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The Nanoparticle - Protein Corona Untold History (1907-2007)

Eudald Casals[a], Michele Vitali[b], and Victor Puntes[b,c,d,*]

[a] *School of Biotechnology and Health Sciences, Wuyi University, Jiangmen, 529020, China*

[b] *Vall d'Hebron Research Institute (VHIR), 08035, Barcelona, Spain.*

[c] *Institut Català de Nanociència i Nanotecnologia (ICN2), CSIC, The Barcelona Institute of Science and Technology (BIST), Campus UAB s/n, Bellaterra, 08193, Barcelona, Spain.*

[d] *Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010, Barcelona, Spain*

** To whom correspondence should be addressed: E-mail: victor.puntes@vhir.org*

Abstract.

The adsorption of biomolecules, primarily proteins, on the surfaces of nanoparticles plays a crucial role in elucidating the safe application of nanotechnology, especially in medicine. Termed the "Protein Corona" since 2007, this phenomenon remains of general interest and continues to be subject to intensive research today. Notably, dating back to the XIX century, a large body of early work has addressed the adsorption of proteins onto different surfaces, from colloids to implants. *Standing on the shoulders* of this historical knowledge could deepen our understanding of the Protein Corona and its biological implications. Herein, we aim to trace the historical trajectory before 2007, combining earlier scientific evidence and findings with contemporary research on the topic. Such insights will contribute to helping and guiding further research into the Protein Corona, thereby accelerating the clinical and societal translation of nanobiotechnology and nanomedicine.

Keywords: Protein Corona, Protein Adsorption, Proteins at Interfaces, Colloids.

Introduction.

The adsorption of proteins onto surfaces, including nanoparticles, has been known since the times of colloidal science and the discovery of proteins in the late XIX century. These studies originated from the observation that proteins, due to their strongly amphipathic nature resulting from a mixture of polar and non-polar chains, tend to concentrate at interfaces, thereby altering the physicochemical properties of both the material surface and the proteins.[1, 2] However, its impact on nanoscience was not fully realized until Dawson, Linse, and co-workers reported in 2006 and 2007 that proteins in biological fluids associate with nanoparticles (NPs) forming the "Protein Corona" (PC) a new entity responsible for the fate of NPs in biological environments.[3, 4] Since then, a variety of multidisciplinary approaches exploiting advances ~~in~~ from engineering tools ~~and~~ to computational modeling, have been employed to elucidate the formation process and the physicochemical and biological characteristics of the PC, with the aim of developing more precise, accessible, and safe NPs.[5-11]

After almost twenty years of intense research, the PC continues to challenge our understanding.[7, 12] The lack of evidence elucidating the phenomena of protein adsorption onto NPs surfaces limits their exploitation in different areas, from the development of nanobiological tools ~~or~~ to nanomedicines[5, 12, 13] to the emerging study of the environmental ~~dimensions~~ nature of the PC, known as the Eco-Corona~~, which examines existing research on the PC~~.[8, 14, 15] ~~The adsorption and stabilization of proteins onto natural or artificial surfaces, including particles and colloids, have been studied and documented for decades.~~ This review aims to analyze the key events that led to different observations on the subject of the protein adsorption to different surfaces before the term Protein Corona was coined, beginning with the first efforts to understand the structural nature of proteins in the first half of the XIX century. Due to the ontological change on particle surface-protein interactions over time (e.g. from mere *protein adsorption* to *proteins at interfaces* to *Protein Corona*) and ~~the way~~how current keyword-research searches scientific information across a plethora of increasing publications, pieces of important information may have been overlooked. Accordingly, b~~B~~y compiling experimental observations reported over these last 150 years, valuable information can be gathered, providing researchers with guidance to address remaining questions related to the different aspects of the PC. **Figure 1** shows the timeline of key events in this history as presented in this review.

