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## Pharmaceutical Nanotechnology

## Nanoparticle Isolation from Biological Media for Protein Corona Analysis: The Impact of Incubation and Recovery Protocols on Nanoparticle Properties

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## ABSTRACT

Nanoparticles are increasingly implemented in biomedical applications, including the diagnosis and treatment of disease. When exposed to complex biological media, nanoparticles spontaneously interact with their surrounding environment, leading to the surface-adsorption of small and bio- macromolecules- termed the "corona". Corona composition is governed by nanoparticle properties and incubation parameters. While the focus of most studies is on the protein signature of the nanoparticle corona, the impact of experimental protocols on nanoparticle size in the presence of complex biological media, and the impact of nanoparticle recovery from biological media has not yet been reported. Here using a non-degradable robust model, we show how centrifugation-resuspension protocols used for the isolation of nanoparticles from incubation media, incubation duration and shear flow conditions alter nanoparticle parameters including particle size, zeta potential and total protein content.

Our results show significant changes in nanoparticle size following exposure to media containing protein under different flow conditions, which also altered the composition of surface-adsorbed proteins profiled by SDS-PAGE. Our *in situ* analysis of nanoparticle size in media containing protein using particle tracking analysis highlights that centrifugation-resuspension is disruptive to agglomerates that are spontaneously formed in protein containing media, highlighting the need for *in situ* analytical methods that do not alter the intermediates formed following nanoparticle exposure to biological media.

Nanomedicines are mostly intended for parenteral administration, and our findings show that parameters such as shear flow can significantly alter nanoparticle physicochemical parameters. Overall, we show that the centrifugation-resuspension isolation of nanoparticles from media significantly alters 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 biologically-relevant shear flow conditions and media composition that can significantly alter particle physical parameters and subsequent conclusions from these studies.

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## Introduction

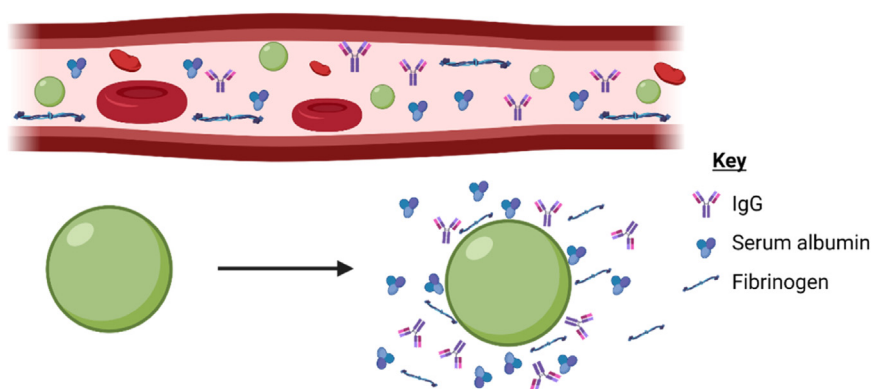
Nanoparticle-based delivery systems have emerged as an attractive approach for the safe and effective delivery of a diverse range of drugs through altered organ biodistribution and controlled drug release.<sup>1</sup> Upon administration to a biological system, colloidal nanoparticles will encounter 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 (Fig. 1).<sup>2,3</sup>

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<sup>4</sup> and biological fluid composition parameters are known to influence this process.<sup>5</sup> Knowledge of the protein corona composition and its impact on nanoparticle physical and chemical characteristics is crucial for understanding protein corona effects on the

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

biological fate of nanomedicines, which can inform the design of novel nanoparticle prototypes with pre-defined target organs, release rates, immune responses, and circulation times.<sup>5–7</sup> 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.<sup>3,8</sup> Though associations between nanoparticle surface charge, morphology and the composition of the protein corona have been reported in numerous studies, 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.<sup>9</sup> Many nanomedicines are intended for intravenous administration,<sup>10</sup> 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.<sup>11</sup> Therefore, understanding the role of haemodynamic parameters as a function of nanoparticle circulation time and techniques used to recover nanoparticles for downstream analysis of protein composition, and their implications for nanoparticle-protein interactions and subsequent cellular effects are important aspects requiring consideration 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 of nanoparticles and downstream analytical methods should be carefully considered and selected.<sup>5–7</sup>

