RSC Advances

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PAPER

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Cite this: *RSC Advances*, 2013, **3**, 1119

Plasma concentration gradient influences the protein corona decoration on nanoparticles†

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Received 10th September 2012,
Accepted 13th November 2012

DOI: 10.1039/c2ra22093h

www.rsc.org/advances

It is now well known that the interaction between nanoparticulate systems and biological fluids leads to deposition of various proteins onto the surface of the nanoparticles (NPs), hence, formation of a protein ''corona''. Arrangement of the associated proteins on the surface of NPs defines the *in vivo* response of material to the surrounding biologic environment. In order to predict the intercellular fate of NPs, therefore, it is essential to have an in-depth insight into the factors influencing the protein corona composition. While remarkable progress has been made in elucidating the factors that affect hard corona composition, the actual intercellular pathways that particles undertake *in vivo* and their dependence on the corona composition have not been investigated. In this study, we demonstrated that variation in plasma concentration can significantly change the biological fate of NPs, through alteration in the composition of the protein shell. For this purpose, sulfonated polystyrene and silica NPs were interacted with human plasma and fetal bovine serum in gradient concentrations. In contrast to the hard coronas formed under conventional static plasma conditions, large differences were observed in the amounts and affinities of proteins when particles were maintained under the plasma gradient conditions. This finding can help scientists to have a better understanding of the nanoparticle–cell interactions *in vivo* and elucidate the safety considerations for biomedical applications, resulting in nano-biomaterials that are ''safe by design''.

Introduction

In the past decade, manufacturing nanomaterials and exploring their properties have attracted growing attention in multiple branches of science including medicine.[1] Interest in nanoparticles (NPs) arises from the fact that their physicochemical properties can be substantially different from those of their bulk counterparts.[1] Surface characteristics of NPs dominate their bulk properties, primarily due to the ultra-large surface to volume ratios of these particles, which results in significant variations in their physicochemical properties. These differences, therefore, are strongly dependent on the size of particles.

Due to their small dimensions, nanoparticles are able to interact with cellular machinery and potentially reach previously inaccessible targets such as the brain tissue microenvironment.[2,3] Accordingly, nowadays, there are numerous biomedical areas where NPs have gained significant scientific and technological interests. This, in turn, has led to increasing concerns for the design of safe nano-biomaterials. It is well recognized that the surface of biomaterials (*e.g.* implants and biomedical devices) is instantly covered by various biomolecules (*e.g.* proteins, natural organic materials, detergents, and enzymes) when they come in contact with a biological medium.[2,4–13] The adsorption of macromolecules onto the surface confers a new ''biological identity'' to the material in the living organism and determines the subsequent cellular and/or tissue responses. In the same way, a complex layer of biomolecules forms on the surface of nanomaterials entering a biologic milieu.[6] Nevertheless, NP–biomolecule interactions are substantially different from those occurring on the surface of the bulk materials. The protein composition and the organization of the associated proteins forming on NPs are noticeably different from those on flat surfaces, resulting in comparatively different biological consequences.[14]

What a biological entity such as cell, tissue, or organ ''sees'' in reality, when interacting with NPs, is completely different from the original pristine surface of the particles. This new

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† Electronic supplementary information (ESI) available: Experimental procedures. See DOI: 10.1039/c2ra22093h

