on laboratory animal, which is a resource intensive process and currently has limited use probably due to the exclusion of PC factor.

6. Diagnostic applications based on protein corona

The blood plasma proteome represents one of the most complex versions of the human proteome present in any sample. The composition of blood proteome changes with the type of disease due to the leakage of tissue biomarkers in blood (Anderson and Anderson, 2002) with the consequence that the bio-identity of NPs in the blood may change depending on the disease type. PC formed in a biological environment may confer visibility to hidden biomarkers which may correlate with the occurrence of pathology and can be used for diagnostic purposes. Surface active maghemite NPs (SAMNs), when introduced into milk samples from animals affected by mastitis demonstrates remarkable specificity towards αs2-casein fragments, which is not present in milk from healthy samples (Miotto et al., 2015). Rapid diagnosis of mastitis in bovine milk can be accomplished by knowledge of PC fingerprints formed on NPs. A significant change in zeta potential with no change in size was observed when liposomes were incubated in plasma obtained from patients with pancreatic cancer and the plasma from healthy volunteers (Colapicchioni et al., 2015; Caracciolo et al., 2014). This may be due to the autoantibodies produced in cancer patients which possibly change the composition of PC. NP-associated PC can, therefore, be a basis for diagnosis in pancreatic cancer patients. PC on NPs can be exploited to develop novel diagnostic tools for cancer. Lipid NPs have been used to diagnose malignant pancreatic cancer due to the formation of an enriched PC when exposed to the blood from patients with pancreatic cancer. The PC was characterized by electrophoresis and the test was able to discriminate between healthy and cancer patients with a high degree of correctness rate (Caputo et al., 2017).

The bio-recognition property of a heteropolymer-NP hybrid has been used for developing a synthetic biosensor and is called as corona phase molecular recognition (Bisker et al., 2016). A heteropolymer (DPPE-PEG(5000)) conjugated with single-walled carbon NP (SWCNT) was screened as a specific corona phase that recognizes fibrinogen with high selectivity. The ability of the construct to detect biological molecules (at clinically relevant concentrations) can help in the development of synthetic sensors and provide an alternative to conventional antibody-based diagnostics.

7. IVIVC: problems and future perspectives

One of the major obstacles in the safe translation of NPs has been the lack of in-vitro methods to predict in-vivo consequences. The IVIVC models have been successfully used in the pharmaceutical industry as a substitute for human bioequivalence studies. The objective of IVIVC is to develop a rational relationship between an in-vitro variable and an in-vivo quantifiable biological response. Unfortunately, in nanomedicine, several promising in-vitro studies have failed in clinical studies (Park, 2014). Despite its great value in pharmaceuticals, the reports on IVIVC in nanomedicine have been very scarce. In one such study, a correlation between in-vitro transfection of siRNA with lipid NPs was successfully correlated with in-vivo hepatocellular transfection (Whitehead et al., 2012). The existing in-vitro tests for efficacy and toxicity testing of drugs and chemicals have not been effectively adapted for the studies on NPs (Dobrovolskaia and McNeil, 2013). The understanding of bio-nano interface as an important determinant of the biofate of NPs has resulted in few predictive models. The interaction of NPs with serum has been used to successfully predict in-vivo biokinetics of NPs (Kreyling et al., 2014). The factors which may be taken into consideration for a IVIVC model based on PC studies is highlighted in Table 3. The major focus of these predictive in-vitro studies has been on the cellular uptake of NPs (Ritz et al., 2015; Caracciolo et al., 2010). The cellular uptake of NPs based on the understanding of specific marker proteins in the PC has given predictive clues of their uptake mechanisms. The PC provides a bio identity to NPs resulting in different intracellular trafficking pathways and different biofate. Immunomapping techniques show that the adsorbed biomolecules may present their functional motifs to their corresponding receptor on cells (Lara et al., 2017) which forms its functional bio-identity (Table 3). The exposure of cryptic epitopes of adsorbed biomolecules on the surface of NPs can provoke a broad range of unanticipated systemic effects (Lynch et al., 2006). We have just begun to realize the reasons for the lack of predictive models in nanomedicine. Unfortunately, there is a dearth of information and lack of clarity on the correlation of in-vitro and in-vivo data in nanomedicine. The factors affecting the PC formation both at the molecular and physiological levels can help us to fully realize the predictive power of the bio-nano interface. Fig. 4 shows a hypothetical scheme for an IVIVC model based on the interaction of the NPs with its microenvironment i.e. the bio-fluid consisting of a plethora of biomolecules.

