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The nanoparticle protein corona: the myth of average

Valérie Forest^{*a}, Jérémie Pourchez^a.

^a École Nationale Supérieure des Mines de Saint-Etienne, CIS-EMSE, LINA EA4624, SFR IFRESIS, F-42023 Saint-Etienne, France.

* Corresponding author: Valérie Forest:

École Nationale Supérieure des Mines de Saint-Etienne

158 cours Fauriel

CS 62362

42023 Saint-Etienne Cedex 2

FRANCE

Email address: vforest@emse.fr - Telephone number: +33477499776

Abstract:

It makes sense that if something is designed based on average measures, it would be appropriate for all, or most of all. But reality is quite the opposite. For example, a seat designed based on the average size of a population will not be suitable for the tallest and the shortest persons. Indeed, by definition an average does not represent extreme values that can be significantly different. The same applies for life sciences as living organisms are made of many cell populations and biological processes are usually investigated globally. Based on a very simplified example, this paper discusses this concept for the particular case of the characterization of the nanoparticle protein corona. Firm conclusions cannot be drawn from the global analysis of samples containing a large number of nanoparticles. It results that in future, different approaches will be required to identify corona subclasses rather than averaging over them. A way to face this challenging task may be through the combined use of different analytical techniques, each shedding light on a different aspect of this complex layer to gain true insight about its nature and dynamics.

Key-words:

Protein corona, nanoparticles, average analysis.

Introduction:

The Myth of Average is a paradigm which is prominent in most sciences. It is the belief that we can use statistical averages to understand particular cases. One of the best historical example was perfectly described by Todd Rose concerning the US Air Force [1]. In 1952, the Air Force was perplexed because they had good pilots flying better planes that benefitted from the most advanced technologies but their performances were worsening. And they didn't know why. It turned out that the problem was actually with the cockpit ergonomic. Indeed, these latter were designed based on 10 average dimensions of the pilots (height, shoulders, chest, waist, hips, legs, reach, torso, neck, and thigh). For a long time, it was assumed that if you design something for the average size person, it would fit for most people. But it was wrong and it was demonstrated 60 years ago by Gilbert Daniels, an Air Force researcher who studied over 4000 pilots. He measured them on the 10 above-mentioned dimensions of size and he asked how many of these pilots were average on these 10 dimensions. The answer was zero, meaning that the "average pilot" does not exist and that the "average cockpit" that should theoretically be suitable for most people actually fitted nobody.

Biological examples:

The same argument can be extrapolated to many fields, especially biology where organisms are made of many different cell populations. Usually the analysis of a biological process is global and conclusions are drawn from average results. As an example [2], gene expression is analyzed in terms of a cell population as information comes from samples containing millions of cells. In other words, the probable state of an average cell in the population is estimated from a complex mixture of cells. But in reality, this supposed "average cell" is a lure and the analysis does not take into account the variation that can be highly significant, among the members of the population. However, recent technological advances have allowed the precise measurement of single-cell transcriptional states to study this variability more rigorously. And it appeared that the way genes are expressed in a population is strikingly different from what was assumed from extrapolating from an average cell.

Another example of increasing importance can be found in the nanotechnology field. It refers to the nanoparticle corona. This dynamic layer of proteins adsorbed at the nanoparticle surface upon contact with biological media alters the initial physico-chemical features and defines the "biological identity" of the nanoparticle. This new interface even impacts the

nanoparticle/cell interactions and consequently the response of the biological systems [3,4]. It is therefore of paramount importance to consider the role of this layer in biological assays. But the protein corona constantly evolves, over time (following a Vroman's effect where more abundant proteins in a medium first adsorb but are progressively replaced by proteins of higher affinity) and also evolves as the nanoparticle travels through different compartments of different composition [5,6]. Consequently, due to this complex and dynamic nature, it is quite challenging to reliably characterize a protein corona. However, many attempts from very simple approaches to much more sophisticated methods have been undertaken, each providing different types of information. For example, nanoparticle size and/or charge changes consecutive to the formation of a protein corona can be detected using dynamic light scattering or transmission electron microscopy. Nanoparticle-protein interactions can be characterized through a wide range of analytical techniques such as isothermal titration calorimetry, fluorescence and ultraviolet-visible spectroscopies, quartz crystal microbalance, surface plasmon resonance, atomic force microscopy, fluorescence correlation spectroscopy, size-exclusion chromatography, circular dichroism spectroscopy, infrared and Raman spectroscopies... [3-6].