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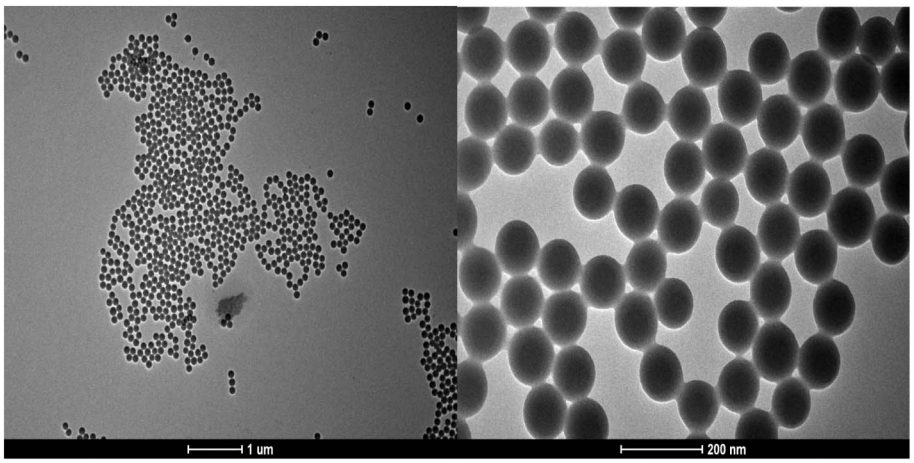
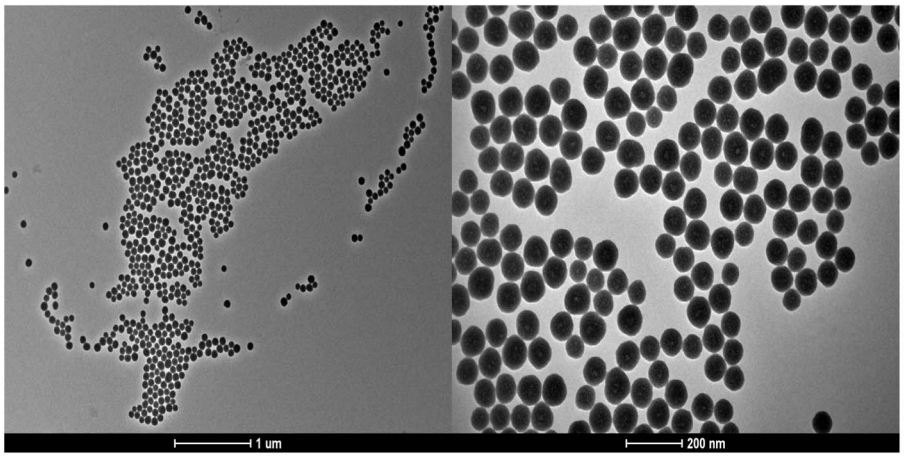


(a)



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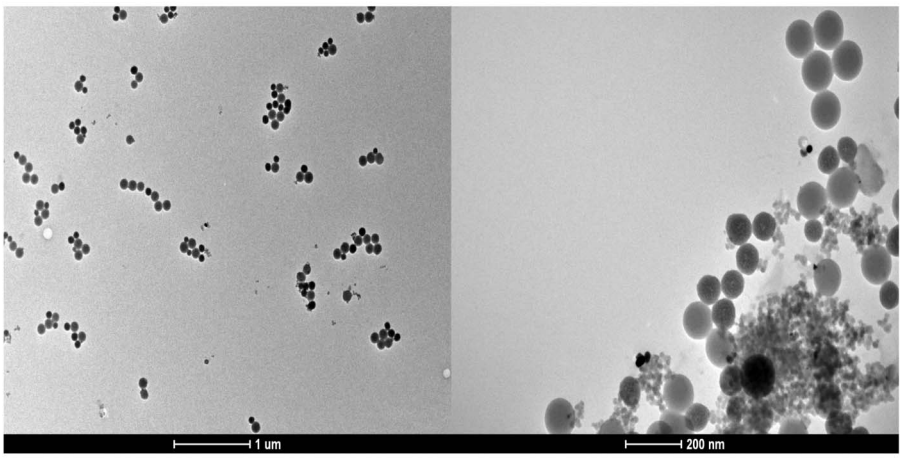

(c)

Fig. 1 TEM images of (a) polystyrene and (b) and (c) silica nanoparticles with a diameter of 100 and 200 nm, respectively, at different magnifications, showing spherical nanoparticles with relatively narrow size distributions.

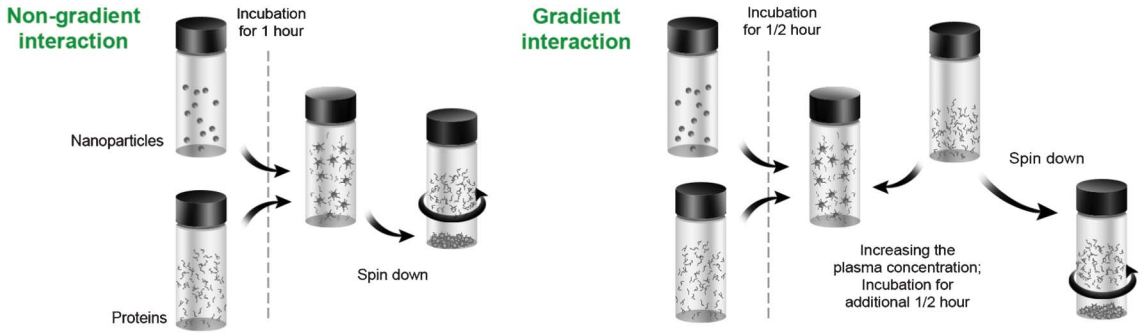

Fig. 2 Schematic representation of the procedure of the preparation of protein–corona coated nanoparticles using both non-gradient and gradient plasma interaction approaches.

