How can we predict behavior of nanoparticles *in vivo*?

“Establishing a database that will contain nanoparticle properties, protein corona fingerprints and corresponding biological outcomes will enable maximizing the use of large datasets.”

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The emergence of nanotechnology and nanoparticles has brought conceptually new possibilities for administration of therapeutic agents. Sustained release, targeting, altered pharmacokinetic, reduced toxicity and increased drug bioavailability make nanoscale drug delivery systems highly attractive for improving the therapeutic index of many drugs. It is quite striking that nanotechnology not simply represents a miniaturization of larger objects, but rather enables preparation of materials at the nanometric scale with physical and chemical properties which dramatically differ from those of bulk materials. The main reason for this remarkable change in nanomaterial behavior is its enormous surface-to-volume ratio, which provides a very large interfacial surface area as driving force for enhanced interaction of nanomaterial with surrounding it molecules. Therefore, upon intravenous administration, nanoparticles inevitably form layers of adsorbed biomolecules (mainly proteins) known as a ‘protein corona’ [1,2].

The adsorption of proteins on the surface of nanoparticle is regulated by protein-nanoparticle binding affinity and by protein-protein interactions. Proteins that directly adsorb to the nanoparticle surface with high affinity (usually characterized by long desorption rates in the order of several hours [3]) form the first layer of tightly bound proteins known as the ‘hard corona’. There

is a second stage, which consists of proteins interacting with this firmly bound protein-nanoparticle complex via low-affinity protein-protein interactions, forming the so-called ‘soft corona’ and consisting of loosely bound proteins. The complex structure of protein corona alters the size and interfacial composition of nanoparticles, conferring them a new biological identity, which is what is ‘seen’ by cells in reality. The biological identity of nanoparticles, which could be significantly different from their original synthetic identity, determines the physiological behavior of nanoparticles influencing their colloidal stability, targeting capability, kinetics of circulation, transport, cellular uptake and organ accumulation, degradation, drug release, signaling and toxicity [4].

Because the relative quantities of the adsorbed proteins on the surface of nanoparticles do not necessarily correlate with their abundance in blood plasma, the composition of protein corona for each particular type of nanomaterial is unique and influenced by multiple factors. The complexity and uniqueness of the protein corona composition in each particular case of nanomaterial does not allow one to reliably predict behavior of nanomaterials *in vivo*, which remains one of the major challenges in achieving a predictable and safe use of nanoparticle-based drug delivery systems in therapeutic applications.

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Commercially available analytical instrumentation as well as custom assays have been able to characterize particle drugs and the protein corona to important, but unfortunately not fully actionable degrees. As pioneering work from Dawson and many others have revealed, the formation of the corona is dictated by key parameters including core particle size, particle charge, temperature and proteins involved in the formation, suspension conditions, time and colloidal stability among other factors [5]. However, while current instrumentation and methodologies have produced important insights on the corona's physical structure and biochemical composition, there is a giant gap in how to use this *in vitro* information of basic particle parameters to predict the outcomes of these drugs *in vivo*.

So what has been measured? The thickness of this biological barrier has been measured using dynamic light scattering, ultracentrifugation, fluorescence correlation spectroscopy, single particle sizing tools and even other more complicated techniques including stained transmission electron microscopy imaging and UV-visible spectroscopy to measure agglomeration [6,7]. Dynamic light scattering, the work horse of particle analysis, provides useful and easy to generate particle size distribution information; but when it comes to measuring the corona thickness, this technique works only with essentially monodisperse particle distributions and faces issues measuring the corona thicknesses for particles greater than tens of nanometers (where the thickness change as a result of protein adsorption is much less than the particle core size itself). The presence of the corona can also be captured using charge measurements such as zeta potential to confirm the adsorption of proteins onto the carrier's surface. While useful and used as a standard measurement for corona formation [3,8,9], zeta potential struggles with biologically complex samples and experience severe difficulty with the predominantly weakly negatively charged protein groups (low zeta).

Biochemical characterization of the protein corona consisted of various methodologies, including western blots, mass spectroscopy, surface plasmon resonance and others, which have been particularly useful in characterizing the hard corona (since this layer is not washed away during gel electrophoresis) [6,10–12]. As described above, great insights have been uncovered showing that the protein corona does not necessarily select for the most abundant proteins in serum [13,14]. The above only captures a small subset of the metrology work on protein corona measurements. As is well documented, all of these analysis techniques have their advantages and disadvantages and have painted a good starting picture of the corona, so what is missing?