So far, the most commonly used strategy for the nanoparticle-protein corona characterization is a multi-step process consisting in: 1) the incubation of nanoparticles with a biological fluid (often serum, *i.e.* a mixture of proteins), 2) a centrifugation to remove unbound proteins, 3) the elution of the proteins adsorbed at the nanoparticle surface using denaturing agents, 4) the separation of the isolated proteins by a one or two dimensional gel electrophoresis and finally 5) the protein identification using mass spectrometry [4,7].

Using this proteomic approach, interesting observations were made. For instance, it was demonstrated that out of the 3700 proteins present in human blood plasma, only a few tens could be found in the protein corona. Most interestingly, the most abundant proteins in plasma were not necessarily the most present in the protein corona clearly indicating that the composition of this latter was not the reflect of the surrounding medium [5,7-9]. It was also observed that the nanoparticle physico-chemical features played a key role in the selective binding of proteins as well as the type of serum [6]. The nature of the proteins bound to nanoparticles is crucial as it directly impacts the nanoparticle fate. Indeed, adsorption of opsonins (IgG and complement factors) promotes phagocytosis with removal of nanoparticles from the bloodstream, whereas adsorption of other kinds of molecules can on the contrary favor cellular uptake. For example, adsorbed apolipoproteins might foster

transport across the blood-brain-barrier, likely through the interaction with low-density lipoprotein receptors [10]. As the protein corona is the interface interacting with cellular systems rather than the nanoparticle bulk material some authors have proposed a new classification of the nanoparticles based on their protein corona nature [3,5,11]. Others have suggested that as this corona formation is not avoidable, it should be found a way to take advantage of it, especially by a rational design of nanomaterials to optimize biotechnological applications and reduce potential nanohazards. In this regard, quantitative proteomic data are needed as a resource for model building, to predict and simulate the kinetics of protein-binding to nanosized objects in physiological systems [11].

Huge progress has been made toward the characterization of the nanoparticle protein corona and protein identification has benefited from proteomic approaches and increased sensitivity of apparatus. However, if some trends can be trustworthy it remains difficult to draw absolute conclusions. One of the main reasons why we should be careful in the interpretation of results from such analytical processes is that analyses are global, therefore they are based on average measures. Let's take a (simplified) example to illustrate our point. As shown in Figure 1, let's imagine that the qualitative and quantitative analysis of a sample of 10 nanoparticles results in a protein corona composed of 30% protein A, 30% protein B, 30% protein C and 10% protein D. From this "average point of view", one could imagine different scenarios. It clearly appears that the biological consequences of these different protein corona configurations would not be the same. And it becomes obvious that firm conclusions cannot be drawn from average analyses, pointing out that in future, different approaches will be required to identify such corona subclasses, rather than averaging over them [5].

In addition, because the corona is generally not at thermodynamic equilibrium, there could be statistical fluctuations in its composition and organization from particle to particle within the same sample [5]. Therefore, protein corona is currently studied through "snapshots", its composition being defined at a specific time point. Instead, analyses should tend to real-time observations to follow the protein corona evolution and to be more accurate in the interpretation of results.

Conclusion and perspectives/future directions:

As protein corona directly interacts with biological systems, the isolation and identification of proteins bound to nanoparticle represents a fundamental prerequisite for nanobiology,

nanomedicine and nanotoxicology. However it is a quite challenging process. Furthermore, the qualitative and quantitative characterization of the protein corona should be integrated in a broader context as the more abundantly associated proteins will not necessarily trigger the most profound biological response. As a corollary, a less abundant protein with high affinity may instead be a key player [8].

In recent years, many analytical developments have allowed to gain sensitivity and improve the protein corona characterization *in vitro*. However these techniques still have limitations or pitfalls. Experimental conditions are crucial and their lack of homogeneity makes difficult the comparison between studies from the literature. Furthermore, as the composition of the protein corona on a given nanoparticle, at a given time, depends on the concentrations and kinetic properties of the proteins found in plasma, it is important to not only determine which proteins are adsorbed onto the surface of the nanoparticle, but also understand the binding affinities and stoichiometries [7].

Despite these limitations, interesting results on the protein corona have been obtained *in vitro*. A new challenge is now to follow it *in vivo* [5,9] and possibly by performing real-time measurements to monitor the evolution in protein binding to nanoparticles.