identity for the NPs is obtained through the creation of a new interface between the particle and the biologic medium which is called the ''bio-nano interface''.[15] It has been recently reported that in addition to the protein corona, the effect of cell observer (*i.e.* cell types) is another crucial factor that determines the fate of the NPs *in vivo*.[16–19] Identical NPs have been shown to have distinct impacts on different cell types. The mechanisms by which this cell-specific response takes place can be related to the numerous detoxification strategies that every particular cell type can utilize in response to the particles (as suggested by Mahmoudi *et al.*[16–19]). The cellular defence mechanism against NPs (*e.g.* activation of different signalling pathways) could be considerably different according to the cell types.

Among several factors that influence corona formation on NPs, particle size and size distribution, shape, composition, surface charge, surface roughness, and solubility play key roles in the interplay between NPs and their surrounding biologic environment.[3,6] Although these interactions have been well addressed, still a greater understanding of how these processes can define the biologic response to the NPs both *in vitro* and *in vivo* seems necessary.[20,21] Using various protein concentrations, Monopoli *et al.*[22] found that the hard corona can evolve quite significantly by replacing the protein concentrations commonly

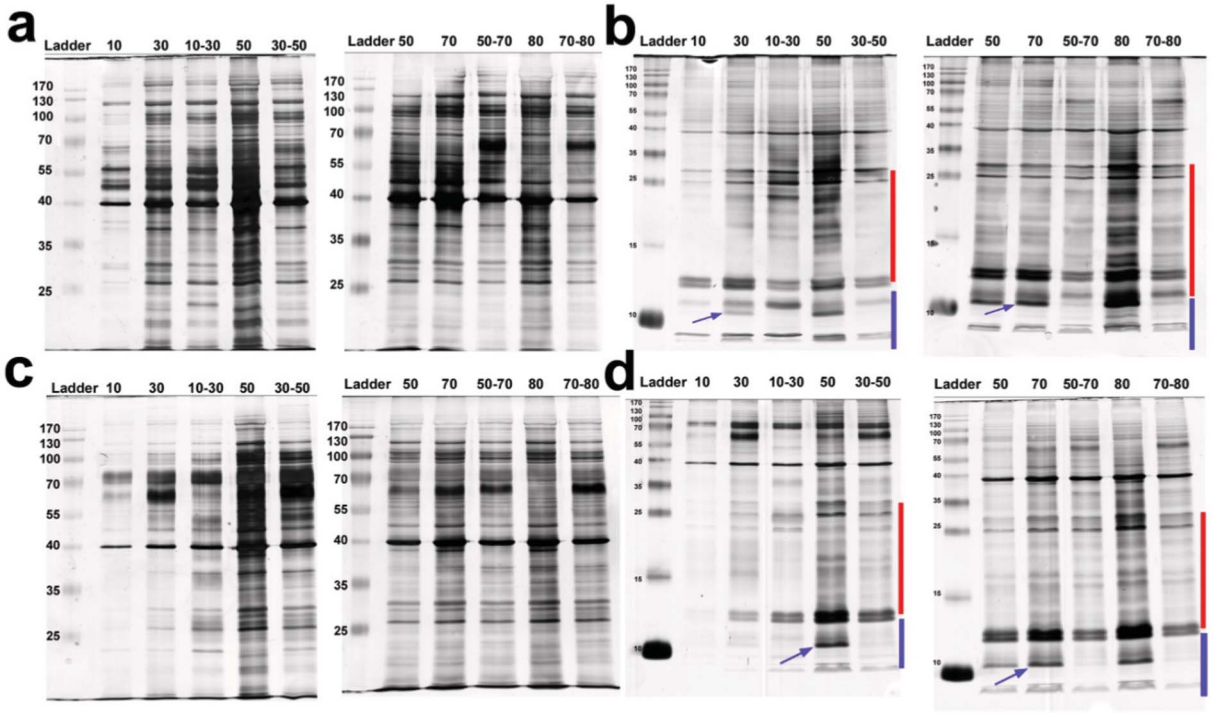

Fig. 3 (a) 15% and (b) 20% SDS-PAGE gel of FBS proteins obtained from polystyrene–protein complexes free from excess plasma following incubation with human plasma at various concentrations (both non-gradient and gradient situations). (c) 15% and (d) 20% SDS-PAGE gel of plasma proteins obtained from silica–protein complexes free from excess plasma following incubation with human plasma at various concentrations (both non-gradient and gradient situations). The molecular weights of the proteins in the standard ladder are reported on the left for reference.

