Evaluating the impact of experimental shear flow parameters on nanoparticle protein corona formation

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Abstract:
The use of nanoparticles has increasingly been implemented in biomedical applications including the diagnosis and treatment of disease. When administered to a biological system, nanoparticles spontaneously interact with their surrounding environment, leading to the surface-adsorption of small molecules and biomacromolecules. The protein component of the surface-adsorbed species, is often referred to as the “protein corona”. The composition of the protein corona is governed by nanoparticle properties, incubation media and parameters related to the environment in which nanoparticle incubations are performed. In this study, we investigated the formation of protein corona on polystyrene nanoparticles which have different surface chemistries and the impact of experimental incubation parameters, including centrifugation-resuspension protocols, incubation duration and shear flow rate conditions. The particle characteristics measured include size distribution, zeta potential and total protein content.

Our findings show significant differences in nanoparticle size following exposure to media containing proteins across the three different nanoparticle surface chemistries. These findings were also confirmed by total protein concentration measurements performed on nanoparticles recovered from bulk media, and measurements of the composition of surface-adsorbed proteins by gel electrophoresis. We also found that exposure to different shear flow conditions alters both the thickness and the composition of surface-adsorbed protein coronas. In parallel to analysis of nanoparticles isolated using the centrifugation-resuspension protocol, we performed *in situ* analysis of nanoparticle size in media containing proteins. Results obtained from these measurements highlight that the recovery procedure is disruptive to the protein corona and therefore the need for investigative methods that do not alter the properties of the nanoparticle coronas.

Nanomedicines are generally intended for administration *via* injection, and our findings show that parameters such as shear flow and media composition can significantly alter nanoparticle physicochemical parameters. Overall, we show that the recovery protocol can significantly alter particle parameters in addition to the overall protein composition of surface-adsorbed proteins. We recommend that nanoparticle characterization pipelines studying bio-nano interactions during early nanomedicine development consider experimental design in the context of biologically-relevant shear flow conditions and media composition because these parameters can significantly alter particle physical parameters and the subsequent conclusions drawn from such studies.

Keywords: Nanoparticle; Polymeric; Protein Corona; Particle Size; Agglomeration

Introduction

Colloidal nanoparticle-based delivery systems have emerged as an attractive approach for the safe and effective delivery [1] of a diverse range of drugs through altered organ biodistribution and controlled drug release. Upon administration to a biological system, colloidal nanoparticles will encounter and interact with biomacromolecules and cells in their immediate environment over time, following which spontaneous surface-adsorption of biomolecules including proteins occurs. The protein corona- has a fundamental impact on the chemical and biological identity of nanoparticles, and their subsequent biological fate [4, 5].

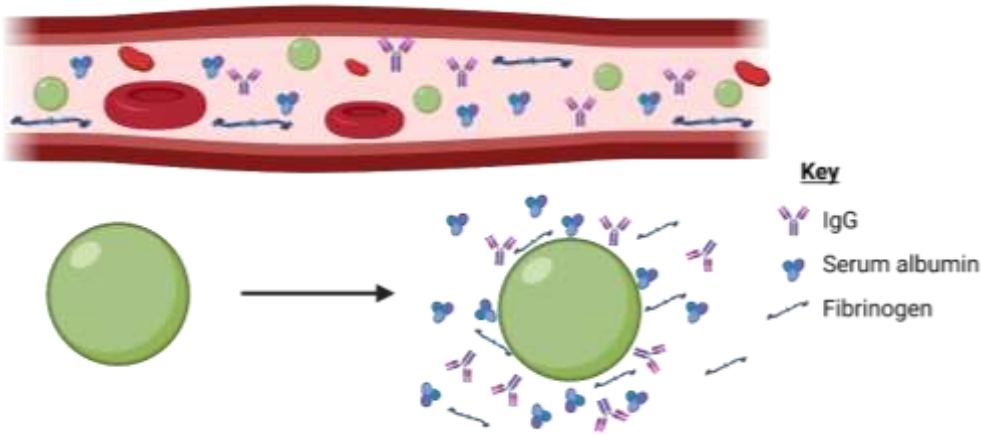

Figure 1. Graphical representation of nanoparticle protein corona formation following introduction to protein-containing media, and interaction with abundant proteins.

Formation of the protein corona is a process governed by nanoparticle physical and chemical properties, and the composition of biological fluids to which nanoparticles are introduced. A variety of nanoparticle [6] and biological fluid composition [7] parameters are known to influence this process. Knowledge of the protein corona composition and its impact on nanoparticle physical and chemical characteristics is crucial for understanding protein corona effects on biological fate of nanomedicine, which can inform the design of novel nanoparticle prototypes with pre-defined target organs, release rates, immune responses, and circulation times [8-10]. Previous work has indicated that cationic (positively charged) nanoparticles are more likely to elicit an inflammatory response in comparison to anionic (negatively charged) and neutral ones [2, 3] . These observations have been attributed to the formation of the surface-adsorbed nanoparticle protein corona. More recently, the non-specific adsorption of other biomolecules (e.g., DNA and sugars) has been considered to impact the composition of the protein corona and lead to observed effects on nanoparticle biological fate [4, 5]. Though associations between nanoparticle surface charge, morphology and the composition of the protein corona have been reported in numerous studies [6-8], a direct comprehensive link between nanoparticle physicochemical properties, composition of the protein corona and biological fate are yet to be established.

The composition of the nanoparticle protein corona is dynamic, complex, and directly dependent on the makeup of the biological media to which the nanoparticle is introduced. The adsorption and desorption rate for each protein on the nanoparticle surface will dictate whether the protein remains as a component of the irreversible corona layer (hard corona) or adsorbs and desorbs (soft corona) in equilibrium with its surrounding environment [9, 10]. Many nanomedicines are intended for intravenous administration, which will subject them to the forces associated with blood flow rates within the circulatory system, as well as biomolecules and cells contained within blood. Therefore, understanding the role of hemodynamic parameters as a function of nanoparticle circulation time and its implications for nanoparticle-

protein interactions and subsequent cellular effects is of significant importance in the early assessment of nanoparticle biological performance.