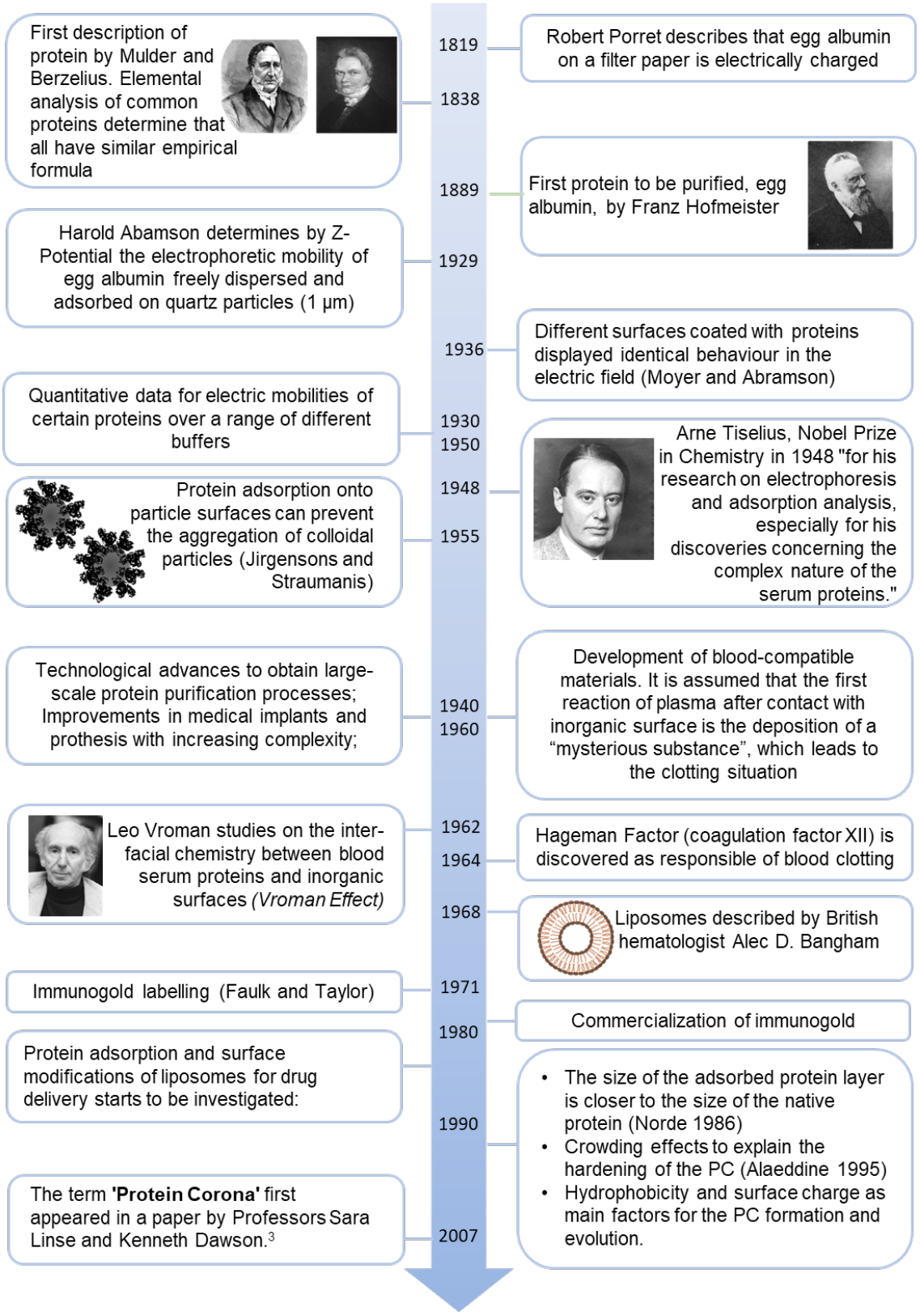


Figure 1. Timeline depicting various studies on the nature and properties of proteins, as well as on the characteristics of proteins at interfaces, as discussed in the text. MIRROR THE TIME LINE!!!

In this line, over the last two decades we have seen how the synergy between knowledge from proteins and nanomaterials has led to the development of advanced nanotools for monitoring and manipulating biological states. Combining NPs and proteins offers opportunities for innovative solutions. For example, conjugating NPs with proteins can yield additive characteristics, such as plasmonic antibodies for detection, magnetic enzymes for recovery, or antioxidant albumin through controlled combinations of NPs and proteins. While drug interactions with serum proteins, notably albumin, are often undesirable in pharmacological sciences, in the case of NPs, protein absorption can be highly beneficial. It safeguards against aggregation and enhances biocompatibility without compromising inherent properties, whether as imaging antennas, drug delivery vehicles, or nanozymes. Reflecting on this historical overview, coupled with dynamic characterizations of PC evolution over time in different physiological compartments, the investigation of subtle modifications in protein configuration, and the detailed characterization of Soft and Hard-PCs should be integral to future research on biomedical applications of NPs. By assimilating insights from past studies and adapting to NP specificities, such comprehensive investigations can pave the way for the future of nanomedicine.

1. Protein adsorption to surfaces.

During the late XIX century and the first half of the XX century, the fascination with proteins and their amphipathic nature triggered ~~the~~ multiple studies of their structure, characteristics, and behavior at interfaces. This is exemplified in this text of Alexander Rother in 1947,[16] where he reviewed the biological activity of films of proteins formed at the water interphase, and on metal and glass slides: *"The importance of surface phenomena in biology has been recognized for a long time. This importance is due to the considerable extension of interphases in the animal body (...), it is to be expected that the interphase offers conditions which may alter reactions involving proteins. Proteins have a very remarkable property in common, that of spreading easily at a water interphase. This spreading usually involves a considerable change in the shape of the protein molecule"*. Of course, there is a long sequence of events that lead to these conclusions~~early studies~~. Proteins were first described by Dutch chemist G. J. Mulder and Swedish chemist J. J. Berzelius in 1838, who coined the term of protein (from the greg *prōteîos*, 'fundamental, principal').[17, 18] Additionally, it is attributed to the work of Robert Porret in 1816 the firsts observations that adsorbed egg albumin on a filter paper wa~~i~~s electrically charged,[2, 19] laying the groundwork for the use of electrophoresis in the analysis of complex protein mixtures a century later by Arne Tiselius.[20, 21]

During those times, early investigations into the nature and properties of proteins at surfaces aimed to explore the structural characteristics of adsorbed proteins on surface films and the influence of the adsorption process on the protein electrical properties. Initial observations were done on particles of glass, which, upon protein adsorption, acquired electrophoretic mobilities more closely resembling those of dissolved protein than of untreated glass ~~particles~~.[22-24] ~~Furthermore~~Comtemporanely, in a study by Harold Abramson and colleagues in 1929, ~~these~~ authors determined~~,~~ through Zeta Potential measurements and by integrating the theories of Smoluchowski, Debye and Hückel, and Henry, that the electric mobility of freely dispersed egg albumin and of microscopic (approximately 1 µm) quartz particles coated with egg albumin were nearly parallel at pH 3.2 and 5.[25] One year later, in 1930, Arne Tiselius, utilizing his advanced electrophoresis method, replicated the experiments on microscopically visible quartz particles coated with a layer of adsorbed egg protein, obtaining similar ~~outcomes~~results.[26]

The agreement between their results enabled scientists to extend the quantitative data on the electric mobilities of certain proteins adsorbed on glass or quartz surfaces across different

buffers. This made available values for changes in charge over a wide pH range, facilitating a preliminary examination of surface-adsorbed protein properties.[27] During this period, surface charge was the primary parameter for studying proteins and their interactions with surfaces. These studies underwent continuous revisions and improvements over the next decades. For example, precise determination were made of the different electrophoretic mobilities (and hence electrophoretic radii and Zeta Potential) of bovine serum albumin and egg albumin, both free and adsorbed on powdered Pyrex glass, and dispersed in solutions of different ionic strengths.[28] These studies reflected a scientific community working on the basis that protein properties were presumably dependent on electric charge, and that this charge was altered when proteins were absorbed onto a surface – a process with significant consequences for their biological activity. Given the importance of these modifications to the ~~electrical properties~~electrostatic properties of absorbed proteins, it is noteworthy that today, alterations in the Zeta Potential and isoelectric points of proteins absorbed on nanoparticle surfaces have received comparatively little investigation compared to other aspects of the Protein Corona. Nevertheless, they may still provide valuable insights into the nature and behavior of the protein corona, including its composition and biological ~~consequences~~impact.

