ELSEVIER

Advances in Colloid and Interface Science 134–135 (2007) 167–174

ADVANCES IN COLLOID AND INTERFACE SCIENCE

www.elsevier.com/locate/cis

The nanoparticle–protein complex as a biological entity; a complex fluids and surface science challenge for the 21st century

Iseult Lynch [a,*], Tommy Cedervall [a], Martin Lundqvist [a], Celia Cabaleiro-Lago [a], Sara Linse [b,c], Kenneth A. Dawson [a]

[a] *School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland*
[b] *Conway Institute for Biomedical and Biomolecular Research, College Dublin, Belfield, Dublin 4, Ireland*
[c] *Biophysical Chemistry, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*

Available online 5 May 2007

Abstract

The major aim of our current work is to develop a deep understanding of biological effects of nanoparticles and how these effects are mediated by proteins that are adsorbed on the nanoparticles under different biological circumstances. Due to their small size, nanoparticles have distinct properties compared to the bulk form of the same materials, and these properties are rapidly revolutionizing many areas of medicine and technology. However, relatively little is known about the interaction of nanoscale objects with biological systems, as this requires quite different concepts from more established nanoscience. Thus, we have argued that in a biological fluid, proteins associate with nanoparticles, and it is the amount and presentation of the proteins on the surface rather than the particles themselves that are the cause of numerous biological responses. It is this outer layer of proteins that is seen by the biological cells, and leads to their responses. We are developing novel techniques to identify and quantify the proteins that are consistently associated with nanoparticles, as a function of the nanoparticle size, shape, and surface properties, and to correlate the adsorbed protein identities with their association and dissociation rates to and from the nanoparticles. We also seek to understand the degree of conformational change that they undergo upon adsorption to the nanoparticles. In essence, we wish to create "epitope maps" of the protein corona that surrounds nanoparticles in biological solutions, as it is the particle–protein complex that is the biologically active entity.

Contents

* Corresponding author.
E-mail address: Iseult@fiachra.ucd.ie (I. Lynch).

0001-8686/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.cis.2007.04.021

1. Introduction

Nanomedicine and nanotoxicology are emerging cross-disciplinary research disciplines, where traditional nanoscience and biology meet. They are now amongst the leading concerns of scientists, physicians, industrialists, legislators, regulators, and indeed governments across the world [1–4]. Nanomedicine recognizes the capacity to treat most of the remaining intractable disease classes (viral, genetic, cancer) using nanomaterials, since only objects of such small sizes are able to gain access to, and operate within, the cell. It is currently considered the single most likely technology direction to improve human health in the coming decades. Nanotoxicology acknowledges that (as with blue asbestos—a nanorod that is non-toxic in bulk form, but the agent for the increasingly common cancer, mesothelioma) there exists a potential for new, serious and unpredictable diseases, originating from the interaction of such small-scale objects with living organisms. Nanoscience could lead to the next generation of products, but unexpected major health risks associated with the development, production or use of the nanoscale structures could lead to public rejection and serious economic damage.

It is a (near) universal rule of materials in biology that the material is always covered by proteins immediately upon contact with a physiological environment, and we believe that this phenomenon will also be the key to understanding much of the bionanoscience world. For surfaces, this has long been known by scientists and the industry involved in the development of biomaterials for use as medical implants, and it is understood that many of the early stage biological responses are determined by the nature of the deposited protein layer [5–9]. Indeed, even much later stage responses are determined by subsequent development of a biopolymer interface between the foreign material and tissue [10]. Strategies in medical device research to minimize protein deposition, such as PEGylation of the surface [11], are well known. The whole arena is, however, complex, and one should not automatically assume that a reduced protein load on the interface is beneficial, and surfaces prepared to achieve this may not be superior in terms of their long-term biocompatibility than those with protein adsorbed in a benign manner.

In the case of nanoparticles we believe that this paradigm will continue to be a key element of the story. In particular, we emphasize that most biology goes on at the surface of foreign materials, and the high surface to volume ratio of nanoparticles means that one is dealing with a very important issue. We are therefore potentially facing an issue that is similar to that of medical devices, but hugely amplified by the amount of surface exposed to living tissue, and in a complex mixture of proteins.