To develop a deeper understanding of the correlation between nanoparticle attributes and their biological fate, sample handling and preparation protocols, including composition of the biological media, incubation conditions (i.e., temperature, agitation) as well as the recovery and analysis methods of nanoparticles should be carefully considered and selected [8-10]. Recent studies using liposome nanoparticles functionalised with an outer polyethylene glycol (PEG) molecular layer have shown that protein corona formation is governed by additional parameters beyond nanoparticle surface charge [11]. In addition, the conditions used for nanoparticle incubation with protein containing media, their recovery procedure and the analytical method need to be considered for the comprehensive assessment of novel nanoparticle prototypes for biomedical applications and to enable comparability across multiple studies.

The development process for nanoparticle-based drug formulations include the *in vitro* screening of cellular cytotoxicity, cellular interactions and uptake, and intracellular trafficking. Routine *in vitro* experiments typically rely on either serum-free conditions or medium supplemented with foetal bovine serum (FBS) to evaluate the rate and extent of nanoparticle cellular uptake. Limitations associated with these experiments are the non-competitive particle uptake when treating cellular systems with serum-free media, and the lack of biologically-relevant cell culture conditions (i.e., flow, 3D structure) [12].

The goal of this study is to investigate the impact of nanoparticle surface chemistry on interactions with FBS and the resulting physical and chemical properties of the nanoparticle. Specifically, we use polystyrene latex nanoparticles as a model drug carrier system and employ sample media which are similar to those used in *in vitro* cellular experiments.

Here, we examine the differences in nanoparticle protein corona impact on the physical and chemical characteristics of nanoparticle samples in response to different experimental protocols used for the isolation of nanoparticles prior to analysis. With most nanoparticles intended for intravenous administration it becomes crucial to understand the impact of physiological shear flow conditions on nanoparticle-protein interactions and subsequent protein corona formation. Here, we investigate the impact of shear flow conditions on nanoparticle parameters

Methodology

Materials

Unmodified (Cat #LB1, Merck, Glasgow, UK), carboxylate- (Cat #F-8803, ThermoFisher, Renfrew, Renfrewshire, UK) and amine-modified (Cat #L9904, Merck, Glasgow, UK) polystyrene latex nanoparticles were used for all the measurements reported in this study. FBS was purchased from Fisher Scientific (Invitrogen, Renfrew, Renfrewshire, UK) and purified via centrifugation to remove any insoluble particles or large agglomerates prior to use. Phosphate-Buffered Saline (PBS) (Cat#BR0014G) was purchased from Fisher Scientific (Invitrogen, Renfrew, Renfrewshire, UK). 2 mM Potassium Chloride (KCl) was used as a dispersant for electrophoretic light scattering (ELS) measurements and sourced from Fisher Bioreagents (Ottawa, Canada). The Mini-PROTEAN TGX Gels (Cat#4561094) and 4X Laemmeli sample buffer (Cat#1610747) were purchased from Bio-Rad (Bio-Rad Laboratories, Watford, Hertfordshire, WD17 1ET) used for SDS-PAGE analysis. The QC Colloidal Coomassie Stain (Bio-rad, Hertfordshire, UK, #1610803) was used for gel staining.

Methods

Sample Preparation and Recovery for Protein Corona Analyses

Polystyrene latex nanoparticles with different surface chemistry (unmodified, amine-modified, and carboxylate-modified) stock were prepared (1 mg/mL) prior to dilution and subsequent analysis for dynamic light scattering (DLS) (20 µg/mL), ELS (20 µg/mL), and particle tracking analysis (PTA) (1 µg/mL).

Size and surface charge analyses were performed for all the nanoparticles (unmodified, amine-modified, and carboxylate-modified) before incubation in protein containing media. Briefly, for size measurements using DLS and PTA, polystyrene latex nanoparticles were dispersed in 0.1XPBS. Measurements of nanoparticle ζ-potential were performed using ELS using 2 mM KCl as a dispersant.

Incubation of Nanoparticles in the absence of flow: for protein corona incubation experiments, polystyrene latex nanoparticles were dispersed in either PBS, or PBS containing FBS (10 vol%) and incubated for 2 hours and 24 hours in a temperature-controlled room at 37 °C using gentle end-over-end agitation to avoid particle sedimentation effects.

Incubation of samples under flow: for samples subjected to biologically relevant flow conditions, a peristaltic pump (Ismatec REGLO, Cole Palmer, Cambridgeshire, UK) was used to control the shear flow and recirculation of nanoparticle suspensions. Nanoparticle suspensions dispersed in either PBS or 10 vol% FBS (in PBS) were introduced to Masterflex silicone tubing (Cole Palmer, #WZ-06411-62, 1.6 mm. inner diameter and 3.2 mm outer diameter) at 37 °C for 2 hours at (0.85 cm/s) and (8.5 cm/s) to mimic physiologically relevant median cubital vein and arterial blood flow conditions, respectively [13, 14]. The tubing was primed with either PBS or FBS-containing media prior to sample introduction to reduce the occurrence of air bubbles within the system.

Nanoparticle recovery from incubation media: following incubation in either PBS or 10 vol% FBS in PBS in the absence and presence of flow, nanoparticle samples underwent three centrifugation-resuspension cycles to remove unbound proteins from the bulk incubation medium. Nanoparticle samples were centrifuged in a pre-chilled centrifuge (4 °C) at 20,000 x g for 30 minutes following each wash cycle. The supernatant was discarded, and the pellet re-suspended using PBS. The process was repeated in triplicate for all samples.

Sample Characterisation

Dynamic Light Scattering (DLS)

A Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK) equipped with a 633 nm Helium-Neon laser was used for all DLS measurements. Nanoparticle spherical-equivalent hydrodynamic diameter and polydispersity index (PDI) were measured prior to and following treatment in incubation media (PBS or 10 vol% FBS in PBS). All measurements were performed using three independent replicates, with each replicate being measured five times. The hydrodynamic diameter was reported as the Z-average with associated standard error. This was performed using the zetasizer software (7.11) from Malvern Instruments.

Electrophoretic Light Scattering

ELS was performed to measure changes in the ζ-potential (mV) of the polystyrene nanoparticles following treatment with incubation media with and Smoluchowski model was used for data processing. All size and zeta potential measurements were set to (25 °C). The

mean and standard error for each sample was determined from five independent measurements. This was performed using the zetasizer software (7.11) from Malvern Instruments.