Another significant discovery was the determination that different particles of different sizes and compositions, coated with proteins exhibited identical behavior in an electric field when the protein concentration was sufficiently high to ensure complete protein layer formation. Laurence Moyer and Harold Abramson conducted these studies using finely powdered quartz particles, Pyrex glass particles, and paraffin oil globules (approximately 1 µm in diameter) dispersed in concentrated protein solutions of gelatine and gliadin (a wheat protein) and in either concentrated or diluted electrolyte solutions.[29] Specifically, the results demonstrated that the ratio of electroosmotic to electrophoretic mobility of all these protein-coated particles in the various electrolyte solutions was nearly identical. One year later, Laurence Moyer confirmed these findings using egg albumin adsorbed on quartz, glass, mineral oil, carbon, cellulose nitrate, aluminum oxide, and silica gel colloids.[27] All of these studies pointed towards a low specificity of the protein adsorption phenomenon for a given material surface and indicated that some general trends could be inferred from their adsorption behavior.

It is worth noting that for these studies, only proteins that could be purified in large quantities, such as gelatin, egg albumin, and those obtained from blood sourced from slaughterhouses, were available for use. Although the first protein to be purified, hen egg-white albumin by Franz Hofmeister in the late XIX century (1889), the situation ~~began~~ did not ~~to~~ change ~~in~~ until

the middle ~~of the XX century due to the Second World War~~ and the increased need for blood proteins for transfusion and medical interventions. Different technological advances in protein fractionation and purification ~~processes were made to~~ enable<u>d</u> large-scale protein purification for commercial purposes. Consequently, this made more proteins, particularly enzymes, available for researchers to study.

In this first part of the historical overview, it is also noteworthy to mention the studies on the stability of colloids. For example, H. B. Weiser detailed <u>in 1933</u> in his book "Inorganic Colloid Chemistry" how the addition of ions compromised the stability of a colloidal gold solution ~~in 1933~~: "*The addition of electrolytes to unprotected red gold sols in concentrations above a certain limiting value causes flocculation of the particles into secondary aggregates, accompanied by a change in color to violet or blue and a slight clouding*".[30, 31] Remarkably, as early as 1955, Jirgensons and Straumanis observed that protein adsorption prevented <u>such</u> particle aggregation by forming a protective layer that stabilized ~~the~~ colloids.[32] <u>This</u> ~~And it~~ has been <u>confirmed recently</u> ~~observed that~~ even in solutions with high ionic strength, <u>where</u> the rapid adsorption of proteins can stabilize nanoparticles against aggregation.[33, 34]

<u>Building upon the work of Jirgensons and Straumanis on the stabilizing properties of protein adsorption to colloidal particles in high ionic strength solutions,[32] Faulk and Taylor identified Salmonella antigens by employing immunogold labeling in their seminal work on immuno-labeling with colloidal gold.[79] These authors noted that "*the addition of strong electrolytes alters the electrochemical environment and allows (the gold sol) to adhere and flocculate. This can be prevented by adding a 'protective' colloid, such as proteins (...) It therefore seemed reasonable to stabilize the gold sol with antibody, and thereby develop the possibility of an immunocolloid method for the electron microscope.*" Initially, they determined the optimal amount of antibody necessary to stabilize the gold particles by exposing the colloids to increasing amounts of antibodies. Subsequently, they studied the stability of the conjugates by adding sodium chloride, a strong destabilizing agent for electrostatically stabilized AuNPs. Once optimal surface passivation was achieved under appropriate antibody concentration conditions, the antibody-labeled colloids were centrifuged to concentrate the conjugates and eliminate unlabeled antibodies. This procedure, described by Faulk and Taylor, is now employed as the typical method for the formation and isolation of nanoparticle-protein complexes.[80, 81] Similar strategies provide nowadays the opportunity to accommodate biomolecules with targeting moieties on the surface of nanomaterials and, as a result, NP-</u>

Protein conjugates can be designed to perform sophisticated biological tasks, such as inducing Natural Killer cells to attack HIV-infected T-cells.[67]

2. Proteins at Interfaces.

In the first half of the XX century, alongside the developments in the purification and large-scale production of proteins, ~~many~~ related medical fields experienced significant advances. One of the most notable advancements was the progress in developing medical implants and prostheses with increasing complexity. However, the need for blood-compatible materials posed a serious challenge due to thrombus formation and cellular adhesion at their surfaces. At that time, it was presumed that the initial reaction of blood or plasma after contact with an inorganic material was the deposition of a mysterious substance, ultimately leading to clotting situations.[35] In 1964, it was determined that this substance was a protein, which was named the Hageman factor after the patient ~~who was the~~ subject of those studies. Hageman exhibited prolonged clotting times due to the absence of this protein, also known as factor XII.[36] Later on, the process of coagulation and implant-induced coagulation, and how it wa~~i~~s triggered by proteins that readily attached to the implant surface—most notably, high molecular weight kininogen and factor XII—were more finely described.