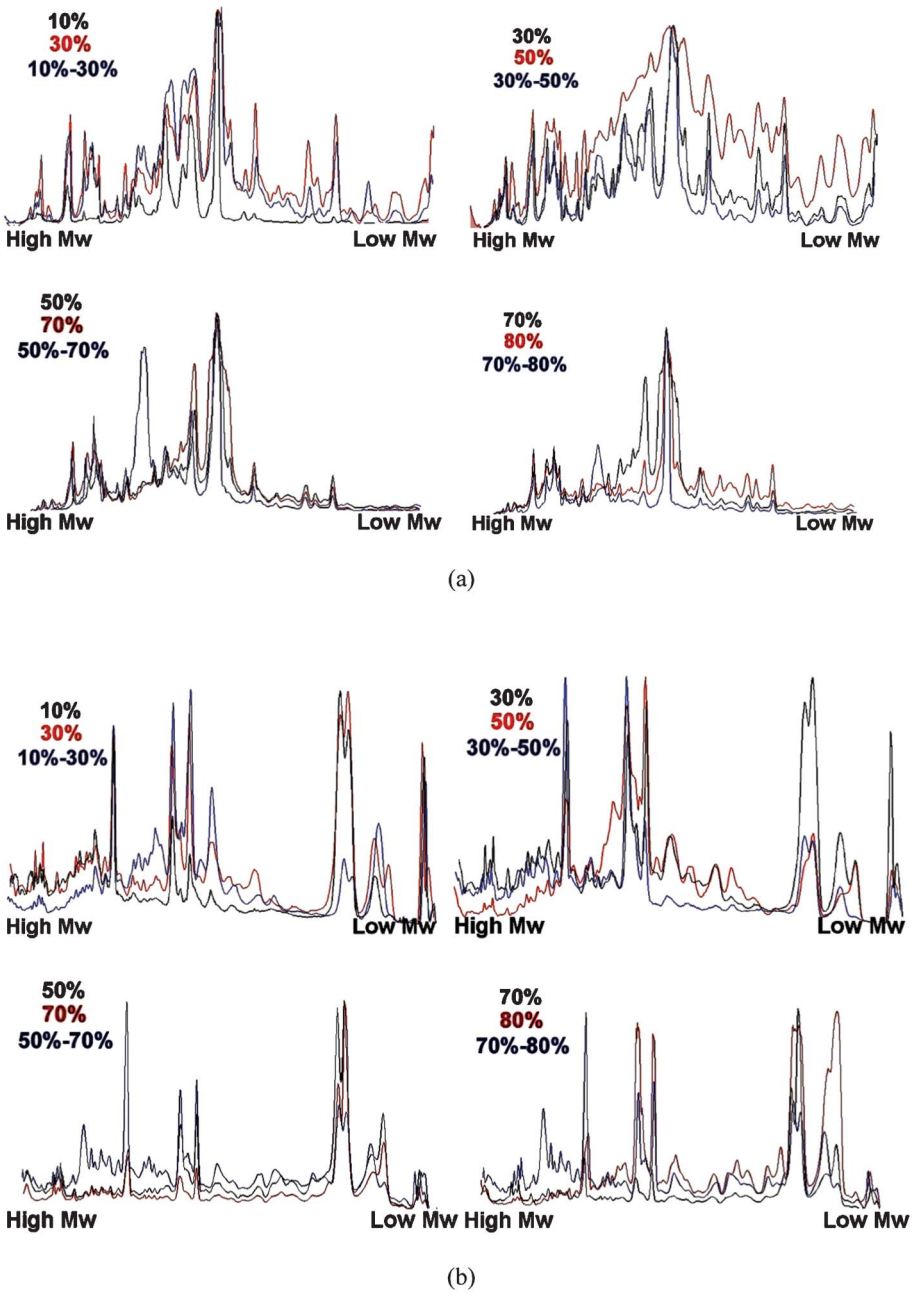

Fig. 4 Comparison of the optical intensity across (a) 15% and (b) 20% gel lanes, for polystyrene particles, between non-gradient (black and red) and gradient (blue) coronas; the *x*-axis corresponds to the run length and is normalized according to how far different proteins in the molecular weight standards lane had moved in each respective gel; the *y*-axis is the normalized intensity of the lanes.