Particle Tracking Analysis (PTA)

PTA measurements were performed to measure the number-based distribution of the spherical-equivalent hydrodynamic diameter of particles suspended in PBS and treatment medium. These measurements were performed using the NanoSight NS300 system (Malvern Panalytical, Malvern, UK) equipped with a 488 nm laser module and a high-sensitivity CMOS camera. Samples were all diluted according to (1 µg/mL) in either particle-free PBS or filtered treatment medium (10 vol% FBS in PBS) for resuspension and *in-situ* measurements, respectively. Five, 60 second videos were acquired for all measurement and averaged. All samples were analysed under constant flow conditions (flow setting 100) and at ambient temperature (~25 °C). The video capture parameters were set at a camera level of 6, with post-processing analyses being performed at a detection threshold of 4. Three independent replicate measurements were performed for each sample which were then used to calculate a mean diameter with associated standard error. Data were analysed using the NTA software (v3.4.0.0.3).

Protein Quantification

Analysis of the total protein content (per mg of polystyrene latex nanoparticle) was performed following a centrifugation-resuspension protocol for the isolation of incubated nanoparticles from treatment media. Nanoparticle samples were treated with Laemmli buffer overnight at ambient temperature (25 °C) under gentle agitation, followed by centrifugation at 20,000 x g for 30 minutes to recover the eluted proteins. The eluted protein content was quantified using the 660-nm protein assay (with ionic detergent, Cat#22663, ThermoFisher, Rockford, USA) as per the manufacturer's instruction. The microplate procedure had a working range between 50 µg/mL and 2000 µg/mL). Absorbance measurements were performed at 660 nm using a FlexStation III Microplate Reader (Molecular Devices, UK).

SDS-PAGE

The composition and relative abundance of proteins eluted from polystyrene latex nanoparticles was analysed with two-dimensional sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following quantification of the total protein content eluted from isolated polystyrene nanoparticles, 20 µL per (1 mg/mL) sample was heated for five minutes at 100 ˚C and loaded onto each lane (across all samples) of a Mini-Protean 4-20 % Pre-cast gel. A parallel measurement was performed with a quantity of 20 µg per sample with 20 µl loaded onto each lane (normalization, across all samples).

The Kaleidoscope weight markers (10-250 kDa, Bio-Rad, Hertfordshire, UK, #1610375) was used for all SDS-PAGE runs. The QC Colloidal Coomassie Stain was used for overnight gel staining at (4 ˚C), and then washed overnight with the water being changed three times as per manufacturer instructions. The gels were imaged using the Bio-RAD Gel Doc EZ imaging system.

Statistical Analysis

Unless otherwise stated, a one-way analysis of variation (ANOVA) was performed to compare the impact of incubation parameters on nanoparticle properties. A paired t-test was used to compare the D50 for nanoparticle samples subjected to flow conditions versus non-incubated baseline conditions. All statistics were performed in Origin 2022 (v. 9.9.0.220).

Results

Nanoparticle characterization following treatment with protein containing media

Table 1. PTA-measured diameter, PDI, ζ-potential and protein content of polystyrene latex nanoparticles in biological media measured by DLS, ELS, PTA and protein quantification analysis. The samples were measured before and after 2 hour and 24-hour incubation in protein media (10vol% FBS in PBS) and isolation using a centrifugation-resuspension process (mean ± standard error, n=3).

Incubation time	PTA-measured Diameter (nm)	Z-average (nm)	PDI	ζ-Potential (mV)	Protein content (μg)
Unmodified					
0 hr	94.3 ± 0.6	118.1 ± 0.5	0.035 ± 0.003	-33.5 ± 0.8	N/A
2 hr	124.3 ± 0.9	147.4 ± 0.3	0.091 ± 0.009	-33.3 ± 0.4	100 ± 0
24 hr	121.5 ± 0.5	157.4 ± 0.2	0.059 ± 0.006	-31.6 ± 0.6	65 ± 4
Amine					
0 hr	84.0 ± 0.5	82.4 ± 0.2	0.041 ± 0.007	50.4 ± 0.8	N/A
2 hr	151 ± 1	273.1 ± 0.8	0.294 ± 0.009	-18.1 ± 0.3	97 ± 4
24 hr	190 ± 20	2020 ± 30	0.56 ± 0.09	-20.3 ± 0.4	92 ± 9
Carboxylate					
0 hr	91.1 ± 0.6	94.9 ± 0.2	0.017 ± 0.002	-34.0 ± 0.5	N/A
2 hr	113.7 ± 0.0	121.9 ± 0.2	0.015 ± 0.001	-24.3 ± 0.4	46 ± 4
24 hr	124.2 ± 0.1	121.8 ± 0.3	0.015 ± 0.002	-24.8 ± 0.4	48 ± 4

Reference characteristics for each nanoparticle type were within the manufacturer specification. In terms of nanoparticles incubated in treatment medium, the mean particle diameter significantly increased from 0 to 24 hour treatment for amine-, carboxylate- modified and untreated polystyrene latex nanoparticles with 126%, 36.2% and 28.7% increases in particle diameters, respectively. The extent of nanoparticle diameter increase observed was most significant between 0-hour and 2-hour incubation measurements. This observed increase in mean particle diameter (69.8%) was accompanied by an increase in PDI for amine-modified nanoparticles, while the extent of diameter increase was reduced in the case of carboxylate- and unmodified nanoparticles. An increase in particle mean size was also observed in PTA-measured diameters, with a corresponding 36.3% (carboxylate), and 28.8% (unmodified) increase in particle size relative to control following incubation.

Total protein content determined using the 660-nm protein quantification assay was the highest following 2-hour incubation measurements for amine-modified nanoparticles, unmodified, and carboxylate-modified nanoparticles. There was no significant difference for protein concentration following 24-hour incubation for amine-, and carboxylate-modified nanoparticles with only unmodified particles showing a (35%) decrease.

The ζ-potential of nanoparticles plays an important role in nanoparticle-protein interactions with ζ-potential changes observed following incubation with protein-containing media for all samples. The most significant observed change in ζ-potential with relative to the reference

particles was in positively charged amine-modified nanoparticles, which showed a change from 50.4mV to -18.1 mV in ζ-potential (following 2-hour incubation, and further decrease following 24-hour incubation to -20.3 mV. A slight increase in ζ-potential was observed for the unmodified nanoparticles from -33.5 mV to -31.6 mV following a 24-hour incubation. The negatively charged carboxylate-modified nanoparticles showed a larger change in ζ-potential from baseline (-34.0 mV) to 2-hour incubation (-24.3 mV) with a similar ζ-potential measured at 24-hour incubation (-24.8 mV). These results overall indicate that nanoparticle interactions with protein occur to a lesser extent with the negatively-charged polystyrene latex nanoparticles.