~~It is now well known that when these proteins bind to the surface, various conformational changes occur, exposing activation sites that initiate the clotting cascade and inflammatory stimuli.[37]. It is worth noting here that NPs do not always elicit a pro-inflammatory response; sometimes, they can go undetected by the immune system, and in certain cases, they may even exhibit anti-inflammatory properties.[38].~~

Those discoveries were not isolated events. At that time, many researchers were pursuing the idea that the main character in this interplay between cells and surfaces were proteins. These studies crystallized in what is known as the Vroman Effect, which sequentially describes the interfacial chemistry between blood serum proteins and inorganic surfaces. The studies of Leo Vroman in the late 50s and 60s, showed how the exposure of hydrophobic inorganic powders to blood plasma resulted in the removal of coagulation factors, as the inorganic surface became more hydrophilic.[39-43] In this process, a competitive adsorption hierarchy was observed: the proteins with high mobility arrived first to the surface, and were then replaced by others with lower mobility but higher affinity, ~~mainly factor V and fibrinogen,~~ in a process that takes up to few hours. Thus, under stagnant conditions, protein deposition was described

to occur in the sequence: albumin, globulin, fibrinogen, fibronectin, factor XII, and high molecular weight kininogen. This phenomenon, described by Leo Vroman in 1962, is acknowledged as the general principle governing the competitive adsorption of a complex mixture of proteins (such as serum) to surfaces.[39, 44, 45] Although this phenomenon was described for nanomaterials in 2010,[46, 47] the characterization of the PC evolution in NPs and drug delivery devices intended for medical applications was often absent in most modern preclinical NPs studies before 2007, when Dawson and colleagues highlighted its significance.[3]

A significant milestone in understanding the interactions between NPs and proteins was the discovery and development of liposomes for drug delivery. Liposomes were initially described by British hematologist Alec D. Bangham at the Babraham Institute in Cambridge.[48] Following this discovery, Bangham and his colleagues[49, 50] along with other researchers,[51-54] reported the ability of liposomes to encapsulate various small molecules and macromolecules, suggesting their potential pharmacological use. However, it was soon observed that the characteristics of liposomes appeared to be altered after exposure to blood, attributed to the absorption of serum proteins, which hindered their clinical implementation. Several studies indicated the reversal of liposome surface charge[55] and increased permeability to entrapped ions when they were dispersed in physiological media,[56, 57] both of which were attributed to the absorption of proteins on their surfaces.

Following the development of liposomes, there was a blossom of new micrometric and submicrometric polymer particles designed for similar pharmacological purposes, which exhibited similar processes of protein adsorption in blood. This trend is exemplified by the special issue of the Annals of the New York Academy of Sciences in 1987, focusing on "Blood in Contact with Natural and Artificial Surfaces,"[58] as well as the series of books on "Proteins at Interfaces" by J. L. Brash and T. A. Horbett,[59, 60] and one of the earliest comprehensive reviews on this topic, also titled "Proteins at Interfaces," by F. Macritchie.[61] While this list of works is not exhaustive, it is representative of the knowledge gained during those early years. **Figure 2** summarizes various materials containing ultrafine particles that were used in these studies.

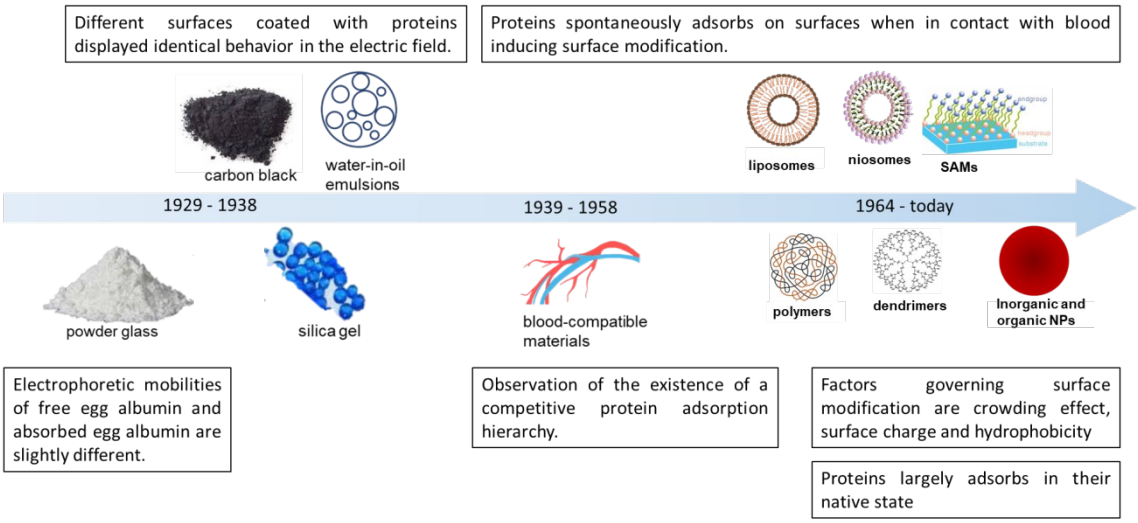


Figure 2. Different materials studied for protein adsorption processes since the XIX[th] century.

After reviewing the literature from the 1960s to the 1980s, it becomes evident that hydrophobicity and surface charge were commonly regarded as the primary factors ~~influencing~~ driving protein adsorption to surfaces. At this times, o~~O~~ne of the most well-known examples illustrating the adsorption of molecules at surfaces wa~~i~~s provided by the work of Prime and Whitesides, who used self-assembled monolayers (SAMs) supported onto gold films.[62] These SAMs consisted of alkane chains with different terminal groups, leading to varying degrees of surface hydrophobicity. It was observed that the more hydrophobic the conjugate, the greater the degree of protein adsorption. Similarly, much higher adsorption of positively charged proteins onto negatively charged polystyrene surfaces, and vice versa, compared to neutral surfaces, was observed by Norde and Lyklema,[63, 64] among many other studies.[60] This confirms the dominance of electrostatic interactions ~~in~~ driving NP-protein interactions before coordination bonds and crowding effects stabilize proteins at surfaces.[65-67] Notably, this increase in effective local protein concentration at the NP surface enhances their chemical activity and may alter the rates and equilibrium constants of the reactions they are involved in.[74, 75]