There are additional complications relating to the particulate nature of nanoparticles, and to the fact that (when sufficiently small) they can access almost every organ [12,13], and then be taken up into cells as opposed to interacting only with cell surface receptors, as is the case with the more traditional biomaterials. Thus, it is the nature of the organization of the adsorbed proteins on the surface of nanoparticles, and any subsequent colloidal instability of either the nanoparticles (e.g. particle aggregation, flocculation, precipitation, etc.) or the adsorbed proteins (such as protein aggregation, clustering, fibrillation, etc.) that determines the initial biological responses to the presence of nanoparticles. The exceptional case of particles becoming so small that they cannot support such a 'coating', but are partially ligated, or even 'bare' we acknowledge to be of importance, but this may be a more exceptional case than expected.

We have recently argued that the effective unit of interest in the cell–nanomaterial interaction is not the nanoparticle *per se*, but the particle and its 'corona' of more or less strongly associated proteins from serum or other body fluids [5,14]. Ultimately it is this corona of more or less disrupted proteins, 'expressed' at the surface of the particle, that is 'read' by living cells. Given the enormous scientific, technological, and economic potential of nanoscience, e.g. nanomedicine combined with the growing awareness of potential risks (nanotoxicology), it is surprising that the particle–protein complex is so poorly understood. If our understanding of protein–nanoparticle interactions and their biological consequences is to be advanced we must identify the proteins that associate to particles in the complex multi-component mixtures that are biological fluids. We require information on the binding affinities and stoichiometries for different combinations of proteins and nanoparticles, ranking of the affinities of proteins that coexist in specific bodily fluids or cellular compartments. In time we believe it will become apparent that a whole conceptual framework, analogous to that pioneered by Langmuir for surfaces, will be required to fully address the challenges. Beyond that, and presenting unique challenges never yet faced by physical scientists, we will need to know the groups of proximate amino acid residues that are expressed at the outer surface of the adsorbed protein layer, for it is this collection of 'epitopes' (the epitope map) that ultimately gives the particle–protein complex its biological identity, not the particle itself.

We conceive of the proteins associated with the particle possessing a very wide range of affinities for the particle surface. In essence we expect a huge range of equilibrium constants (one for each protein) representing the quite different (and competitive) binding mechanisms present. This means that we see the proteins associated to the particle as a 'corona', rather than a solid fixed layer. The composition of the protein corona at any given time will be determined by the concentrations of the over 3700 proteins in plasma, and the kinetic on and off rates (or equilibrium binding constants) of each protein for the particular nanoparticle. This corona may not immediately reach equilibrium when exposed to a biological fluid. Proteins with high concentrations and high association rate constants will initially occupy the nanoparticle surface but may also dissociate quickly to be replaced by proteins of lower concentration, slower exchange and higher affinity. These relaxation processes may also be important when particles redistribute from one compartment or organ to another, such as upon receptor-mediated endocytosis from the extracellular environment into the primary endosomal cavity, or from the cytosol to the nucleus. For example, a tightly associated protein that exchanges slowly may follow the nanoparticle as it endocytoses

from the extracellular fluid into an intracellular location, while a protein with fast exchange will be replaced by an intracellular protein during or after such transfer. The biological outcome may also differ depending on the relative protein exchange between nanoparticles and cellular receptors.

An additional complicating factor in studies of this nature is the very large natural variation in protein composition and expression of different individuals, even amongst healthy individuals. For example, the protein composition of blood (serum) has been shown to vary significantly between individuals, with many of the proteins that are considered the wild-type not being the one present in the majority of individuals [15].

It is clear that, in *understanding* how particles will interact with cells, these issues which are currently almost unstudied, are amongst the most fundamental. Many of these issues have never before been addressed systematically. No single case of a particle whose outer exposed surface is characterised in biologically relevant conditions exists yet in the literature, and rational attempts to relate nanoparticle characteristics to biological response (except in simple cases where the chemical substance is patently toxic) have not yet been successfully attempted. Success in this element, of fully characterising the particles in biological context, will require the most advanced physical, chemical, and biochemical approaches, as well as refinement of existing techniques to take into account the complex and dynamic nature of this new biological entity—the nanoparticle–protein complex.

The present article summarizes some of the recent developments from our group towards characterising the nature of the protein corona that associates to nanoparticles in biological solutions. It should be seen as a 'status report', for this is a field that is still at early stages. We are working towards producing a map of nanoparticle-induced protein surface expression, or an "epitope map". An epitope map is seen by us as the "biological identity" of the particle–protein complex, and based on the nature of this, the nanoparticle interacts with the cell, or is trafficked to numerous different cellular locations. Thus, not only nanoparticle uptake into cells, but also the determination of the nanoparticle final destination (and activity there) in a cell is determined mainly by the "epitope map", or the nature of the particle associated protein corona. This associated corona then is, as we have noted above, its true biological entity.