Measurement of nanoparticle attributes following incubation under various shear flow conditions.

Unmodified, amine-modified, and carboxylate-modified polystyrene latex nanoparticles were incubated for 2 hours and subjected to shear flow of 0.85 cm/s (flow rate mimicking the median cubital vein blood flow) and 8.5 cm/s (flow rate mimicking arterial blood flow) and recirculated using a peristaltic pump mimicking physiological conditions as previously described [15]. Nanoparticles were then isolated using the centrifugation-resuspension protocol (Table 2, Figure 2).

Table 2. PTA-measured diameter, size distribution (PDI), ζ-potential and protein content of polystyrene latex nanoparticles in biological media determined by DLS, ELS, PTA, and protein quantification analysis. The samples were measured before and after incubation in protein media (10 vol% FBS in PBS) under 0.85 cm/s and 8.5 cm/s flow rate, and following isolation using a centrifugation-resuspension process (mean ± standard error, *n*=3).

Flow rate	PTA-measured Diameter (nm)	Z-average (nm)	PDI	ζ-Potential (mV)	Protein content (μg)
Unmodified					
Untreated	94.3 ± 0.6	118.1 ± 0.5	0.035 ± 0.003	-33.5 ± 0.8	N/A
0.85 cm/s	123 ± 2	141.4 ± 0.4	0.072 ± 0.005	-25.1 ± 0.2	70 ± 2
8.5 cm/s	140.0 ± 0.3	179 ± 1	0.152 ± 0.007	-23.9 ± 0.2	68 ± 1
Amine					
Untreated	84.0 ± 0.5	82.4 ± 0.2	0.041 ± 0.007	50.4 ± 0.8	N/A
0.85 cm/s	181 ± 4	375.4 ± 0.4	0.28 ± 0.02	-25 ± 2	79 ± 3
8.5 cm/s	178 ± 5	333 ± 6	0.26 ± 0.03	-7.6 ± 0.3	78 ± 5
Carboxylate					
Untreated	91.1 ± 0.6	94.9 ± 0.2	0.017 ± 0.002	-34.0 ± 0.5	N/A
0.85 cm/s	112.9 ± 0.4	122.1 ± 0.5	0.015 ± 0.001	-22.3 ± 0.4	45 ± 4
8.5 cm/s	110.2 ± 0.5	119.9 ± 0.4	0.015 ± 0.002	-23.1 ± 0.4	49 ± 3

A significant increase in mean nanoparticle diameter was observed for all particles studied following incubation within protein media under physiological shear flow conditions (median cubital vein) with a 31% increase in mean diameter observed for unmodified particles, and a 24% increase for carboxylate-modified nanoparticles. The most significant increase in mean

diameter was observed for amine-modified particles with a 118 % increase. A further increase in mean particle diameter (49%) was observed for unmodified particles when incubated under higher shear flow conditions (8.5 cm/s)

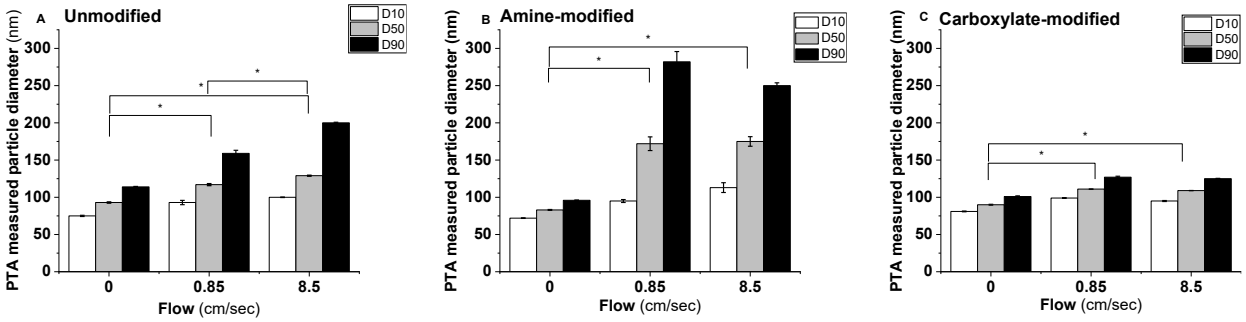

Figure 2. Shear flow conditions differentially impact polystyrene nanoparticles with different surface chemistry. Corresponding mean PTA-measured particle diameter for A) unmodified B) amine-modified, and C) carboxylate-modified polystyrene latex nanoparticles isolated using the centrifugation-resuspension method used median (D10, D50, D90) values (mean ± standard error, *n*=3). Polystyrene latex nanoparticles were incubated for 2 hours with protein media under 0.85 cm/s and 8.5 cm/s flow speeds. *P<0.05* deemed as statistically significant (student's t-test for D50 measurements). Samples with *p-values* not shown had no significant differences.

An increase in the median (D50) unmodified nanoparticle size was observed following incubation under the higher shear flow conditions with a statistically significant increase in the D50 nanoparticle diameter when incubated at 0.85 cm/s (25.8% increase) and 8.5 cm/s (38.7% increase), and significant differences in measured particle diameter were observed between the 0.85 and 8.5 cm/s shear flow conditions. A similar trend was observed with amine-modified nanoparticles at 0.85 cm/s (107% increase) and 8.5 cm/s (110% increase) flow for D50, with a significant increase in particle diameter observed following incubation under shear flow conditions. There were no significant differences between particle sizes measured for amine-modified nanoparticles incubated under 0.85 and 8.5 cm/s shear flow conditions.

The results suggest a change in particle-protein interactions when incubated under shear flow conditions with overall trends in size analysis showing differences in particle size across nanoparticle surface chemistry in response to different shear flow rates. This is further supported by the shift in zeta potential from (-25 ± 2) at 0.85 cm/s to (-7.6 ± 0.3) at 8.5 cm/s for amine-modified nanoparticles which indicates a change in the protein corona composition.

The impact of centrifugation-resuspension recovery process on nanoparticle size

Time-based incubations

Unmodified, amine-modified, and carboxylate-modified polystyrene latex nanoparticles were incubated at 37 ˚C for 2 and 24 hours with protein media, and then isolated using the centrifugation-resuspension method. In parallel, nanoparticle samples were incubated with treatment media for 2 and 24 hours and analyzed with PTA without recovering the nanoparticles from protein medium (**Figure 3**).

