Probing the interactions of proteins and nanoparticles

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Despite the hype concerning the vast and imminent commercial potential of nanotechnology, there is little doubt that nanoparticles offer real and radically new opportunities in fields such as biomedicine and materials science (1, 2), among others. Such particles are small enough to enter almost all areas of the body, including cells and organelles, potentially leading to a new approach to medicine (nanomedicine), including rational approaches to targeted intracellular delivery of genes and other therapies (see, e.g., ref. 3). However, there have been widespread concerns that their use could involve some biological hazard (4–8) and a growing consensus that our understanding of the interaction of nanoscale objects with living matter, even at the level of single cells, has not kept pace with the explosive development of nanoscience in the past decades. A significant step in this area has been taken by Cedervall *et al.* (9) in this issue of PNAS on development of methods for probing the association of proteins to nanoparticles: Such association is almost always a first step when nanoparticles enter a biological fluid, so that when we think of the interactions of nanoparticles with a living system, we are really speaking of protein-coated particles. Adsorption of the proteins onto the particle surface can lead to altered conformation, exposure of novel "cryptic" peptide epitopes, perturbed function (caused by structural effects or local high concentration), or avidity effects arising from the close spatial repetition of the same protein (10). Thus, although protein adsorption has been studied classically both on planar surfaces and in colloidal dispersions (11–13), the focus by Cedervall *et al.* on the specific binding rates and affinities of different plasma-related proteins to nanoparticles is a welcome development in this field. Proteins compete for the nanoparticle surface, leading to an adsorbed protein layer or "corona" that largely defines the biological identity of the particle. Underlying all of these effects is the fact that nanoparticles have a very large surface-to-volume ratio, so that even small amounts of particles present extremely large surface areas available for protein binding.

A better understanding of the biological effects of nanoparticles requires knowledge of the binding properties of proteins (and other molecules) that associate with the particles from realistic mixtures of proteins such as those in biological fluids. However, the isolation and identification of particle-associated proteins is not a simple task. The methods proposed by Cedervall *et al.* (9) are able to identify both major and minor particle-associated proteins and to study the competition between proteins that bind when the system is under kinetic or thermodynamic control. The challenge is to use nonperturbing methods that do not disrupt the protein–particle complex or induce additional protein binding.

Most of the methods applied by Cedervall *et al.* (9) have not been used before for studying nanoparticle–protein affinity but, happily, are nonetheless based on established techniques, including size-exclusion chromatography (SEC), isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR), which should make them widely accessible. These approaches are applied to a set of model copolymer nanoparticles of two different sizes that allow for systematic investigation of the effect of the composition (hydrophobicity) and size of the particles on their interaction with proteins. In particular, a new approach (based on SEC-gel filtration; see below and Fig. 1) is introduced that can yield both the identity of the proteins on the nanoparticles and the rates of exchange with plasma proteins. This method is less perturbing of protein–particle complexes than centrifugation and other approaches (9). Cedervall *et al.* also show that ITC can be used to assess the stoichiometry and affinity of protein binding, and SPR studies (in which nanoparticles are linked to gold by a thiol anchor) yield additional data on protein association/dissociation from nanoparticles.

Nanoparticles have a very large surface-to-volume ratio.

The results indicate that many proteins form transient complexes with nanoparticles and that there is a clear dependence of the binding and dissociation parameters on protein identity and the particle surface characteristics. The resulting corona then effectively constitutes the new nanoparticle "surface," while its shape and size also may play an important role in its interactions with cell surfaces. An important role of the nanoparticles also may be in giving a "ride" to the corona proteins across a cell or organelle membrane, where they are then dissociated.

A remarkable observation made by Cedervall *et al.* (9) is the large variation in dissociation rates for proteins on nanoparticles, implying that, depending on the experimental procedures and times, different sets of proteins may be identified as part of the particle–protein corona. Also, the concentrations of particles and biological fluid will influence the outcome of identification experiments. The total protein concentration in bodily fluids, especially in intracellular environments, can be up to 35% (0.35 g/ml), representing several thousand different proteins spanning a wide range of concentrations. As a result, there will be competition between the proteins for the available nanoparticle surface area in a typical biological environment. Human serum albumin (HSA) and fibrinogen may dominate on the particle surface at short times but will subsequently be displaced by lower abundance proteins with higher affinity and slower kinetics (in the case of the hydrophobic particles studied here, for example, apolipoprotein A-I is much more important). In contrast, when the available nanoparticle surface area is in excess over the total available protein, lower-affinity proteins such as albumin also may be found in isolation experiments. To identify a set of associated proteins that more closely reflects the situation *in vivo* will require the protein mixture (e.g., plasma) to be in excess over the available particle surface area, and at best that the particle concentration used reflects a true biological situation (such as a typical therapeutic or

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imaging particle concentration; see, e.g., ref. 14).