2. Discussion

2.1. The nature of nanoparticles

The term nanoparticles typically refers to particles with diameters in the size range 1–200 nm, and is actually a more recent term for what have long been called colloids. The important aspects of nanoparticles are their small size, and extremely high surface to volume ratios, which means that the importance of surface cannot be overemphasized when considering nanoparticles. For example, a nanoparticle with a diameter of 70 nm at a concentration of 0.01 mg/ml provides a total surface area of 0.8 m^2/l, whereas a particle with a diameter of 200 nm at the same concentration (0.01 mg/ml) has a total surface area of 0.3 m^2/l, illustrating the dramatic increase in surface area with decreasing particle size, at constant weight fraction.

It is also their small size that makes nanoparticle interactions with cells of interest - particles of this scale are small enough to be taken up into cells. Particles (or species, e.g. viruses) less than 100 nm can enter cells via receptor mediated endocytosis [16], as shown schematically in Fig. 1. Although the nanoparticle shape issue is not yet settled, it is believed that cylindrical objects can pass membranes more easily, as they can harness the clathrin structure easily [17]. This is one of the reasons for the present concern about the biological effects of carbon nanotubes [18], and may be a factor in the toxicity of asbestos particles, especially blue asbestos, which is implicated in the particularly nasty form of cancer, mesothelioma [19]. However, the source of the largest biological disruptions is likely to be the effect of different nanoparticle surface compositions on the nature and conformation of the adsorbed protein layer. For example, if a protein is tightly bound, and looses much of its conformational identity, it will no longer function as it should, meaning that essential cellular processes or functions could be directly disrupted as a result of the protein-nanoparticle interaction. Additionally, proteins that the adsorbed protein normally interacts with may now not be

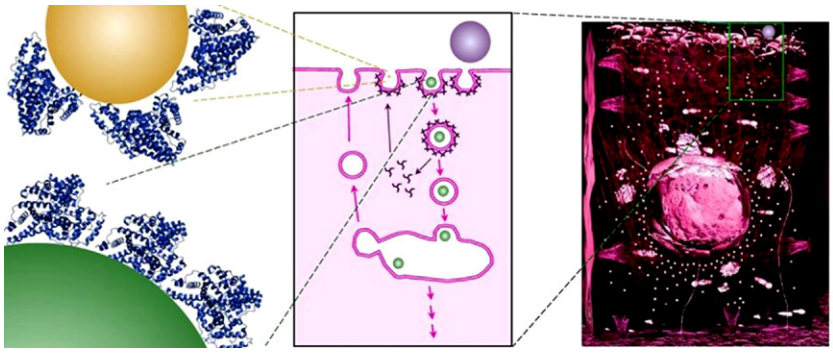

Fig. 1. Receptor mediated endocytosis: nanoparticles are coated by a protein corona. Depending on their size, nanoparticles either remain at the cell surface or are taken up into the cell via receptor mediated endocytosis, mediated by the triskeletal protein, clathrin. The picture on the right is a computer generated image of a cell, taken from "The Dynamic cell", by Springer Verlag.

activated, resulting in disruption of downstream cellular signalling pathways. Thus, it is clear that it is not the nanoparticle per se that is the biologically relevant species, but instead the nanoparticle–protein complex.

For the purpose of our initial studies, we have been focusing on polymeric nanoparticles composed of mixtures of *N*-isopropylacrylamide (NIPAM) and *N-tert*-butylacrylamide (BAM). By varying the ratio of the two monomers we can control the hydrophobicity of the nanoparticles in a systematic manner, and by varying the synthesis conditions (the amount of added surfactant) we can control the size of the particles. Additionally, we have considerable experience working with this copolymer system, and have studied in detail the interaction of flat surfaces of these materials with cells, including determining the phenotypic and genotypic changes induced by interaction of cells with these materials. Here we describe some of our recent experiments using two different nanoparticle sizes (70 and 200 nm, i.e. one size particle that is small enough to harness the process of receptor mediated endocytosis, and one that is too big to enter cells via this mechanism), and three different monomer ratios, 85:15, 65:35 and 50:50 NIPAM: BAM, which have increasing hydrophobicity as indicated by contact angle and phase transition measurements [20].