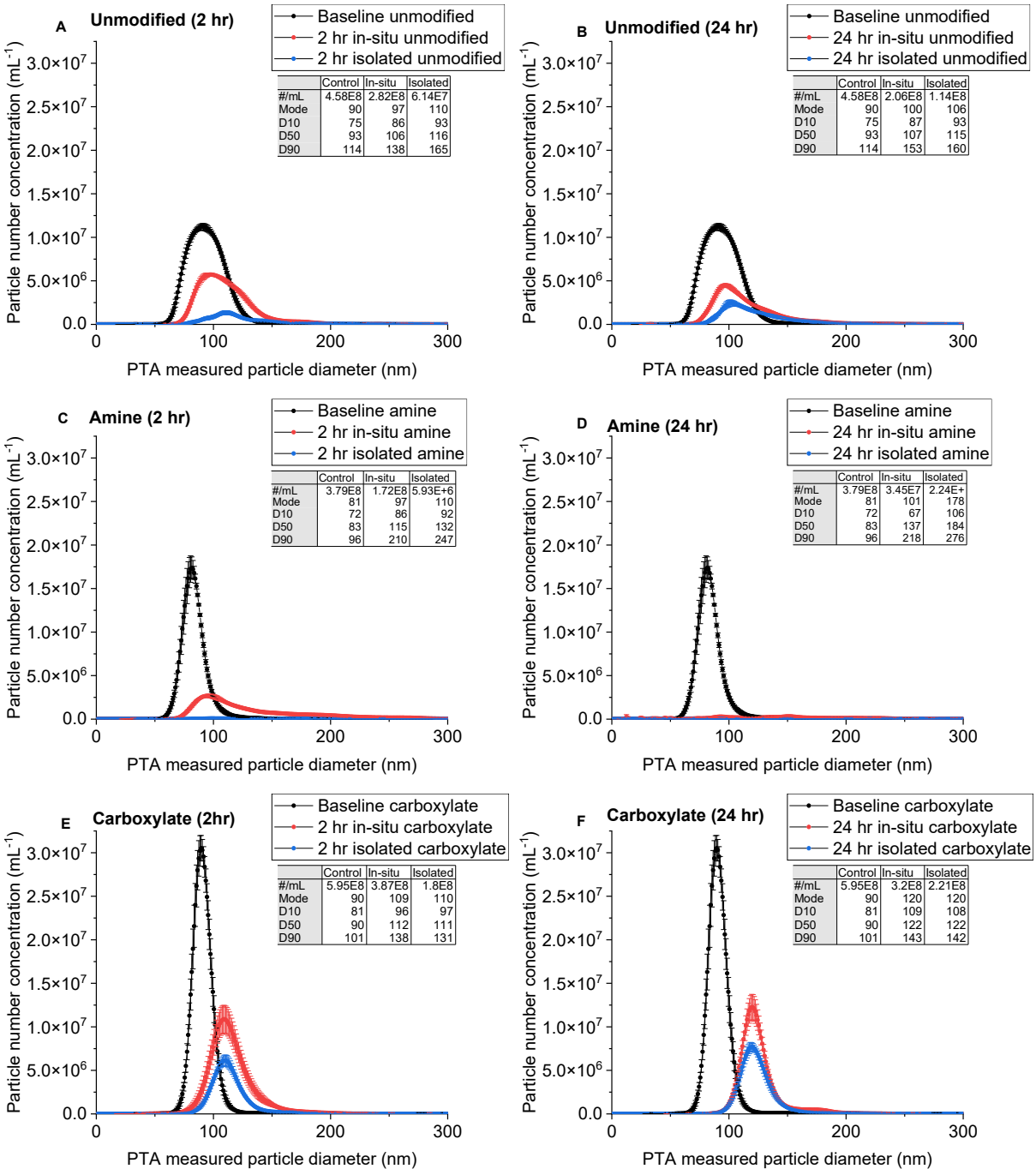

Figure 3. Nanoparticle recovery using centrifugation-resuspension protocols alters nanoparticle concentration and particle size distribution. PTA particle size distributions for **A**) unmodified (2 hour), **B**) unmodified (24 hour), **C**) amine- (2 hour), **D**) amine- (24 hour), **E**) carboxylate- (2 hour), and **F**) carboxylate-modified (24 hour) polystyrene latex nanoparticles measured following incubation with treatment medium (mean ± standard error, *n*=3). The size distributions represented in each graph are control measurements (black), *in-situ* measurement (red) and nanoparticles isolated using centrifugation-resuspension process (blue).

For all polystyrene latex nanoparticle samples, the *in-situ* analysis of particle size distribution using PTA showed a reduction in nanoparticle concentration across all sample types following 2-hour and 24-hour incubation in protein media at 37℃. Consistent with this observed reduction in particle concentration, peak broadening of the size distribution to higher sizes was observed across all nanoparticle types examined. This observation is consistent with particle agglomeration, which resulted in lower particle concentration and the emergence of nanoparticles at higher size distributions. It is also likely that protein-particle agglomerates were formed and precipitated prior to measurement. This observed effect was absent for samples incubated in PBS without protein (supplementary information). When comparing the particle concentration detected by PTA between *in situ* measurements and nanoparticles recovered using the centrifugation-resuspension protocol, a notable reduction in particle concentration was observed across all samples for particles isolated from protein-containing treatment media. Of all polystyrene latex surface modifications examined in this work, amine-modified polystyrene nanoparticle size was the most susceptible to effects of the particle recovery protocol as can be seen from the flat size distributions (Figure 3). This is likely caused by large agglomerates formed by nanoparticle-protein and protein-protein interactions which cannot be measured using PTA or may have precipitated.

Incubation under shear flow

Polystyrene latex nanoparticles with different surface chemistries were incubated for 2 hours at 37 ˚C in 10 vol% FBS medium under shear flow conditions mimicking the median cubital vein (0.85 cm/s) and arteries (8.5 cm/s). The nanoparticles were then isolated using the centrifugation-resuspension method. In parallel, samples were treated under the same conditions and directly analyzed with PTA in the protein medium (Figure 4).