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The study of the absorption of proteins at surfaces, a phenomenon ubitquitous in nature, has received strong impetyus with the realization that Random Sequential (disordered) vs Clustering (cooperative) absorption models provide a good basis for describing the processes [https://www.ncbi.nlm.nih.gov/pubmed/9962592]. Cooperative absorption means that a

protein absorbed to a surface binds more strongly to it if surrounded by similar proteins, thanks to the possibility of build compact arrangements with identical building blocks. Similarly, when a cluster of proteins is made, to move one, many have to be moved, increasing thus coating stability (cooperative effect) as the protein domain grows. In this conditions, proteins which are identical pack better than proteins that are different, therefore favoring the formation of homongeneous coating domains from a mix of coating molecules, as Stellachi previously showed with diblock co-polymers and other organic molecules [https://www.nature.com/articles/nmat1116]. Thus, if we start from a perfect stable mixture where NPs and antibodies/PROTEINS are perfectly soluble, NPs and Abs will remain isolated in solution. As the solubility of NPs and proteins decreases, manifested in an increase of attraction forces and consequent aggregation, the system evolves towards a totally aggregated composite state. In between, intermediate states can be stabilized. In the case where the NP instability is greater than the protein instability this leads to NP Protein Monolayer Coronas. Now, this corona can be populated randomly by proteins or cooperatively. This is determined by the interactions hierarchy. The interactions hierarchy depends on the employed building blocks, what is determined by their own nature, their relative concentration and the electrolytes (ions) present in solution which controls interaction between solvated species surrounded by different ionic species by altering the NPs and proteins water envelope (following Debye length or Hoffmeinster series, where small ions with high charge expulses water "salt-out" and large ions with small charges "salt-in" the proteins or NPs).

As it is well known, proteins rapidly form a soft protein corona around the NP formed of loosely bound proteins, in equilibrium with free proteins in solution. Due to rearrangements and crowding effects, this evolves towards the formation of a hard corona of proteins permanently attached to the NP surface that remains associated to the NP even after precipitation and resuspension into protein free media [CASALS AND CASALS]

This stability related to crystallinity (or packing density) is also refered as the Crowding effect, where to move one Ab implies to move many (all of them packed against it and the other packed against the packed against it and so on...).

This protein crowding, characterized by dense packing, is a known effect explained by the limited mobility of the proteins when they are confined. This confinement causes them to behave in a radically different manner compared to free proteins in solution. On one hand, the intermolecular forces of attraction that hold protein tertiary structure together are weak non-covalent interactions. Thus, at room temperature, there is enough thermal energy available to allow proteins to break and reform their tertiary configuration. Consequently, they possess a set of interchangeable conformational states with different free energies, described by the so-called energy potential landscape.[83, 84] This landscape is essential for various protein functions, such as catalysis or ligand binding.[85], and this flexibility is diminished when the

proteins are absorbed to surfaces. On the other hand, the crowding of proteins on a surface modifies their dissociation constant, favoring association. This phenomenon explains the increased stability of the packed units at the surface to which they are attached.[86-88]

3. Hard and Soft Protein Coronas.

Studies of interface phenomena involving proteins have also identified their adsorption to surfaces as an irreversible process starting with loosely absorbed proteins at the NP surface evolving towards a permanent dense protein coating, the PC. The stable PC is known as the Hard-PC, to distinguish it from the Soft-PC, which consists of loosely attached proteins reversibly adsorbed at the NP surface. Over time, different proteins irreversibly adsorb until the entire NP surface is coated, forming the Hard-PC, which does not detach from the NP surface after purification and dispersion in protein-free media.[63, 76] The mechanism by which proteins adhere to surfaces primarily involves hydrophobic and electrostatic interactions initially, followed by hydrogen and coordination bonds, all of which are often rather weak. However, the summation of these weak interactions between the NP surface and aminoacids, results in strong absorption, which becomes irreversible after ~~molecular~~ structural rearrangements and protein clustering at the surface. Consequently, attempts to fit protein adsorption data to the Langmuir equation have largely failed.[68]. Detailed studies have provided further insights into these mechanisms. Contrary to what might be expected, proteins do not always distribute randomly on surfaces.[69-71] ~~Instead, quite often, once the first proteins are attached, an initial cluster of proteins forms around them, stabilizing them. This mechanism is repeated until the entire surface is filled, resulting in densely packed protein coronas growing under cooperative, rather than random, absorption~~ Instead, ~~quite often,~~ once the first proteins are ~~attached~~absorbed onto the surface, an initial cluster of proteins forms around ~~them~~, stabilizing them. This mechanism is repeated until the entire surface is ~~filled~~covered, resulting in densely packed protein coronas growing under cooperative, rather than random, absorption.[67]

Over the long term, NPs inside the body traverse through media with different molecular compositions, pH levels, and ionic strengths (e.g., from the blood to the endosomes, lysosomes, and to the extracellular matrix), where various processes of adsorption, desorption, and resorption may occur.[78] As a result, within living systems, various Soft-PCs may exist at different time points in different body compartments. However, the detailed characterization of these Soft-PCs remains a challenge due to the lack of analytical separation methods.[77] Only a

few studies have addressed the modifications of both the Hard and Soft Coronas after long-term exposure in physiological environments.