2.2. Identification and quantification of consistently adsorbed proteins

One of the major goals of our initial studies has been to measure the relative amounts and identity of different proteins adsorbed to nanoparticles from biological fluids such as plasma or intracellular fluids (see Fig. 2). Until now the preferred method to separate the nanoparticles from plasma has been by centrifugation; however, this method has significant limitations that have not been recognized previously. Firstly, the outcome of a centrifugation assay will be affected by the duration of washing steps and the solution volumes used in these steps. A protein in high abundance in plasma may be retrieved because of insufficient washing, or because it is truly associated with the particle. Sedimentation of large proteins, protein aggregates, and co-precipitation may further complicate the picture, depending on the centrifugation rate and duration.

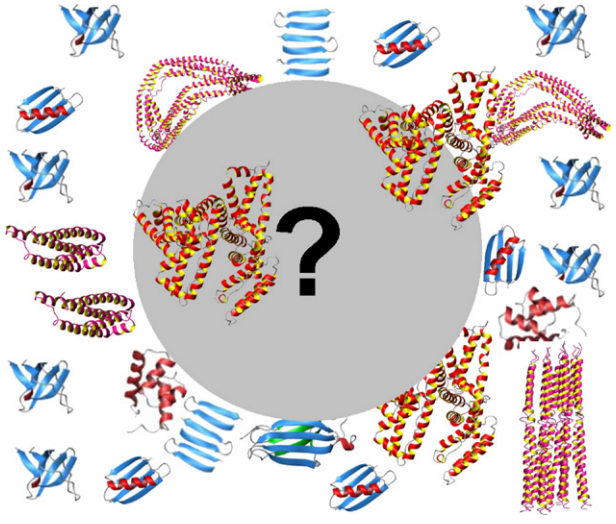

Fig. 2. Protein–nanoparticle complex. Examples of the range of proteins contained in human plasma or serum. Depending on the affinity of the proteins for the nanoparticles, and the lifetimes of the associations, the nature of the protein-particle complex can be considered as a dynamic state which changes over time and as a function of the nanoparticle's location.

Despite these problems, centrifugation assays are still the most efficient way to retrieve enough proteins for safe identification of proteins using mass spectrometry, where it is well known that the quality of the identification depends greatly on the amount of material available [21]. We have recently shown that centrifugation assays can be made reliable and reproducible if conducted with care and when accompanied by other methods to exclude false positives and to assess the relative rates of protein association and dissociation (see next section) so that a true picture of the kinetic and equilibrium situation in a biological fluid is obtained [22]. One such approach, which we have recently pioneered, is the use of size exclusion chromatography (gel filtration) to separate nanoparticle-associated proteins from complex mixtures such as plasma, and to determine the strength and lifetime of the association of the proteins to the nanoparticles [14]. In this case, only proteins that are associated with the nanoparticle for the entire duration of passage through the column are recovered in the same fraction as the nanoparticles, and the further behind the nanoparticle fraction that a protein is eluted, the weaker its, association for the nanoparticles. Further details of this process are given below in Determination of the kinetics of protein association/dissociation with/from nanoparticles.

The polymer nanoparticles described above were incubated with plasma, keeping the particle concentration low to ensure a large excess of protein over available nanoparticle surface area (as would be expected for nanoparticles in vivo), and the adsorbed proteins were retrieved by centrifugation with a total washing time of 20 min in order to retain proteins with relatively high affinity and slow exchange. The hydrophobicity of the nanoparticle surface was found to influence both the amount and the identity of the proteins bound to the nanoparticles [22]. Virtually no protein was retrieved when plasma was incubated with the less hydrophobic copolymer particles, whereas significant amounts of six different proteins were consistently bound to the hydrophobic particles, with the amount of HSA bound being very low given its high abundance suggesting lower affinity than for the other six proteins [22,23]. The amount of bound protein varied with size in the range of 70 nm to 700 nm, although this was at constant weight fraction of particles rather than at constant nanoparticle surface area, and so was to be expected. We are currently repeating these experiments at constant particle surface area for the different sized nanoparticles. However, the pattern of the adsorbed proteins is the same for all nanoparticle sizes. This indicates that, for these cases and this size range, the surface curvature is not a major determining factor for the relative affinities of proteins for the particles. Ongoing efforts include varying the contact time between the plasma and the nanoparticles, and varying the washing time in order to determine the conditions

under which irreversible adsorption involving protein denaturation occurs. This will help us to evaluate and distinguish between kinetics and thermodynamic processes involved in protein adsorption. Additionally, we will compare the protein adsorption with protein adsorption on planar surfaces, both surfaces of identical composition as our nanoparticles, and with data from the extensive literature on planar surfaces regarding thermodynamic and kinetic effects which will also be extremely useful in distinguishing between the different mechanisms and driving forces.