used in the *in vitro* cell culture studies with those applicable to the *in vivo* conditions. Moreover, Lundqvist *et al.*[23] studied the variation of protein corona composition when particles transferred from the plasma medium into the cellular cytosolic fluid. However, the interaction between protein concentration gradients and different NPs, which would recapitulate the actual NP pathways in the human body, has not yet been investigated. Before reaching to cells, NPs will be exposed to a variety of biologic fluids which contain different protein compositions and concentrations. NP pathways *in vivo* depend on the manner by which the particles are administered into the body (*e.g.*, subcutaneous, intradermal, intramuscular, intravenous, intraosseous, intralumbar, and inhalation); for instance, in the case that NPs enter the body through an inhalation process, they will first ''see'' the lung cell barrier, with a completely different protein medium compared to the blood plasma proteins. Therefore, the composition of the protein corona formed on the surface of NPs, which defines the specific body response to the exogenous particles, will be highly dependent to the pathway taken by the particles. Consequently, evaluation of the protein

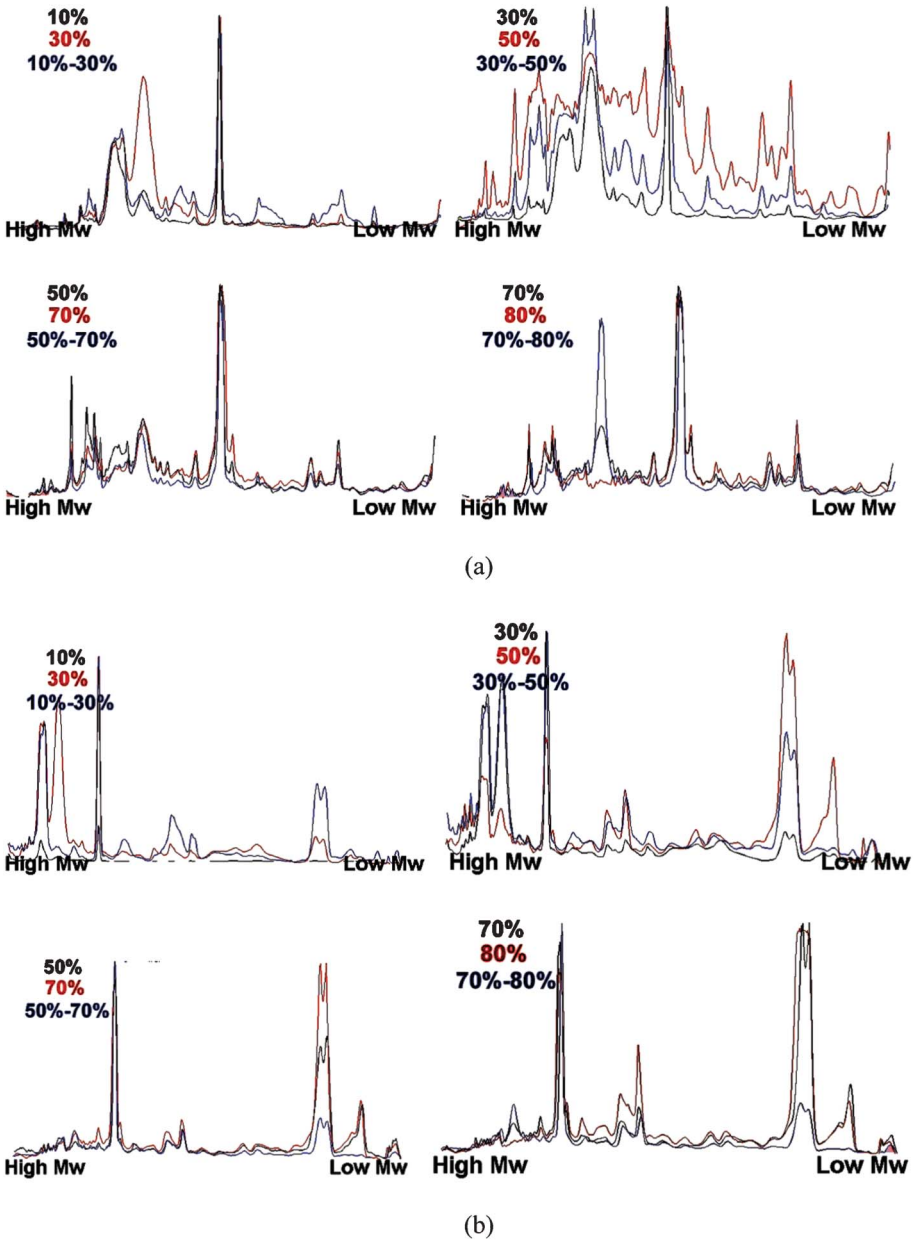

Fig. 5 Comparison of the optical intensity across (a) 15% and (b) 20% gel lanes, for silica particles, between non-gradient (black and red) and gradient (blue) coronas; the *x*-axis corresponds to the run length, normalized according to how far different proteins in the molecular weight standards lane had moved in each respective gel; the *y*-axis is the normalized intensity of the lanes.