The increased stability of the adsorbed proteins, leading to the hardening of the protein corona, has significant implications, particularly in the case of complex mixtures of proteins such as blood. For example, Brash and later Hove reported that an increase in plasma fibrinogen concentration proportionally increased the amount of fibrinogen adsorbed on different inorganic surfaces tested.[72] When the concentration of competing proteins was increased, the amount of fibrinogen on the surface decreased. Conversely, when the concentration of competing proteins was increased, the amount of fibrinogen on the surface decreased. Therefore, the irreversible adsorption of proteins to surfaces was found to depend not only on binding affinities between NPs and proteins, but also on relative concentrations, relative mobilities, and relaxation rates of the initial adsorbates of competing species.[73] ~~This increase in effective concentration at the surface enhances their chemical activity and may alter the rates and equilibrium constants of the reactions they are involved in.[74, 75]~~

Despite these findings, it can be argued that small NPs with a high curvature radius and similar size to proteins may alter ~~complicate~~ and ~~alter~~ complicate the process of stabilization and packing of proteins at their surfaces, especially for NPs smaller than proteins. ~~However~~Same limitations and ~~,~~similar trends have been observed but at different rates. The crowding effect has been observed in NPs as small as 10 nm when exposed to human or bovine serum.[66] Below this critical size, NPs and proteins interact, forming various complexes, but not fully formed coronas.[34] ~~The stable PC is known as the Hard PC, to distinguish it from the Soft PC, which consists of loosely attached proteins reversibly adsorbed at the NP surface. Over time, different proteins irreversibly adsorb until the entire NP surface is coated, forming the Hard PC, which does not detach from the NP surface after purification and dispersion in protein-free media.[63, 76]~~ Piella et al. showed the significant role that size plays at the nanoscale in the kinetics of this protein absorption process.[34] In this work, it is demonstrated how the thickness and density of this protein coating are strongly dependent on the finite NP ~~particle~~ size, allowing for the identification of different transition regimes as the size of the particles increases: (i) NP-protein complexes (or incomplete corona), below 10 nm NP diameter, (ii) the formation of a near-single dense protein corona layer, between 10 and a few 100 nm NP diameter, and (iii) the formation of a multilayer corona over a few 100 nm NP diameter.

~~This protein crowding, characterized by dense packing, is a known effect explained by the limited mobility of the proteins when they are confined. This confinement causes them to behave in a radically different manner compared to free proteins in solution. On one hand, the intermolecular forces of attraction that hold protein tertiary structure together are weak non-covalent interactions. Thus, at room temperature, there is enough thermal energy available to allow proteins to break and reform their tertiary configuration. Consequently, they possess a set of interchangeable conformational states with different free energies, described by the so-called energy potential landscape.[83, 84] This landscape is essential for various protein functions, such as catalysis or ligand binding.[85]~~ It is now well-known that when these proteins bind to the surface, various conformational changes occur, exposing activation sites that initiate the clotting cascade and inflammatory stimuli.[37] It is worth noting here that NPs do not always elicit a pro-inflammatory response; sometimes, they can go undetected by the immune system, and in certain cases, they may even exhibit anti-inflammatory properties.[38]

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Over the long term, NPs inside the body traverse through media with different molecular compositions, pH levels, and ionic strengths (e.g., from the blood to the endosomes, lysosomes, and to the extracellular matrix), where various processes of adsorption, desorption, and resorption may occur.[78] ~~As a result, within living systems, various Soft-PCs may exist at different time points in different body compartments. However, the detailed characterization of these Soft-PCs remains a challenge due to the lack of analytical separation methods.[77] Only a few studies have addressed the modifications of both the Hard and Soft Coronas after long-term exposure in physiological environments. Over the long term, NPs inside the body traverse through media with different molecular compositions, pH levels, and ionic strengths (e.g., from the blood to the endosomes, lysosomes, and to the extracellular matrix), where various processes of adsorption, desorption, and resorption may occur.[78]~~

In line with this, Norde and Anusiem reported the phenomenon of conformational memory of BSA adsorbed to silica surfaces and subsequently desorbed. Interestingly, the desorbed BSA exhibited greater affinity for silica surfaces than native BSA that had not undergone this process.[63] Another study suggested that the attachment of BSA to the silica surface led to an increase in BSA internal entropy and a modification of its structure. As a result, the desorbed BSA was found to be more stable on the silica surface than BSA that had not been previously adsorbed. Once again, this highlights the importance of examining the modifications that

proteins undergo on the surface of nanoparticles in both the short and long term, as these changes can have significant biological consequences.

For biological and medical implications, the dense packing of proteins at a surface typically prevents their denaturation, as the compact folded protein occupies less volume than an unfolded one. A notable example is the development of solid-phase immunoassay methods, such as those used in paper strips for antigen tests for COVID-19 based in AuNP-Antibody conjugates. These methods demonstrate that at least a portion of the proteins (antibodies) attached to a surface maintain their native and active state for long~~er~~ periods.

~~Building upon the work of Jirgensons and Straumanis on the stabilizing properties of protein adsorption to colloidal particles in high ionic strength solutions,[32] Faulk and Taylor identified Salmonella antigens by employing immunogold labeling in their seminal work on immuno-labeling with AuNPs colloidal gold.[79] These authors noted that "*the addition of strong electrolytes alters the electrochemical environment and allows (the gold sol) to adhere and flocculate. This can be prevented by adding a 'protective' colloid, such as proteins (...) It therefore seemed reasonable to stabilize the gold sol with antibody, and thereby develop the possibility of an immunocolloid method for the electron microscope.*" Initially, they determined the optimal amount of antibody necessary to stabilize the gold particles by exposing the colloids to increasing amounts of antibodies. Subsequently, they studied the stability of the particles by adding sodium chloride, a strong destabilizing agent for electrostatically stabilized gold NPs. Once optimal surface passivation was achieved under appropriate antibody concentration conditions, the antibody-labeled colloids were centrifuged to concentrate the conjugates and eliminate the unlabeled antibodies. This procedure, described by Faulk and Taylor, is now employed as the typical method for the formation and isolation of nanoparticle-protein complexes.[80, 81] Similar strategies nowadays provide the opportunity to accommodate biomolecules with targeting moieties on the surface of nanomaterials. As a result, NP-Protein conjugates can be designed to perform sophisticated biological tasks, such as inducing Natural Killer cells to attack HIV-infected T-cells.[67]~~

In addition to the success of immunoassay methods in the solid phase, Norde et al., and subsequently confirmed in other studies, demonstrated that the size of the protein layer adsorbed to different surfaces is closer to the size of the native protein.[82] However, other research suggests that the crowding effect may induce some misfolding of proteins due to their aggregation,[83-85] leading to conformational changes that can result in protein aggregation

at the NP surface and malfunction in vivo.[86-89] Hence, potential subtle conformational changes should be further investigated in more detail to predict the biological behavior of the NP-PC. In their 2006 seminal report, Lynch et al.[4] discussed how protein adsorption at the surface of NPs might result in the exposure of protein residues that are typically buried in the core of the native protein, ~~the~~ exposing "cryptic epitopes," which can be recognized by cells and trigger inappropriate cellular signaling events.