Finally, to address the problem of average, nanoparticles should ideally be characterized individually. A parallel can be made with flow cytometry for cell analysis; indeed, this technique allows the individual multi-parametric analysis of cells. In this context the use of nanoparticle tracking analysis (NTA) could represent a useful technique [9]. However, the individual analysis of nanoparticle protein corona may be a utopia due to the complexity of the task. An alternative solution may lie in the development of new methods or in the combined use of different available analytical techniques to analyze simultaneously several aspects of nanoparticle-protein interactions [4,5,12].

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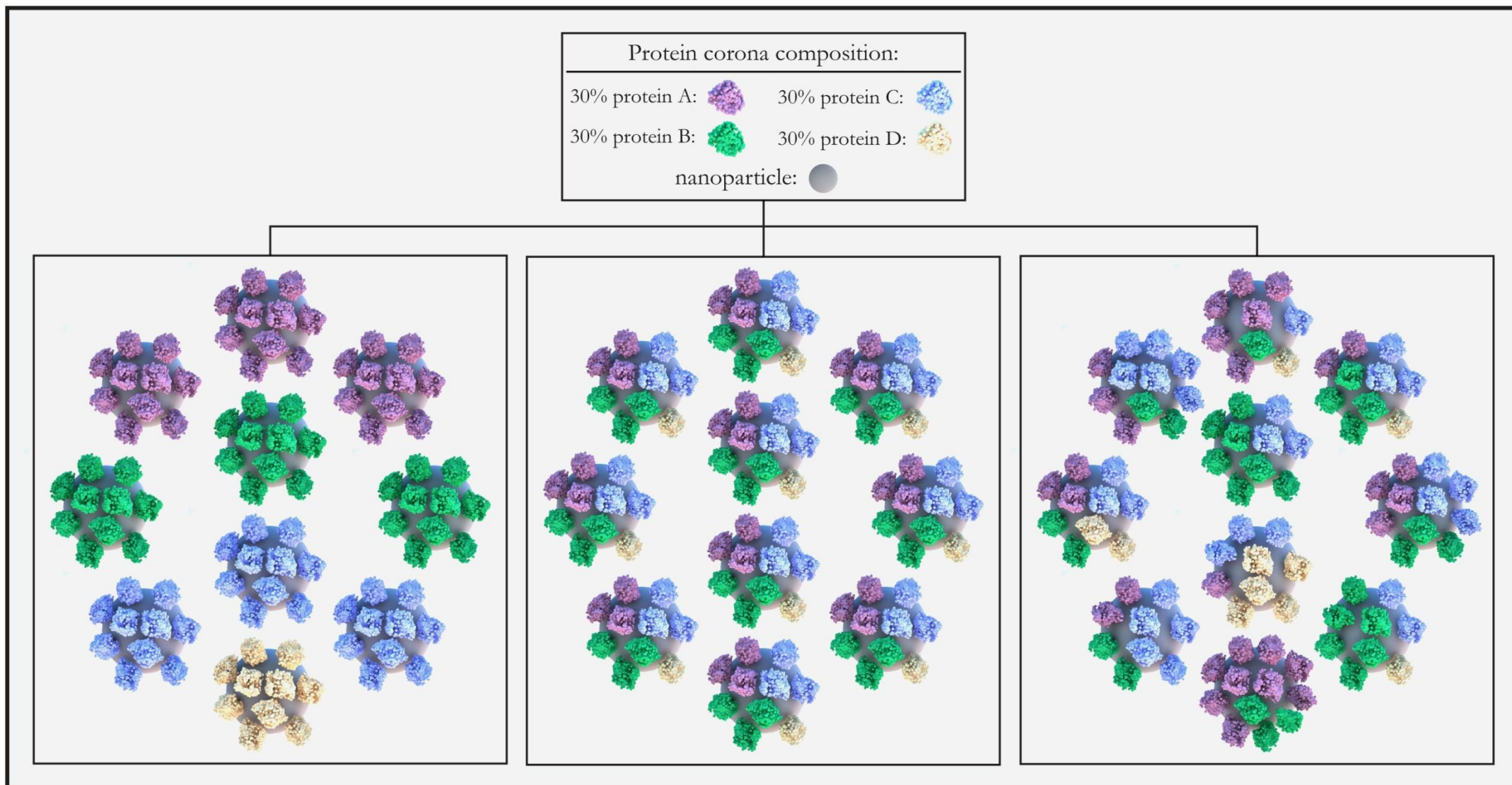
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Figure legends:

Figure 1 – Possible scenarios after the proteomic analysis of the protein corona of a sample of 10 nanoparticles.

Figure 1



For 10 particles, 3 examples of possible scenarios