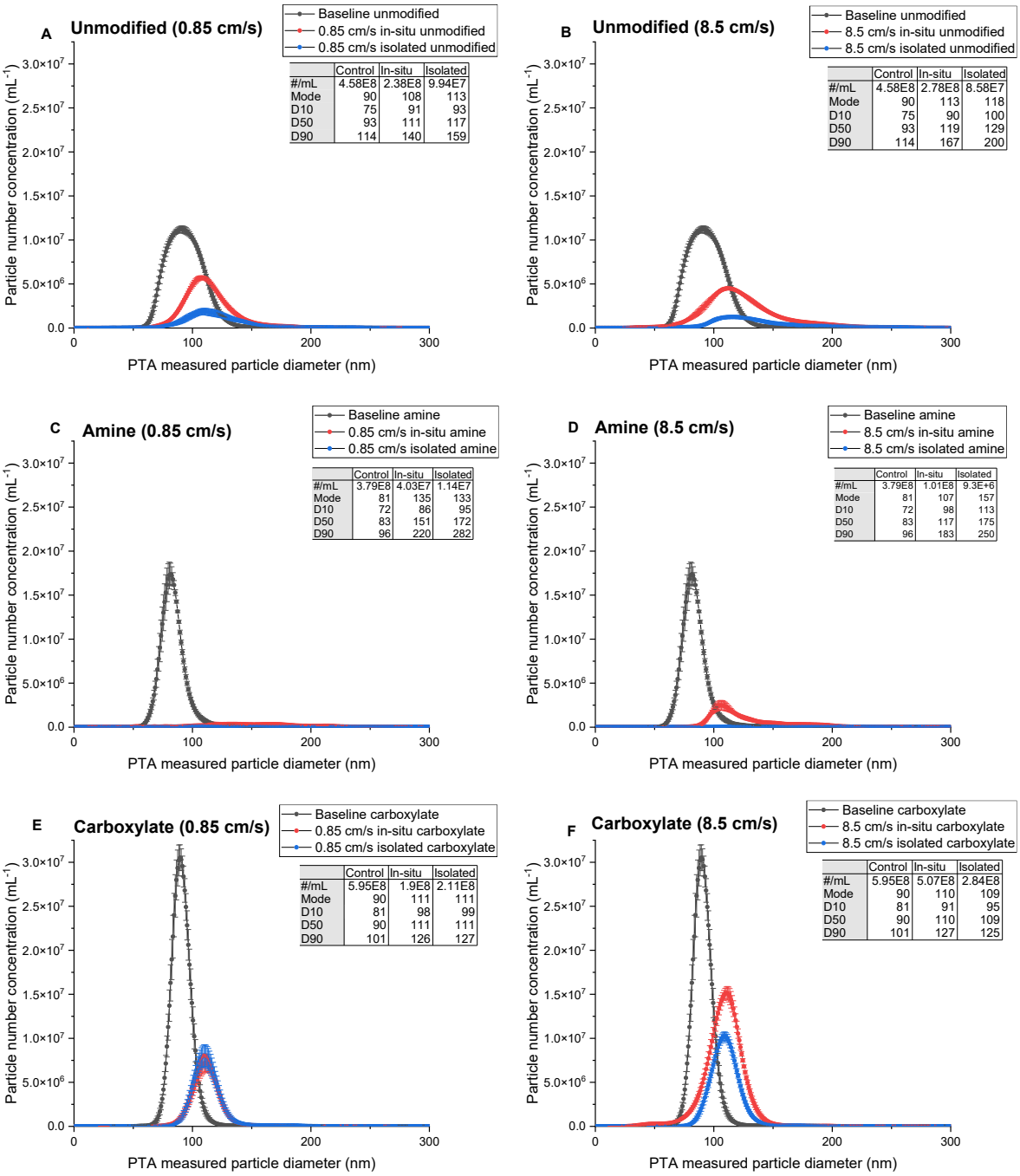

Figure 4. The centrifugation-resuspension isolation protocols alters the nanoparticle concentration and particle size distribution. With **A**) unmodified (0.85 cm/s), **B**) unmodified (8.5 cm/s), **C**) amine- (0.85 cm/s), **D**) amine- (8.5 cm/s), **E**) carboxylate- (0.85 cm/s), and **F**) carboxylate-modified (8.5 cm/s) polystyrene latex nanoparticles measured after 2 hr incubation with 10 % v/v FBS at shear flow speeds of 0.85 cm/s and 8.5 cm/s, (mean and standard error, *n*=3 independent replicates and n=5 measurements per replicate). One-way ANOVA with Tukey's comparison test was performed to determine significant difference.

Corresponding traces represented are control measurements (black), *in-situ* measurement (red) and nanoparticle isolated using centrifugation-resuspension process (blue).

The *in-situ* analysis of particle size distributions using PTA following incubation at 37℃ for 2 hours under physiologically relevant shear flow conditions (0.85 cm/s and 8.5 cm/s) showed an overall reduction in particle concentration across all surface modifications, with amine-modified particles showing the most significant decrease in the number of particles detected. As shown with previous measurements, this trend is accompanied by an increase in mean particle diameter and broadening peaks for the size distributions across all particles (unmodified, amine-modified, and carboxylate-modified). This observation likely arises due to particle-protein and protein-protein interactions and the formation of particle agglomerates. Particles incubated in PBS in the absence of protein did not show this effect (supplementary data). There was a further decrease in particle concentration detected by PTA measurements across all polystyrene latex nanoparticles (unmodified, amine-modified, and carboxylate-modified) following particle recovery using the centrifugation-resuspension method. This was once again most significantly observed for amine-modified nanoparticle.

PTA analysis for samples at (8.5 cm/s) (Figure 4) showed a shift to larger size ranges when compared to samples incubated at 0.85 cm/s and 2-hour static (Figure 3) conditions across all particle surface modifications. Further PTA analysis showed a widening of the particle size distribution span for unmodified nanoparticles with a baseline span of 0.42, which increased to 0.62 (isolated), and 0.49 (*in-situ*) following 2-hour incubation. Under shear flow conditions an increase in particle size distribution span to 0.45 (*in-situ)*, and 0.56 (isolated) was observed following incubation at (0.85 cm/s). A similar increase was seen for particles incubated at (8.5 cm/s) with a particle size distribution span increase to 0.65 (*in-situ*) and 0.78 (isolated). Amine- and carboxylate-modified nanoparticles showed a decrease in particle size span in comparison to static incubation (2 hours) following incubation under shear flow conditions (0.85 cm/s, 8.5 cm/s) (see **supplementary information**).

This observation is likely a consequence of an increase in particle-protein interactions due to increased contact between particles and protein under shear flow conditions leading to an emergence of particle sub-populations within the sample and the formation of large agglomerates which precipitate and can no longer be measured using PTA.