4. The Nanoparticle Protein Corona

The development of nanoscience in the late 90s and early 2000s, with the possibility of creating small NPs that can interact with biological structures in unique ways, presented a situation similar to the advent of liposomes. This situation ~~involved~~ requieres high pharmacological capabilities and the necessity to assess risks. Although the nonspecific adsorption of proteins on NP surfaces had been addressed in previous works,[90-94] it gained broader attention through the studies of Dawson, Linse, and their colleagues published in Science Signaling,[4] and subsequent works.[3, 95] In fact, it is attributed to these authors the introduction of the term ~~of the~~ "Nanoparticle-Protein Corona" as the *"evolving collection of proteins that associate with nanoparticles in biological fluids, which is, in fact, the 'biological relevant' entity that interact with cells"*, first ~~studied~~ published in ~~the works of~~ Cedervall et al.,[3] Sahoo et al.,[96] and Lundqvist et al.[97]

Following these initial papers, a plethora of reports, papers and reviews on the "Nanoparticle-Protein Corona" have emerged over the last decade, investigating different aspects such as NPs morphology (size and shape), composition, surface characteristics including charge or adsorbed/conjugated molecules, and the type of proteins present in the media, and considering factors such as whether the patient's blood is infected or not. Five years later, the formation of the NP-PC was described in a diversity of nanomaterials, including AuNPs[46, 98-100] and AgNPs;[66, 101] hybrid metallic (FePt) NPs;[102] metal oxide NPs such as SiO_2,[78, 103] Fe_3O_4, CoO and CeO_2,[66] TiO_2 and ZnO NPs,[104] Quantum Dots,[96, 102] as well as non-metal NPs like polymers[3, 97] and carbon nanostructures.[105-108] These early studies were followed by numerous others, which progressively refined our understanding of the NP-PC.[109-112] Many reviews on the characterization and comprehension of the NP-PC can also be found in the literature.[5, 6, 10, 11, 33, 80, 113-117]

After decades dedicated to the study of NP and proteins, dDifferent from the previous history of protein adsorption studies, the substrates for study, NPs, are not fixed like an implant or prosthesis, but they move rapidly (Brownianly) in solution. They Small NPs have similar dimensions to proteins, which may facilitate their escape from opsonization and the Reticuloendothelial System. They Nanoparticles have a high curvature radius, thus altering the accessibility of proteins to their surfaces. All of these factors may modify the kinetics of the encounter between the nanoparticle surface and proteins, the mechanisms of attachment, and ultimately, the biological outcome. However, many similarities between the results obtained in these works and the previous history can be observed. For instance, the specificities of the corona evolution with time, reminiscent of the Vroman Effect, were later described in metal NPs,[46] and polymeric NPs[47] The stability, hardening, and crowding effects of the proteins in the corona, already described decades ago,[75, 118] were observed again in works from 2011 for metal and metal oxide NPs.[66] As well, studies on the effects of the size and surface properties of NPs to determine the protein corona formation process (and the implications for biological impacts) were similarly described in previous works. Similarly, studies on the effects of the size and surface properties of NPs on the protein corona formation process (and the implications for biological impacts) were similarly described in previous works. These and other overlaps indicate that a careful examination of other experimental data already generated can help fill some of the gaps or propose new hypotheses to be tested for a complete understanding of the NP-PC.

5. Outlook and Future Perspectives.

The early works on the nature and structure of proteins laid the groundwork for studying how proteins behave when they encounter different surfaces and how they modify them. Following the notion attributed to chemist Frank Westheimer, the attributed to the chemist Frank Westheimer, *"Why spend a day in the library when you can learn the same thing by working in the laboratory for a month?"*, considering this previous valuable information can contribute to a better understanding of current investigations on the NP-PC. An analysis of recent literature reveals significant efforts devoted to understanding the interactions of NPs with proteins from various perspectives. A recent review identifying and analysing what has been reported on the NP-PCby Hajipour et al.,[119] categorized thesc different governing aspects, identifying and analysing what has been reported on the NP-PC, including NP compositions, morphology, physicochemical properties, methodology, purification of the protein corona, analytical methods utilized for characterization, protein sources utilized, and the number and identity of

proteins detected in the protein corona composition, along with its relation to the protein sources. However, there are still some aspects to be clarified, which can benefit from the large body of early work on the adsorption of proteins to different surfaces.

For instance, while most of the research has focused on modifying nanomaterial surfaces and understanding the biological consequences,[120-122] less effort has been devoted to analyzing the modifications of proteins in the protein corona.[112] Looking at the efforts to understand protein interactions with material surfaces before 2007, we could identify some parameters that might be missing in recent Protein Corona studies. Memory effects such as those described by Norde and Anusiem[63] have not been extensively explored again, nor has the detailed analysis of protein surface charge and its relation to different ion Debye lengths (or Hoffmeister series). Subtle changes in protein conformation at the nanoparticle surface have not been deeply studied, while both extremes, epitope conservation and dramatic denaturation leading to protein aggregation and sample loss, have been reported. Similarly, increased enzymatic activity and loss of selectivity have been observed,[123] related to minute alterations in protein structure and accessibility when forming the densely packed Hard-PC.