corona composition and quantity obtained from gradient complex media is crucial in order to find out what the cells precisely "see" *in vivo* when interacting with particles. In this study, we investigated the adsorption of plasma protein onto the surface of two commercially available NPs (hydrophobic carboxylated polystyrene ($PSOSO_3$) and hydrophilic silica (SiO_2) particles). NP–protein complexes were characterized by utilizing one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D PAGE), liquid chromatography mass spectrometry (LC-MS/MS), dynamic light scattering (DLS), zeta-potential, differential centrifugal sedimentation (DCS), and transmission electron microscopy (TEM) techniques. Based upon the obtained results, one can follow the total binding isotherms for the NPs, hence identifying the nature and the quantity of the most relevant proteins as a function of the plasma gradient concentration. Thereupon, the degree to which the biomolecule corona may change when interacting with biologic media can be determined.

Results and discussion

Two different types of NPs with a diameter of 100 nm (carboxylated-polystyrene and plain silica) and 200 nm (silica

Table 1 List of the proteins existing under the gradient plasma condition. The amounts of the proteins were significantly lower in comparison to that under the non-gradient plasma condition (SD obtained from three individual evaluations)

Protein	Reduction percentage
Apolipoprotein A-I precursor	19.3 ± 2.3%
Apolipoprotein A-II precursor	14.7 ± 1.2%
Apolipoprotein C-I precursor	15.2 ± 1.3%
Apolipoprotein C-II precursor	11.6 ± 2.1%
Apolipoprotein C-III precursor	5.4 ± 0.4%
Plasma retinol-binding protein precursor	7.2 ± 1.7%
Transthyretin precursor	9.5 ± 0.9%
Isoform 1 of haptoglobin-related protein precursor	8.7 ± 1.2%
b-2-Microglobulin precursor	23.2 ± 1.8%
Serum amyloid A protein precursor	17.1 ± 0.8%
Serum amyloid A protein precursor	14.3 ± 1.7%

particles) were interacted with human plasma at protein concentrations of 10, 30, 50, 70, and 80%. These specific particles were selected because of their importance as the first group of materials that was evaluated for safety issues at the nano scale.[22,24,25] Morphology and size distribution analysis of the NPs by TEM demonstrated the presence of spherical particles with considerably narrow size distributions (Fig. 1) which were relatively appropriate for the protein corona evaluation assay. The concentration of particles in the whole medium was precisely set at 100 μg ml^{-1} for all of the batches. For the gradient plasma experiments, NPs were incubated in media containing a gradient concentration of plasma proteins (10–30%, 30–50%, 50–70%, or 70–80%, see Fig. 2 and experimental section† for details). Subsequently, the particles covered with ''hard'' corona proteins were collected, according to the protocol described in Materials and Methods†, and analyzed by SDS PAGE. Fig. 3 shows the lanes from silver stained gels for both polystyrene and silica NPs in different plasma concentrations and protein gradients. It is evident that the SDS-PAGE gel patterns of the NPs coated with a protein corona of gradient plasma proteins were significantly different from the ones formed under the non-gradient conditions.

In order to obtain a better insight into the protein corona variations, two types of 15% and 20% SDS-PAGE gel were examined. Studying the obtained protein patterns for both particles, it can be concluded that the protein gradient media had significant effects on both the composition and the amount of associated proteins within the formed corona. In particular, low molecular weight proteins (<25 kDa) played a crucial role in alteration of the protein corona composition interacting with gradient protein concentration (Fig. 3b and d). Although these proteins could still be found within the corona structure, their quantity was significantly reduced when compared to that in the corona forming under the non-gradient plasma condition. It is noteworthy to mention that the 50% or 80% plasma displayed a completely different size distribution compared with the other gradient or non-gradient plasma; this is due to the fact that protein coated particles, at concentrations approximating 50%, tend to lower their surface energy by exchanging proteins with the whole set of proteins that diffuse to the surface, in shorter time scales, as proposed by Monopoli *et al.*[12] The faint bands of the proteins interacted with gradient plasma indicated that low molecular weight proteins left the structure of the corona and consequently, the corona composition was relatively diluted from those proteins. The proposed phenomenon can be seen more clearly in Fig. 4 and 5, demonstrating the intensity profiles of coronas corresponding to the gel lanes shown in Fig. 3, where the difference between the curves became significant at the low protein weight ranges. Gels were scanned using a Biorad GS-800 calibrated densitometer scanner and gel densitometry was performed using the ImageJ software (version 1.410).