We are now extending this work to enable us to identify and quantify the proteins that are associated with the nanoparticles after uptake of the nanoparticles into cells. This will involve refining the current methods of separating cellular fractions, cross-checking the results using different isolation and recovery techniques, to ensure that the same proteins are consistently recovered using the different methods, and correlation of identified adsorbed proteins with their association and dissociation rates (see below) to ensure that the findings are consistent, and that proteins are not being identified as adsorbed simply due to their high natural abundance, or as a result of the separation technique.

2.3. Determination of the kinetics of protein association/dissociation with/from nanoparticles

In terms of the biological response elicited by a nanoparticle–protein complex, it is not necessarily the more abundantly associated proteins that will have the most profound effect. A less abundant protein with high affinity and specificity for a particular receptor may instead be a key player in determining the biological response to nanoparticles. It is thus essential to develop methods to determine the lifetimes of protein-particle complexes, and to study the competition between proteins of different abundances to bind when the system is under kinetic or thermodynamic control.

Surface plasmon resonance (SPR) studies have traditionally been used to study protein–protein interaction kinetics. We have developed a modified version of the technique which yields data on protein association to and dissociation from nanoparticles. In order to utilise SPR for determination of the rates of protein association and dissociation, it is necessary to link the nanoparticles to the gold surface to ensure that they are not washed away. This has been achieved using a thiol anchor to covalently attach the nanoparticles to the gold surface. A protein solution can then be flowed over the nanoparticle-modified surface and the binding kinetics determined. Rinsing with buffer then shows the dissociation kinetics. Examples of the association and dissociation curves are shown in Fig. 3. Both the association and dissociation rates are clearly dependent on the hydrophobicity of the particles (Fig. 3). The data for plasma (Fig. 3C) could not be fitted assuming a single process with a uniform dissociation rate constant. Neither could the association data be fitted assuming a single process with uniform association and dissociation rate constants (Fig. 3B). Rate constants for association and dissociation were therefore estimated by curve fitting using the following equations,

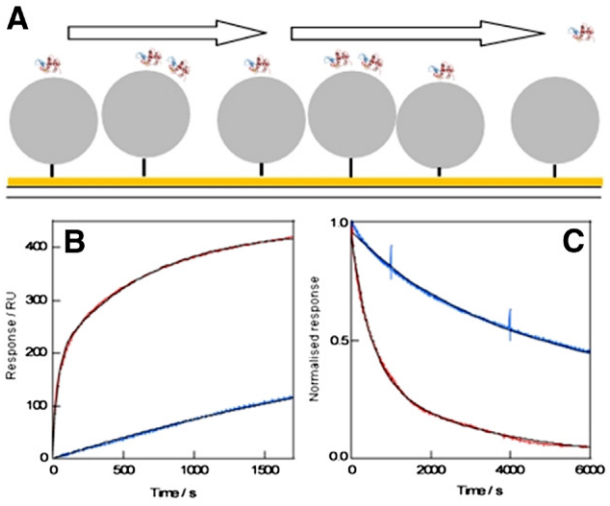

Fig. 3. SPR. Surface plasmon resonance studies of plasma. (A) Cartoon of a gold surface with thiol-tethered particles and associated protein over which buffer is flown. (B and C) SPR data of plasma proteins injected at 60-fold dilution over 70 nm 85:15 NIPAM:BAM (blue) or 50:50 NIPAM:BAM (red) for 30 min (B) followed by buffer flow for 24 h (C, first 6000 s shown). The data show the response as a function of time which reflects the change in refractive index as the amount of protein bound to the surface increases (B) or decreases (C). The black lines are computer fits using Eqs. (1) and (2) for the association and dissociation (washing), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which account for two uncorrelated association/dissociation processes (for two different classes of plasma proteins):

$$R(t) = C1(k^{1on}/(k^{1on} + k^{1off}))(1 - \exp(-(k^{1on} + k^{1off})t)) + C2(k^{2on}/(k^{2on} + k^{2off}))(1 - \exp(-(k^{2on} + k^{2off})t)) \quad (1)$$

$$R(t) = A1\exp(-k^{1off}t) + A2\exp(-k^{2off}t) \quad (2)$$

$R(t)$ is the response as a function of time, which reflects the change in refractive index as molecules are bound at a surface and in case of protein binding studies it is proportional to the amount of protein bound at the surface. Data for single proteins were fitted assuming single processes, i.e. C2 and A2 were set to zero. Note that Eq. (2) refers to the washing experiment where protein-free buffer is flown over the surface directly after the association phase. Eqs. (1) and (2) are derived for first and second order reactions as described previously [24], assuming baseline subtraction and summation of the responses for two independent processes.