7.1. Effect of blood hydrodynamics and physiological temperature

Blood-related assays for characterizing PC may not encompass factors like hydrodynamic forces, hemorheology and blood cell interactions of the physiological blood flow environment. Blood is always in a highly dynamic state in the body which affects the margination of NPs based on the size and shape of NPs (Müller et al., 2014). The binding efficiency of vascular targeted NPs is influenced by the RBCs present in the blood (Namdee et al., 2015a). The PLGA NPs shows a 90% reduction in vascular adhesion relative to adhesion in simple buffer flow, which depends on the particle size and the adsorption of immunoglobulins. Depletion of immunoglobulin restores the vascular adhesion of PLGA NPs (Sobczynski et al., 2014). In a dynamic physiological environment, the PC formed on NPs depends on the surface chemistry, the exposure time of NPs and results in altered cellular uptake (Palchetti et al., 2017).

A mathematical model which fits with human physiology would require a comparative study between human and animal physiological environment. The ratio of particle size to RBC size is an important

Table 3
Factors to be considered for IVIVC models based on protein corona studies.

	Ref.
Identification of a marker biomolecule in the protein corona responsible for a biological effect	
Presence of fibrinogen in the corona may be responsible for the inflammatory effect of NPs	Deng et al. (2012), Thevenot et al. (2008), Nonckreman et al. (2010)
Adsorption of apolipoprotein E mediates the delivery of nanoparticles to brain	Müller and Keck (2004), Gessner et al. (2001), Shamenkov et al. (2006)
Binding of vitronectin promotes efficient uptake of nanoparticles to pancreatic cancer cells	Caracciolo et al. (2013)
Presence of thrombin in corona results in procoagulation behavior of nano formulations	Li et al. (2014)
Identification of specific proteins which can enhance (like apolipoprotein ApoH) or suppress (like ApoA4 or ApoC3) cell uptake of NPs.	Ritz et al. (2015)
Effect of a dynamic environment on protein corona and in vivo behavior of NPs	
Protein corona of a different nature is formed when physiologically relevant dynamic models are used	Samuel et al. (2012), Freese et al. (2014)
Dynamic model of circulating blood results in margination of nanoparticles	Namdee et al. (2015a, 2015b)
Dynamic flow models results in different binding property of vascular targeted NPs to endothelial cells	Sobczynski et al. (2014)
Effect of personalized protein corona	
The blood proteome of animals is different than human being which may effects protein corona composition	Gessner et al. (2001), Namdee et al. (2015a, 2015b)
Blood proteome changes in disease state resulting in change in composition of corona	Colapicchioni et al. (2015), Caracciolo et al. (2014), Hajipour et al. (2014, 2015)
Some drugs may change the composition of blood proteome	Laimer et al. (2006), Reid et al. (2010)
Effect of physiological differences among human and animal models on protein corona	
Effect of blood volume, circulation time and basal metabolic rate of the animal model to be tested	Riviere (2013)
Effect of body temperature difference among animals and circadian rhythm	Hasday and Singh (2000)
Effect of change in blood proteome among animals	Gessner et al. (2001)
Effect of hemorheology on protein corona and cell uptake	Namdee et al. (2015a, 2015b)

factor which governs binding of vascular-targeted carriers (VTC) in blood flow (Namdee et al., 2015a). A comparative study on the effect of blood plasma of different animals on the adhesion of VTC shows that porcine plasma has a significant negative effect on the vascular targeting with the formation of different PC compositions from other species (Namdee et al., 2015b). Variations in the blood physiology among different animal models can seriously limit any attempt to develop a model for IVIVC. Appropriate characterization and control of NP properties and the identification of variables governing the dynamics at the bio-nano interface can accelerate the development of optimized NPs.