The bands located in the range of low molecular weight proteins (<25 kDa) were analyzed using LC-MS/MS technique (regions defined in Fig. 3b and d: non-gradients (30, 50, 70, and 80) and gradient (30–50 and 70–80) lanes). It should be noted that to obtain more accurate results from the LC-MS/MS technique, the proteins were stained with the coomassie blue staining method, instead of the silver stating approach. Table 1 lists the proteins with reduced concentrations under the gradient plasma condition, as compared with the non-gradient plasma environment (see SI for details on the LC-MS/MS analysis†). Remarkably, the majority of the diminished proteins were apolipoprotein-based precursors. Apolipoprotein-based precursors are an assembly of proteins, lipids, and cholesterol that transport lipids and cholesterol in the blood stream. They

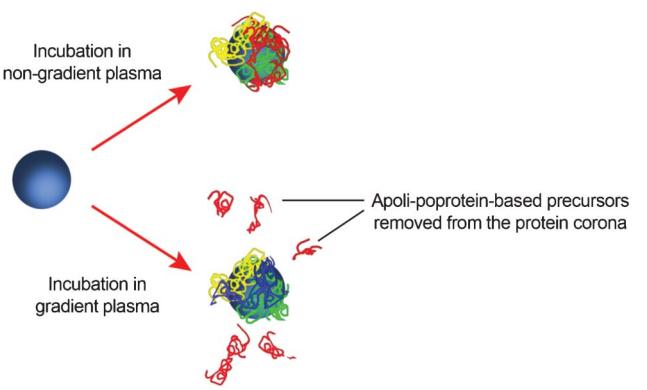

Fig. 6 Schematic representation of the importance of NP trafficking on catching apolipoproteins in the corona composition.

can therefore play a crucial role in defining the biological fate of NPs. According to the previous reports, apolipoprotein A-I always participates in the composition of protein corona of different materials.[23,25–27] However, our results indicate that the contribution of these precursors to the composition of protein corona is highly related to the NPs pathway inside the human body (Fig. 6).

Apolipoprotein-based precursors have a specific function in lipid metabolism; hence, they possess particular value in monitoring lipid-lowering therapies where lipoproteins are less predictive of further cardiovascular events.[28] Lipoproteins (with a core of neutral lipids and shell of polar lipids) are responsible for the transportation of cholesterols and triglycerides *in vivo*. Apolipoproteins are structural components that can be integral to the outer shell, or bind to the exterior of the lipoprotein-based peptides. This could be the major hypothesis for the removal of apolipoproteins from the protein corona in the gradient plasma situation. Considering the fact that apolipoproteins have a crucial role in interacting with transport proteins and lipoprotein receptors as well as being co-factors for lipolytic enzymes,[29,30] their existence in the protein corona structure seems vital for the cellular uptake of the NPs.

In order to probe the effect of NP size on the removal of apolipoprotein-based precursors due to the interaction with gradient plasma proteins, silica NPs with an average size of 200 nm were employed. Results confirmed that there is a significant change in the quantity of low molecular weight proteins within the corona structure in comparison to the corona formed on the 100 nm NPs (Fig. S1 in SI†). Thus, one can conclude that particle size, rather than particle composition, can be considered as a key factor to determine the biologic fate of the NPs (which is in agreement with the report by Tenzer *et al.*[31]).

In order to probe the interactions between the NPs and human plasma proteins *in situ* (*i.e.* in the presence of excess plasma proteins), under both non-gradient and gradient conditions, the differential centrifugal sedimentation (DCS) method was employed. Complete details of the DCS approach are given in the SI†. It is notable that DCS is one of very few techniques that can be applied to complex biological systems, without the need for fluorescent labels and other such devices, or the need for extreme NP dilutions. The DCS results (Fig. 6 and Fig. S2 in the SI†) were remarkably reproducible and obtained at a high precision. The main peak for the polystyrene particles was shifted to the larger values after interaction with human plasma proteins. This was due to the fact that the density of human plasma proteins (1.125 g ml^{-1}) was higher than polystyrene NPs (1.052 g ml^{-1}). In contrast, for silica NPs (density of 2.2 g ml^{-1}) the main peak was shifted to smaller values when interacted with human plasma proteins (Fig. 7a and b for polystyrene and silica NPs, respectively). In both cases, the thickness of the protein corona was directly related to the extent of the shift. Based upon these results (Fig. 7), there were significant differences in the extent of the peak shift between non-gradient and gradient