Another very interesting and, to my mind, unexpected observation is that for a given level of nanoparticle hydrophobicity, there is a distinct difference between the degrees of surface coverage of the nanoparticles depending on their size, with the larger degree of coverage on the larger particle. This finding suggests that as little as a 3-fold difference in nanoparticle sizes can result in a marked curvature-induced suppression of the protein adsorption [as pointed out by Cedervall *et al.* (9)], even when the nanoparticles themselves, of diameters 70 and 200 nm, are much larger than the adsorbing proteins (of order 10 nm for the globular HSA studied). One speculation is that the curvature of even smaller nanoparticles, say, 30 nm or so in diameter, may entirely suppress the adsorption of certain (presumably larger) proteins: thus, the nanoparticle size, and not only the chemical composition of its surface, may itself be an important parameter in determining the composition of the protein corona.

From a methodological point of view, the gel filtration approach looks promising. The simple, but effective, idea underlying this approach is that the nanoparticles, which are much larger than the proteins that attach to and detach from them, move through the gel faster and elute from it sooner than free proteins, which, en route to elution, sample many of the smaller gel cavities from which nanoparticles are excluded. Thus, proteins may hitch a fast ride on the nanoparticles as long as they are attached to them but migrate sluggishly once they dissociate, as illustrated in Fig. 1. A fraction-by-fraction comparison of the proteins eluting with and without particles allows the identification of both slowly and more rapidly dissociating proteins, and their exchange rates may be estimated from their elution profiles. With careful choice of column dimensions and relative concentrations of protein and particles, the technique isolates both major and minor particle-associated proteins, and modern methods of proteomics may be applied to identify them. Further development of the technique, using a wide range of column designs, lengths, and other parameters, could be expected to make the approach increasingly flexible and contribute significantly to our understanding of the particle–protein corona.

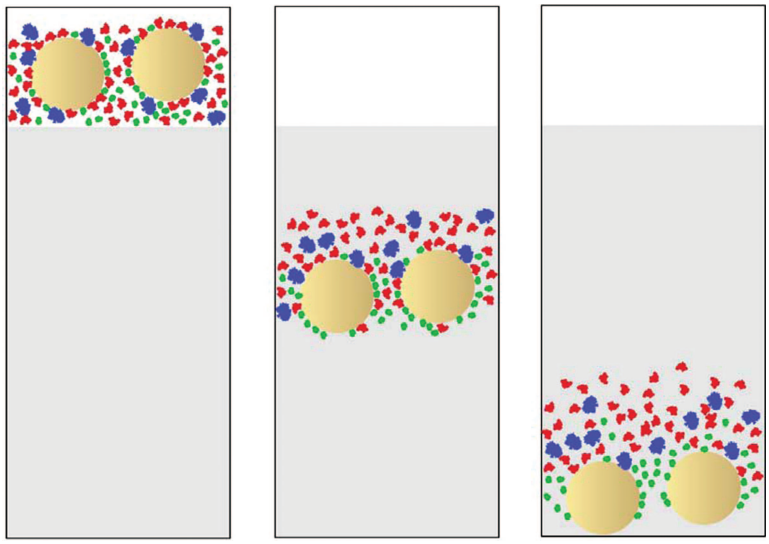

Fig. 1. Gel filtration of a nanoparticle protein mixture. Nanoparticles (beige) are applied to an SEC column in a protein mixture, symbolized by a large blue protein of low abundance and medium off rate, a red protein of high abundance and higher off rate, and a green protein of high abundance and low off rate. In the eluate, the green protein is preferentially enriched on the particles, whereas the faster dissociating proteins elute later.

The novelty and importance of the work by Cedervall *et al.* (9) is that it imaginatively adapts familiar methodologies to examine the issue of nanoparticle–protein interactions and the resulting coating of the particles by a protein layer (so-called corona). This issue will be of increasing relevance to the fields of nanomedicine and, in particular, nanotoxicology, which recognizes the potential for harmful interactions between living tissues and submicrometer- or nanometer-scale objects in a way that may differ qualitatively from more familiar, larger-scale particles with which living organisms have evolved (15). Further advances in these fields will require not only a more quantitative and systematic study of the composition of the nanoparticle–protein corona but also a study of how that corona actually interacts with and affects the well-being of living cells, using, among other approaches, the highly sophisticated methodologies that have been developed to measure surface and intermolecular forces directly (16).

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