Of course, there are more than two kinds of proteins in plasma, but the very large improvement in the fit going from one to two processes does not motivate the inclusion of additional rate constants. The important results are that the particles bind more than one kind of protein from plasma, and that these proteins differ from one another in terms of their association and dissociation rates. Assessment of association rate constants requires knowledge of exactly which proteins are involved and their concentration in plasma. For plasma proteins on the 70 nm 85:15 NIPAM:BAM particles, the two

dissociation rate constants are $3.7.10^{-4}$ s^{-1} and $6.1.10^{-5}$ s^{-1} and for plasma proteins on the 70 nm 50:50 NIPAM:BAM particles, the rate constants are $2.0.10^{-3}$ s^{-1} and $3.4.10^{-5}$ s^{-1}. A similar difference is seen between the 200 nm particles with 85:15 and 50:50 NIPAM:BAM. SPR studies with pure Human Serum Albumin (albumin) and fibrinogen show dissociation rate constants consistent with the fast dissociation event, suggesting that these proteins account for the faster of the observed kinetic processes. Again for albumin and fibrinogen, we observe faster dissociation from the more hydrophobic compared to the more hydrophilic particles. Thus, the dissociation rate is dependent on the nature and properties of both the protein and the nanoparticle.

The rates of exchange of protein on nanoparticles can also be estimated from size exclusion chromatography (gel filtration) which we have recently introduced as a novel, non-perturbing method for isolating nanoparticle-associated proteins, and determining the affinity and lifetime of the particle–protein interaction. The method is shown schematically in Fig. 4. The elution profile of a single protein in a protein–nanoparticle mixture is dependent on the dissociation rate, the chromatographic run time and flow rate, and other factors. If protein exchange from the particles is very slow, with a residence time several times longer than the separation time, one fraction of the protein would elute with the particles and one at the same position as for protein injected alone. If the exchange is very fast, the protein would elute at the same position as without particles. Intermediate dissociation rates produce divided or broadened peaks and the detailed elution pattern will be determined by the rates of protein-particle exchange.

Human serum albumin mixed with 200 nm 85:15 NIPAM:BAM nanoparticles was shown to elute earlier than albumin without particles [14]. The shift is small and with particles, albumin elutes behind the top of the particle peak, indicating that albumin dissociates from the nanoparticles during passage through the column. Data for albumin with the 200 nm particles of composition 85:15, 65:35 and 50:50 NIPAM:BAM reveal that more protein elutes early with the more hydrophilic particles, implying that human serum albumin has a longer residence time on these nanoparticles. With the most hydrophobic nanoparticles, a large fraction of the protein elutes later than human serum albumin alone. Fibrinogen with 200 nm 65:35 NIPAM:BAM elutes as a double peak with elution times equivalent to the free protein and earlier than albumin on the same particles, suggesting that fibrinogen dissociates at a lower rate. Fibrinogen mixed with 50:50 NIPAM:BAM particles also elutes as a double peak with elution times equivalent to the free protein and later than the free protein. Our data imply that single purified proteins associate with the NIPAM:BAM copolymer nanoparticles and that the rates of association/dissociation are very different from protein to protein, depending on the nanoparticle composition.

By comparing our experimental data with simulated chromatograms for different dissociation rates [25], we can estimate the dissociation rate to be around 4.10^{-4} s^{-1} (half life 30 min) for the complex of albumin with 200 nm 85:15 particles. The dissociation rate needs to be approximately 10-fold higher to yield a protein peak at the same position as for protein alone. The observed elution time for albumin in a mixture with the 200 nm 50:50 nanoparticles is later than the elution time of free albumin. Therefore, the exchange rate for albumin from these more hydrophobic particles can be inferred to be at least one order of magnitude faster than from the more hydrophilic particles.