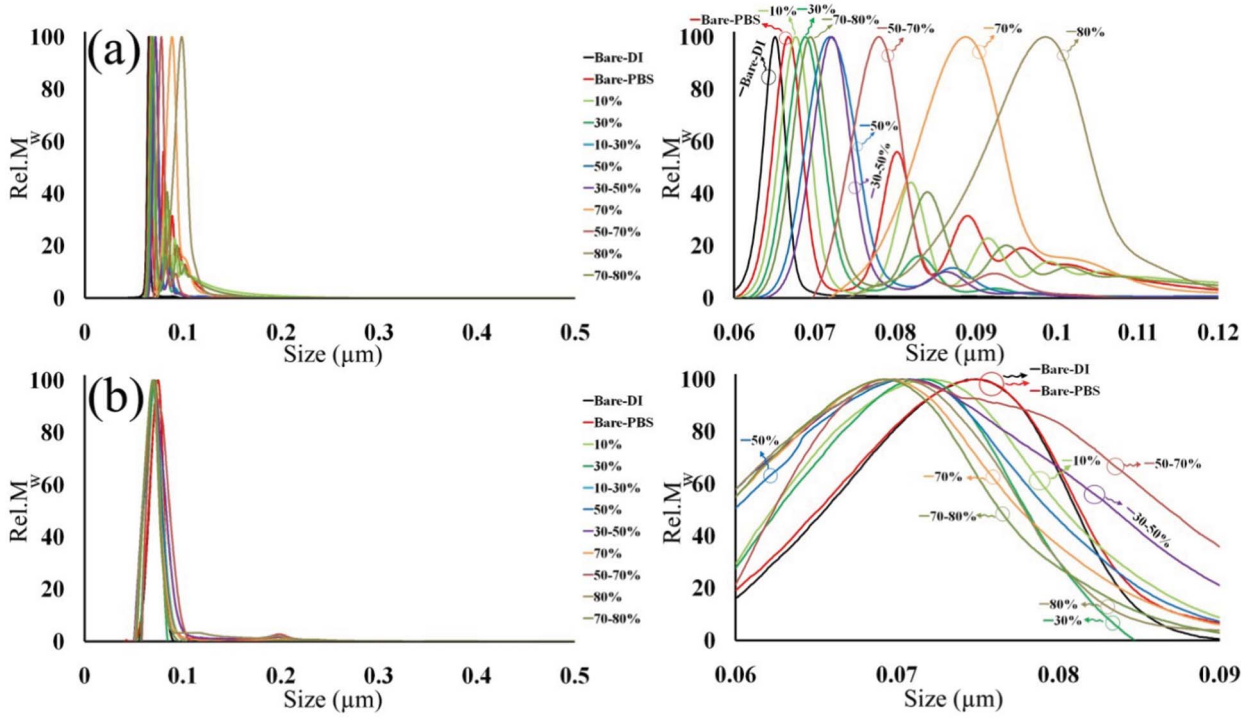

Fig. 7 DCS results for (a) polystyrene and (b) silica (100 nm) nanoparticles interacting with human plasma proteins at various non-gradient and gradient conditions *in situ*. Right graph is an enlargement of the main peak areas of the DCS graphs to highlight the shifts to bigger and smaller size after interaction with plasma proteins for polystyrene and silica nanoparticles, respectively.

conditions, which were in agreement with the gels and mass spectroscopy data. Moreover, the DCS results (Fig. S2 of SI†) of the larger silica NPs (200 nm) confirmed that there were no significant changes in the protein thicknesses (*i.e.* the extent of the shift for the main peak) in non-gradient and gradient cases, which was in agreement with the previous results.

Conclusions

Considering the various approaches by which nanoscale objects can be administered into the human body, NPs frequently encounter complex microenvironments containing variable concentrations of proteins. In this study, plasma protein gradient was introduced as a critical factor with unprecedented influence on the fate of the particles *in vivo* by inducing a number of alterations in the composition of the protein corona. The main corona variations were observed in proteins with lower molecular weights. A greater understanding of the complex interactions between nanoparticulate systems and biomimetic plasma conditions can usher in a new wave of studies to further extend the application of various NPs *in vivo*.

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