Changes in the composition of the nanoparticle protein corona in response to sample incubation and isolation conditions

For comparison of the composition of surface-adsorbed proteins across the particle chemistry and incubation parameters, SDS-PAGE analysis was performed following the incubation of nanoparticle samples for pre-defined times (2 and 24 hours) and shear flow conditions (0.85 and 8.5 cm/s). Nanoparticles were recovered from incubation media using the centrifugation-resuspension process, and the surface-adsorbed proteins eluted using a detergent. The composition of surface-adsorbed proteins was compared for incubations under shear flow conditions representing venous (0.85 cm/s) and arterial (8.5 cm/s) blood flow (Figure 5).

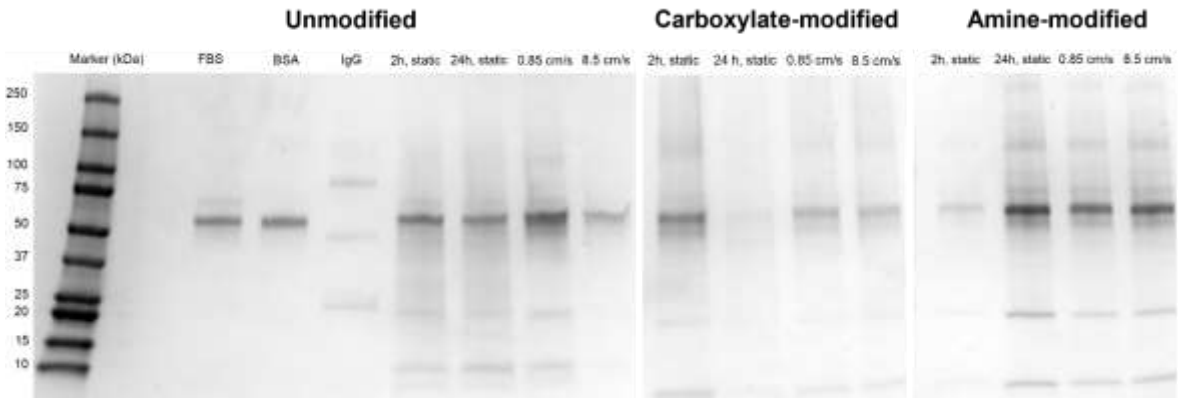

Figure 5. Protein isolated from polystyrene latex nanoparticles after 2- and 24-hour incubations with treatment medium, under static conditions and shear flow (0.85 and 8.5 cm/s). Corresponding SDS-PAGE (230 V, 25-30 mins) gels for 20 µL of sample added to each lane. All gels were stained overnight with QC Colloidal Coomassie Stain and imaged using the BioRad Gel Doc EZ Imager.

Multiple protein bands were detected for each sample type, predominantly occurring at 60 which was likely bovine serum albumin [16, 17], and 75 kDa which may potentially be bovine serotransferrin [18]. Bands corresponding to lower molecular weight species were also observed at 12 and 25 kDa. In the case of amine-modified latex nanoparticles, a larger number of high-intensity bands were observed, which is in agreement with the observed higher total protein content of surface-adsorbed protein.

Similar observations were found for gels which were normalized and loaded in each lane based on total protein content (i.e., 20 µg per lane, see **supplementary information**). Across all polystyrene nanoparticle types examined, a change in the protein corona composition was observed following incubation under various shear flow conditions. Across all nanoparticle chemistry examined, the introduction of shear flow conditions at 0.85 cm/s led to an increase in the number of bands observed and their corresponding intensity, suggesting the importance of examining protein corona formation under physiologically relevant flow conditions. We also observed time-dependent changes in the intensity of gel bands and relative composition of protein between 2- and 24-hour incubation timepoints. This observed effect was more pronounced for unmodified and amine-modified nanoparticles.

Discussion

The nanoparticle protein corona plays a key role in the biological fate of nanoparticles following administration to biological systems. Regulatory bodies including the European Medicine Agency (EMA) recommend the characterisation of nanoparticle physicochemical properties and their interaction with biological media across different stages of the nanomedicine drug development life cycle including *in vitro* and pre-clinical *in vivo* studies during early development [19]. The adsorption of proteins onto the particle surface leads to changes in the nanoparticle physicochemical parameters including size, ζ-potential, shape, surface chemistry, surface charge and colloidal stability. These parameters influence nanoparticle-cellular interactions and changes in these parameters will subsequently alter the nanoparticle biological fate [20, 21]

Our goal in this work was to assess the role of nanoparticle surface chemistry in the physical fate of nanoparticles exposed to protein containing media under conditions mimicking cell culture protein content. Here, we studied the role of nanoparticle physical parameters in the range of nanoparticle-protein interactions using polystyrene latex nanoparticles (unmodified,

amine-, and carboxylate-modified) as model nanoparticles. It is well known that proteins adsorb onto nanoparticle surfaces to form a protein corona, however there is a current lack of understanding on how the corona formation impacts nanoparticle size and size distribution, and how this subsequently governs the physical and compositional role of the nanoparticle protein corona in dictating nanoparticle pharmacological activities [21, 22]. Conversely, there is also a lack of understanding on how the protein corona contributes to an altered biological fate in the context of whether this is primarily dictated by the composition of the protein corona, or changes occurring in nanoparticle physicochemical parameters (i.e., size and charge) following corona formation. While the focus of many studies to-date has been on the quantitative compositional analysis of the protein corona, there has been a limited focus on the quantitative physical changes occurring in nanoparticle systems in response to protein treatment [23]. As such there is a clear need to develop a novel bioanalytical pipeline for the reproducible measurement of nanoparticle parameters in response to exposure to biological media as a crucial step in the development and translation of novel drug delivery systems [24].

Here, we assessed the impact of nanoparticle surface chemistry on changes in particle size and size distribution in response to treatment with media containing FBS. The conditions selected were used to mimic typical protein concentrations encountered in *in vitro* cell culture experiments, which are normally used for the early *in vitro* evaluation of nanoparticle interactions with biological media.

In most cases, we saw an increase in mean nanoparticle size following (unmodified, amine-modified) polystyrene latex particle incubation with protein-containing media and a further increase in nanoparticle size during exposure to protein-containing medium for up to 24 hours (**Figure 3**). This trend correlates with previous findings where gold nanoparticles showed an increase in particle size following prolonged incubation within protein containing media [25]. Previous work has suggested that the increase in nanoparticle size is likely due to the displacement of lower affinity proteins with high abundance by higher affinity proteins with lower abundance that form the tightly bound hard corona (Vroman effect), coupled with an increase in protein adsorption (protein concentration) over time [26].