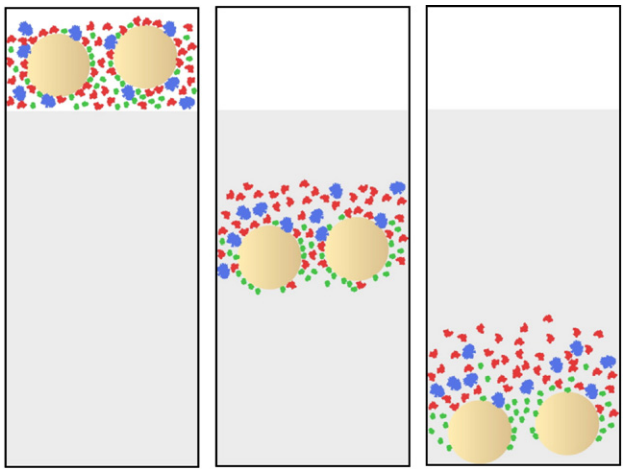

Fig. 4. Size exclusion chromatography of plasma proteins. Cartoon showing the principle of the method. Nanoparticles (beige) are applied to a size exclusion column in a protein mixture, symbolised by a large blue protein of low abundance, medium off-rate, a red protein of high abundance and higher off-rate and a green protein of high abundance and low off-rate. In the eluate, the green protein is preferentially enriched on the particles while the faster dissociating proteins elute later. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Combining the identities of the adsorbed proteins in the different cellular compartments, with data relating to the abundance of the proteins and their affinities, association and dissociation constants will give a much more accurate understanding of the nature of the adsorbed protein layer and indeed the nature of the protein-particle complex.

The next step in completing the “epitope map” will be to connect this information to the degree of conformational change experienced by the proteins, and to the functionality (or otherwise) of the adsorbed proteins. This information will combine to give us a deeper understanding of the (potential) biological consequences of nanoparticles *in vivo*.

2.4. The particle–protein complex as a biological entity

Once we can connect the nanoparticle characteristics to the nature of the protein layer adsorbed onto the particle, and the details of the “epitope map”, we begin to be in a position to correlate the epitope map to the biological responses resulting from the presence of the nanoparticle *in vivo*. In principle, it is the nature of the adsorbed protein coat that determines uptake and intercellular trafficking of nanoparticles. Thus, in principle, we can begin to think of designing nanoparticles to adsorb specific proteins in order to determine the final cellular location of the nanoparticles, i.e. to make nanomedicine a reality. In

principle, we should be able to "post" a nanoparticle to anywhere in the cell, simply using the protein coat which will be "read" by the cellular machinery, and direct the nanoparticle to the desired location according to the instructions "printed" on the nanoparticle surface, via the "epitope map". It is clear that the final cellular location of a nanoparticle will determine the range of cellular pathways and processes that the particle can potentially disrupt, and thus reaching different intracellular locations leads to different functional responses. Thus, it becomes apparent that in order to fully understand the potential biological effects of a particular nanoparticle, it is first necessary to understand where the nanoparticle will end up in the cell, and in order to do that, we need to understand the nature of its protein corona, bringing us back to our original assertion, that it is the particle–protein complex that is the biological species, and which determines the biological response.

3. Conclusions

We have two broad sets of conclusions. The first set concerns the status of our (and related) scientific work in this field. The second ranges more fully across the whole development of the scientific arena.

We believe that the central role of the particle–protein corona is now becoming recognized, and expect scientists to turn to that issue increasingly. We have presented our view that the challenge is ultimately to determine the epitope map of this protein corona in all relevant organ and cellular compartments, only then being in a position to predict the types of biological activity of the particle. This we consider to be the central challenge of the field.

We have developed novel methodology to enable measurements of protein–nanoparticle interactions at equilibrium, as well as the rates of association and dissociation, as a function of protein and particle type. Thereby, we have for the first time shown that (in biological fluids) many proteins are associated with particles, with a very wide range of affinities. Some exchange relatively quickly, some much more slowly, so that a set of proteins may be thought of as preferentially adsorbed on the particles. We have begun to be able to read off these proteins in order of the amount of time they spend on the particle, thereby approaching the issue of characterising the corona. However, the next key point we wish to make is that, while these steps are promising, the field is yet in its infancy and will require considerable coordinated efforts to rise to the challenges being set by developments on a larger scientific, industrial and societal scale.