Furthermore, while most Protein Corona experiments have focused on standard in vitro cellular conditions, exploration of kinetic aspects through time and temperature incubation experiments at different ratios and in various physiological conditions remains incomplete. Similarly, in vivo kinetic evaluation of Protein Corona formation, identification of its components, and understanding how it affects cellular interactions with NPs, and how we can harness this knowledge for clinical benefits, are only beginning.[65, 120, 124-126] Similarly, while ~~other~~ numerous Protein Corona research efforts have aimed to understand its relation to the safety or toxicity of nanomaterials,[9, 127-129] fewer works have sought to harness this phenomenon to design useful tools.[10, 11, 130] One simple example is the albuminization of NPs prior to administration, which enhances their stability and biocompatibility without compromising their activity, or the design of viral protein coronas for immunization.[131]

(TO THE EDITOR: the objective of this paper was not to study the pharmacological interactions between protein coronas and durgs during therapeutic intervention but the study of the interactionds of NP andproteins forming PROTEIN CORONAS... considering the reviewrs comment we have added the following text in page XXXX)

Interestingly, the study of interactions of proteins and drugs during therapeutic intervention is a major field of pharmacology. While plasma is made majorily of albumin with high

absorption tendency and antibodies with low absorption tendency (other then to their cognate antigens), other important and minoritary proteins are present and they have tendency to interact and modify the fate of drugs, as is the albuminization of cisplatinum after administration [COMENGE]. It is expected that protein coronas will also interact with small drugs in the blood stream in a rather similar way as free protein does. In this context, other related construct is Abraxane, where albumin is aggregated into the a NP trapping paclitaxel in ist interior showing good results permeating tumor stroma as in the case of Pancreatic Ductal Adeno Carcinoma… [https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5583572/]

~~In this line, over the last two decades we have seen how the synergy between knowledge from proteins and nanomaterials has led to the development of advanced nanotools for monitoring and manipulating biological states. Combining NPs and proteins offers opportunities for innovative solutions. For example, conjugating NPs with proteins can yield additive characteristics, such as plasmonic antibodies for detection, magnetic enzymes for recovery, or antioxidant albumin through controlled combinations of NPs and proteins. While drug interactions with serum proteins, notably albumin, are often undesirable in pharmacological sciences, in the case of NPs, protein absorption can be highly beneficial. It safeguards against aggregation and enhances biocompatibility without compromising inherent properties, whether as imaging antennas, drug delivery vehicles, or nanozymes. Reflecting on this historical overview, coupled with dynamic characterizations of PC evolution over time in different physiological compartments, the investigation of subtle modifications in protein configuration, and the detailed characterization of Soft and Hard-PCs should be integral to future research on the Protein Corona~~biomedical applications of NPs~~. By assimilating insights from past studies and adapting to NP specificities, such comprehensive investigations can pave the way for the future of nanomedicine.~~

~~l'ultim paragraf l'he reorganitzat de la següent manera:~~

Sumarizing, i~~I~~t is worth noting that newly developed nanomaterials tailored for medical applications offer opportunities for more precise diagnostics and enhanced therapies with targeted delivery, lower toxicity, and reduced side effects. However, despite substantial investments and preclinical successes, effective clinical translation remains elusive. Key factors

contributing to this delay include unclarified aspects and sometimes contradictory data concerning their pharmacokinetics and safety.[132] The study of protein adsorption on the surface of nanomaterials administered to living systems or dispersed in biological fluids plays a crucial role in elucidating their activity and fate in biological systems. This is because associated layers of absorbed proteins determine the NP's biological identity, including cellular recognition, cellular uptake, immune responses, biodistribution, and bioactivity. The current research on NP-PC confirms the exploration undertaken previously, which can shed light on neglected parameters such as surface charge and electrostatics, and modifications of protein configurations, sometimes subtle, which were pivotal in previous surface-protein studies.

~~aquest era el paràgraf original~~

~~In summary, all those early works on the nature and structure of proteins laid the ground to study how proteins would behave when they encounter other surfaces and how they would modify them. Basically, current research confirms what was explored previously. The surface charge and electrostatics seems the only neglected parameter in comparison with initiall surface protien studies. Alteration of the isoelectric points in the Protein Corona, such modification of protein configurations, sometimes subtle, has been little investigated in the case of nanoparticles due to their high colloidal mobility, limited surface araea and high curvature radii. The revision of this history, along with the dynamic characterization of the Protein Corona evolution over time in different physiological compartments, the study of subtle modifications of protein configuration, and the detailed characterization of the Soft and Hard Protein Coronas, should be part of future integral work on the Protein Corona that, by looking on what has been done and adapting to nanoparticle specificities, can pave the way for the future of nanomedicine. Newly developed nanomaterials designed for medical applications provide opportunities for more precise diagnostics and enhanced therapies with targeted delivery, lower toxicity, and fewer side effects. However, despite the large investments and preclinical successes, it can be acknowledged that little progress has been made towards their effective clinical translation.1-5 Identified key factors accounting for this delay are the unclarified aspects, and sometimes contradictory data, of their pharmacokinetics and safety.6-10 The study of the adsorption of proteins on the surface of the nanomaterials administered to living systems or dispersed in biological fluids plays a major role in elucidating their activity and fate in biological systems.11 This is because associated layer(s) of absorbed proteins will determine the NP biological identity, that is, cellular recognition, cellular uptake, immune responses, biodistribution, and bioactivity.12-18~~

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NOTES:

Pauli 1912 is a reference from another reference.... I never found the original one.

Brossa 1915, abstract at: https://chemport.cas.org/cgi-bin/sdcgi?APP=ftslink&action=reflink&origin=npg&version=1.0&coi=1%3ACAS%3A528%3ADyaC2MXptlah&md5=107d6750d2a212d405021f0b1f2ab9f5