Here, we show the development of a pipeline for the robust characterization of nanoparticles prior-to and following introduction to protein containing media. We have addressed some of the key challenges facing nanoparticle-protein interactions as shown by our findings. These data suggest that surface chemistry plays a significant role in governing nanoparticle-protein interactions, where we demonstrated that the carboxylate-modified nanoparticles adsorbed the least amount of protein, accompanied with the lowest increase in particle size following incubation across all conditions (2 hour, 24 hour, 0.85 cm/s, and 8.5 cm/s). We show that amine-modified particles are positively charged before incubation (Table 1), and adsorb more proteins onto the particle surface following incubation when compared to the negatively-charged carboxylate-modified polystyrene latex nanoparticles. This is the result of the incubation medium containing predominantly negatively-charged proteins at physiological pH [22]. The unmodified polystyrene latex nanoparticles, despite having a similar charge to carboxylate-modified nanoparticles showed more protein adsorption when compared to carboxylate-modified nanoparticles and possessed a higher mean particle size. This is due to the exposed carboxyl groups found on the particle surface following functionalisation which impacts nanoparticle-protein interactions [27]. Similar observations were made by Lundqvist et al. [28], which also compared polystyrene latex nanoparticles with similar surface chemistry incubated within human serum.

Centrifugation and resuspension of nanoparticle pellets following nanoparticle treatment with protein-containing media remains the most frequently used approach to isolate nanoparticles

for analysis. Here, we examined the role of sample isolation using this approach on measured particle characteristics, since it is well known that the centrifugation-resuspension approach for nanoparticle recovery from bulk media, significantly alters nanoparticle physicochemical properties (particle size and concentration) when compared to *in-situ* sample measurements [29]. Our findings show that (**Figure 3**, **Figure 4**), there was an increase in mean nanoparticle size for protein-treated nanoparticles following recovery using this method when compared to particles measured within protein-medium. This was most likely due to protein aggregation and particle agglomeration induced by high-speed centrifugation [30].

Furthermore, there was an observed significant loss in nanoparticle concentration measured by PTA for particles subjected to the centrifugation-wash method and *in-situ* measurements, which was likely caused by the formation of large agglomerates which precipitate or fall outside the PTA dynamic range in both cases. When compared with *in-situ* measurements of nanoparticle concentration (in incubation media), however, there is a clear higher loss for samples subjected to the centrifugation-wash methods which can be explained by the loss of sample during the centrifugation and resuspension steps. The resuspension step could potentially lead to the dissociation of nanoparticle and protein agglomerates, rendering the accurate assessment of nanoparticle size distribution challenging following recovery from incubation media. Therefore, the nature and range of techniques used for nanoparticle isolation from protein-containing media becomes a key step in the pipeline for nanoparticle characterisation and analysis of their interactions with biological systems. Our findings have implications for the design of protein corona studies, suggesting the need for more gentle separation techniques such as field-flow fractionation [31], or *in-situ* analytical approaches for measuring changes in nanoparticle parameters to minimize sample disruption through dissociation of particle agglomerate or particle-protein complexes, and subsequent alteration of the protein corona composition.

Most nanoparticle formulations are intended for intravenous administration and will result in administered nanoparticles experiencing shear flow conditions in the circulatory system [32, 33]. Here we examined the role of shear flow conditions in nanoparticle parameters following treatment with protein containing media, mimicking arterial and venous blood flow. Our findings showed that under flow conditions, total surface adsorbed protein content under flow was equivalent to the same levels seen following a 24-hour incubation with end over end rotation (see Table 2), conditions. The composition of proteins adsorbed onto nanoparticle surfaces were found to significantly vary depending on nanoparticle surface chemistry, but shear flow did not change the number of bands across particle samples (Figure 5).

A fingerprint of the protein corona composition was obtained for samples incubated under various biologically-relevant incubation conditions using SDS-PAGE analysis. We demonstrated that there were changes in protein corona composition when incubated under shear flow as opposed to static conditions across all surface chemistry (unmodified, amine-modified, carboxylate-modified). Unmodified polystyrene nanoparticle data strongly correlates with a study conducted by Jayaram et al. [15] in which similar particles were used, particularly with the trend observed at ~65 kDa where an increase in band intensity is observed at 0.85 cm/s followed by a decrease for corona composition at (8.5 cm/s). A similar change was also demonstrated across all surface chemistries for time-based incubations, which correlates with trends observed for multiple nanoparticle prototypes including co-polymers [34], and spherical nucleic acids [35]. SDS-PAGE analysis suggests the identity of adsorbed proteins is influenced by the surface functionalisation of particles, with our results showing similar trends in composition to studies [28] performed with the same nanoparticles.

Overall, our results suggest that the biorelevant assessment of nanoparticle-protein interactions requires a consideration of multiple factors that include both nanoparticle and environmental parameters, the intended route of administration for nanoparticles, and the incubation medium and conditions. Current approaches to the analysis of the nanoparticle protein corona are disruptive in nature, where sample handling steps induce either particle dissociation, agglomeration, and alteration of loosely bound surface proteins (the soft corona) as such we recommend the use of gentle isolation techniques such as field-flow fractionation or *in-situ analysis*. Furthermore, current sample handling protocols and analytical processes compromise the purpose of surface modification of nanoparticles. As shown from the results, the surface modified (amine-modified, carboxylate-modified) nanoparticles are more monodisperse with a higher number concentration before incubation as measured by PTA. However, upon exposure to protein-containing medium the functionalised particles (particularly amine) show a more significant change in particle size and size distribution. In comparison, unmodified particles appear more stable following protein corona formation in terms of particle size and size distribution.

Conclusions

The nanoparticle protein corona alters the biological fate of nanoparticles. Experimental parameters during analysis such as environmental incubation variables alter the rate and extent of nanoparticle-protein interactions. This change is dependent upon the physical and chemical properties of nanoparticles. Here, we examined the role of incubation conditions and variables in model nanoparticle systems. We show that nanoparticles with different surface modifications are differentially susceptible to experimental parameters such as shear flow and incubation duration. Furthermore, we show that the method utilised to isolate particles prior to analysis impact the resulting particle agglomeration and protein corona properties. Our findings demonstrate that careful consideration is needed in the design of sample handling and analysis of nano-bio studies where there is a need to understand nanoparticle behaviour under physiologically relevant conditions.

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