3.1. Remaining challenges

In the future it seems to us that nanoparticles will have to be classified in part by the manner in which they interact with proteins. This conclusion will hardly be of surprise to the medical device specialists in our audience—but it is of some surprise to nanoparticle scientists. We believe that the central role and relevance of the protein corona or particle–protein complex is only now becoming fully appreciated in the nanoscience world and (given the pressing need to advance bionanoscience for reasons of human health) this delay now constitutes a serious, perhaps even the most serious, limitation on the field.

Having recognized this, the implications are clear, and striking. To understand such problems will require collaborative teams that extend far beyond nano- and other particle scientists, including biocolloidal science (thereby understanding the whole issue of colloidal stability in biological fluids), protein chemists, biophysical, complex fluids and other scientists. This will be required purely to deal with the particle–protein science alone; to go beyond and make the explicit connection to biological activity will require extended groupings of molecular and cell biologists, as well as the whole 'omics' specialization from modern biotechnology. Such alliances have been talked about for decades, and are notoriously difficult to form and maintain, given the great differences in culture and languages in these different communities. Now as we enter the nanocentury in full force, the issues facing us are significant, touching the interests of all developed economies, and impacting on human health (for good or ill) for many decades to come. A concerted research effort is now required from a wide range of disciplines to address the issues.

Acknowledgements

This work was supported by the Marie Curie RTN CIPSNAC, Enterprise Ireland, EPA, and Science Foundation Ireland (Walton Fellowship and RFP-Spatio-temporal aspects of nanoparticle interaction with cells).

References

[1] Colvin VL. Nat Biotechnol 2003;21:1166.
[2] Donaldson K, Stone V, Tran CL, Kreyling W, Born PJA. Occup Environ Med 2004;61:727.
[3] European Commission. Nanotechnologies: A Preliminary Risk Analysis on the Basis of a Workshop Organized in Brussels on 1–2 March 2004 by the Health and Consumer Directorate General of the European Commission; 2005.
[4] Royal Society of Chemistry and Royal Academy of Engineering. Nanoscience and Nanotechnologies: opportunities and uncertainties, 2005.
[5] Lynch I, Dawson KA, Linse S. Sci STKE 2006;327:pe 14.
[6] Gray JJ. Curr Opin Struct Biol 2004;14:110.
[7] Anderson JM. Annu Rev Mater Res 2001;31:81.
[8] Shen M, Garcia I, Maier RV, Horbett TA. J Biomed Mater Res A 2004;70:533.
[9] Norde W, Lyklema J. J Biomater Sci Polym Ed 1991;2:183.
[10] Welt FGP, Rogers C. Arterioscler Thromb Vasc Biol 2002;22:1769.
[11] Norde W, Gage D. Langmuir 2004;20:4162.
[12] Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, et al. Inhal Toxicol 2004;16:437.
[13] Semmler M, Seitz J, Erbe F, Mayer P, Heyder J, Oberdorster G, et al. Inhal Toxicol 2004;16:453.
[14] Cedervall T, Lynch I, Lindman S, Berggård T, Nilsson H, Thulin E, Linse s, et al. Proc Natl Acad Sci 2007;104:2050–5.
[15] MuMuthusamy B, Hanumanthu G, Suresh S, Rekha B, Srinivas D, Karthick L, et al. Proteomics 2005;5:3531.
[16] Gao H, Shi W, Freund LB. Proc Natl Acad Sci 2005;102:9469–74.
[17] Kam NWS, Jessop TC, Wender PA, Dai HJ. J Am Chem Soc 2004;126:6850–1.

[18] Chiu-Wing Lam C-W, James JT, McCluskey R, Hunter RL. Toxicol Sci 2004;77:126.
[19] Manning CB, Vallyathan V, Mossman BT. Int Immunopharmacol 2002;2:191.
[20] Lynch I, Blute IA, Zhmud B, MacArtain P, Tosetto M, Allen LT, et al. Chem Mater 2005;17:3889.
[21] Baldwin MA. Mol Cell Proteomics 2004;3:1.
[22] Cedervall T, Lynch I, Foy M, Berggard T, Donnelly S, Cagney G, et al. Angewandte Chemie Int. Ed., in press.
[23] Lindman S, Lynch I, Thulin E, Nilsson H, Dawson KA, Linse S. Nano Lett 2007;7:914.
[24] He X, Shen L, Malmborg A-C, Smith KJ, Dahlback B, Linse S. Biochemistry 1997;36:3745.
[25] Stevens FJ. Biophys J 1989;55:1155.