We believe that there are three main obstacles that must be overcome so that metrology can help researchers understand and develop better particle-based drugs, given the ever present reality of the protein corona. First, there is a need for tools capable of measuring the weak interactions that are augmented in the nanoscale in high-throughput. Most systems are designed to detect strong interactions, like strong electrostatic forces, or require lots of material (bulk-based measurements) to operate. Weak interactions have been ignored or been out of the reach of most existing instrumentation. Second, particle analysis technologies that work more effectively in complex fluids like blood and serum under little dilution. This capability is a big challenge to systems optical and electronic alike, but chemical methods are fair better. Third, we have to challenge the idealistic concept of what we think the particle-based drugs look like after formulation versus what they really look like. There is a need to measure and validate what we claim.

To address these challenges, novel comprehensive surface characterization technologies have to be developed. For example, the Optofluidics NanoTweezer Surface Analysis System is designed to tackle the first and third challenge by enabling high-throughput colloidal surface analysis. Colloidal analysis is in dire need of greater surface characterization since the surface is the vehicle of interaction for these particles, it is where the corona is formed and where it changes with the environment and time, but such measurements have been largely disregarded by current analytical techniques. To measure surface properties, including the corona and other surface coatings, the system captures the total interaction potential of weak and strong interactions (the summation of the electrostatic, hydrogen bonding, steric and other forces) at the individual nanoparticle level [15] through the use of microfluidics and nanophotonic waveguides. Its outputs include particle repulsion strength and characteristic interaction length, and maps how the particles interact against different reference surfaces, allowing one to screen for specific interactions. Greater insights of these weak interactions can enable better understanding of corona formation and dissolution, for example, the steric contribution in protein–protein interactions present in the corona, yielding information key to understanding the biophysical basis, and one step closer to answering which forces are involved in determining whether a targeted nanoparticle is cleared by the immune system or reach the target tumor site. Also, by being able to measure subpopulations (one particle at a time), one can start to assess surface heterogeneity and examine whether all particles exhibit the same surface characteristics (e.g., are all my particles coated with PEG and how uniform is that protein corona?).

In addition to the advancement of novel analytical techniques enabling high-throughput colloidal surface analysis and investigation of protein corona composition, quantitative models capable to accurately predict biological interactions of nanoparticles in physiological milieu are needed to accelerate clinical translation of nanoparticle-based drugs. Recently, Chan and coworkers have proposed a novel approach to predict the cellular interaction of gold and silver nanoparticles based on quantitative structure–activity relationships, which link the composition of the formed protein corona with the bioactivity of nanoparticles [14]. The composition of the protein corona ‘fingerprint’ (based on the relative abundance of the serum proteins adsorbed on the nanoparticle surface) was used to predict the cell association of about a hundred-member library of chemically and physically diverse nanoparticles. Cell association (nanoparticle surface binding and uptake) was chosen as a model biological interaction due to its relevance to inflammatory response, biodistribution and toxicity. This work has shown that the quantitative model that uses the protein corona composition fingerprint was 50% more accurate than a model that uses physicochemical properties of nanoparticles such as size, aggregation state and surface charge [14]. While the above work has demonstrated that protein corona fingerprints contribute more significant information than the physicochemical properties to make an accurate prediction of biological behavior of nanoparticles, the overall prediction can be further improved if the model will use both corona composition and relevant physicochemical properties [16].

It is of note, that blood serum does not comprise an ideal model reflecting a realistic *in vivo* environment, because key blood coagulation factors are absent. To make more accurate predictions for nanoparticles behavior *in vivo*, the future correlation models may consider using nanoparticles exposed to a whole blood or even injected into the blood stream with subsequent isolation and characterization that will reflect a more realistic corona composition. This strategy may contribute to our understanding whether there is a dramatic difference in corona composition when particles exposed to real conditions *in vivo* and how this affects nanoparticle biological behavior and fate.

Using the quantitative structure–activity relationships that will link the information obtained by the

most advanced colloidal surface analysis techniques with the protein corona composition data (originated from ideal *in vivo* conditions) and corresponding biological outcomes, the researchers will be able to make more accurate predictions of nanoparticles association with various relevant cells types, such as circulating immune cells, endothelial cells (lining the lumen of the blood vessels) and organ-related hepatocytes and splenocytes. Because the nanoparticle–cell interactions influence multiple downstream cellular responses, the predictions could be extended to characterize signaling pathways, cytokine secretion, gene expression and toxicity. This knowledge can be further extended to predict the realistic *in vivo* pharmacokinetics, biodistribution and organ response. Using the recently proposed alphanumeric nomenclature system, nanomaterials possessing specific chemical and physical properties can now be categorized and indexed [17]. Establishing a database that will contain nanoparticle properties, protein corona fingerprints and corresponding biological outcomes will enable maximizing the use of large datasets [14]. Ultimately, such databases will allow the broader research community to model biological interactions without the need for extensive experimentation, which will lead to establishment of predictive models that will guide the design of nanoparticle-based systems for diverse therapeutic applications.

Disclaimer

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