Variation in physiological body temperature can vary in different parts of the human body and intracellularly due to various factors like human circadian rhythm, physical activity and hyperthermia (Hasday and Singh, 2000). PC composition on NPs was found to substantially vary with slight change in temperature and hence the biological effect of its uptake by cells may vary accordingly. Molecular modeling studies show a change in conformation of adsorbed protein molecule due to high local temperatures on gold nanorods. Importantly, a hyperthermic (Gautherie, 1980) and acidic microenvironment (Gerweck and Seetharaman, 1996) is found in cancer tissue due to excessive cellular metabolism which may induce conformational changes in the adsorbed proteins on NPs. Animals have a different thermoregulatory response curve than humans (Speakman and Keijer, 2013) which can affect the extrapolation of animal data to humans.

7.2. Standardization of in-vitro protocols

The current battery of analytical techniques for toxicity testing has to be validated to nullify the interference of NPs, which otherwise

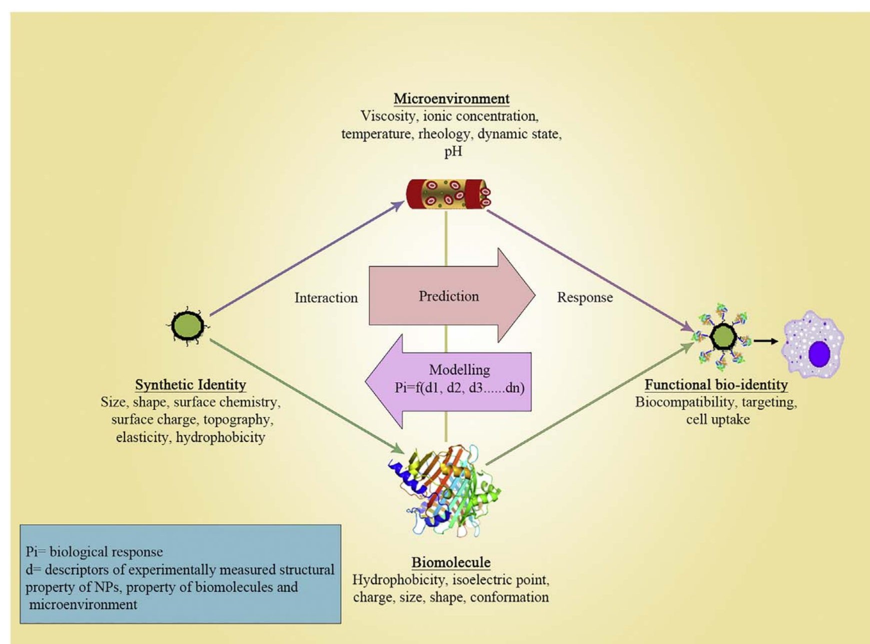

Fig. 4. The goal of IVIVC is to understand the physicochemical correlates of biocompatibility or functional bio-identity (corona specific signature biomolecules responsible for bioactivity or toxicity) to develop functionality-by-design or a safety-by-design framework in nanomedicine.

results in frequent overestimation and underestimation of toxicity and makes it difficult to predict the in-vivo results (Monteiro-Riviere et al., 2009). There have been several reports of the interference due to the presence of NP in standard toxicity protocols such as MTT and LDH viability assay, enzyme inhibition assay etc. (MacCormack et al., 2012; Han et al., 2011; Holder et al., 2012; Zaqout et al., 2012). The high reactive potential and unique physicochemical property of NPs affect the biochemical tests used in toxicity evaluation, leading to data artifacts and consequent over or underestimation of toxicity. Understanding the degree of interference due to the presence of NPs is required for a successful IVIVC model.

8. Conclusion

Decoding the PC fingerprint is essential to understand the biological fate of NPs. Development of a comprehensive database based on PC fingerprints can accelerate the translation of NPs. A multivariate model developed by Dell'Orco et al. using a bioinformatics-inspired approach represents a simple and effective dynamic model for the interaction of proteins with NPs in human plasma based on protein affinity, stoichiometry, and rate constants of protein adsorption (Dell'Orco et al., 2010). The PC fingerprint can predict the cellular interactions of NPs as compared to a model which incorporates physicochemical parameters (Walkey et al., 2014). A mathematical relationship that predicts the biological responses may be possible only by understanding the fundamental mechanisms of the interplay of biomolecules at the nano-bio interface. This can replace trial and error methods to controlled development of NPs.

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