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.<sup>12</sup> 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 includes the *in vitro* screening of cellular cytotoxicity,<sup>13</sup> cellular interactions and uptake<sup>14</sup>, and intracellular trafficking<sup>15</sup>. 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).<sup>7</sup>

The goal of this study was to investigate the impact of nanoparticle surface chemistry on interactions with media containing foetal bovine serum concentrations used in routine cell culture conditions and the resulting physical and chemical properties of the nanoparticle. Previous work has shown the *in situ* agglomeration of polymeric nano- and micro-particles following administration to pre-clinical species, an aspect that has not been investigated during the *in vitro* assessment of nanoparticle interactions with biological media. Specifically, we use polystyrene latex nanoparticles as a model nanoparticle system (due to minimal polymer hydrolysis effects) and employ sample media with the same protein content and composition as those studied for *in vitro* cellular experiments of nanoparticle cellular uptake and biocompatibility evaluation. We investigated the centrifugation-resuspension approach for recovering nanoparticles from biological media, since this is the most routinely-used method in the literature.

We report the differences in nanoparticle protein corona impact on the physicochemical characteristics of nanoparticles in response to different experimental protocols used for the isolation of nanoparticles prior to analysis. With most nanoparticles intended for parenteral 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 existing nanoparticle recovery approaches and shear flow conditions on nanoparticle parameters following exposure to protein containing media. To our knowledge, while extensive evaluation of the protein corona composition has been performed in nanoparticles to-date, the impact of co-incubation with protein has been investigated to a lesser extent.

## 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 centrifuged to remove any large agglomerates prior to use. Phosphate-Buffered Saline (PBS) was purchased from Fisher

Scientific (Invitrogen, Renfrew, Renfrewshire, UK). Mini-PROTEAN TGX precast Gels (Cat#4561094) and 4X Laemmli 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

Polystyrene latex nanoparticles (100 nm particle diameter for all surface chemistries) with different surface chemistries (unmodified, amine-modified, and carboxylate-modified) stock were prepared to maintain the same polymer equivalent concentration of 1 mg/mL (corresponding to a total polystyrene latex nanoparticle number of  $\sim 1.83 \times 10^{12}$  particles/mL) across all samples studied. The following equation was used to determine the total particle concentration used in every experiment for consistent normalization of sample concentration relative to protein across all three sample chemistries studied.

$$N = \frac{(6 \times 10^{10}) \times S \times P_L}{\pi \times P_S \times d^3} \quad (1)$$

Where  $S$  is the concentration of solids expressed in % w/w,  $d$  the diameter in  $\mu\text{m}$ ,  $P_L$  the density of latex in  $\text{g/mL}$ , and  $P_S$  the density of the bulk polymer in  $\text{g/mL}$ . The concentration of foetal bovine serum (FBS) selected (10 % v/v, corresponding to 3.9 mg/mL total protein concentration), was used to represent the composition and concentration of protein used in cell culture-based experiments.

**Incubations in the absence of shear flow** for protein corona incubation experiments, polystyrene latex nanoparticles were dispersed in either PBS (control), or PBS containing 10 % v/v FBS and incubated for 2 hour and 24 hour periods in a low protein-binding microcentrifuge tube in a temperature-controlled room (37°C).

**Incubation of samples under flow** for samples subjected to biologically relevant flow conditions, a peristaltic pump (Cole Palmer, Cambridgeshire, UK) was used to control flow and recirculate nanoparticle suspensions dispersed in either PBS or 10 % v/v FBS (Cole Palmer, #WZ-06411-62, 1.6 mm inner diameter and 3.2 mm outer diameter) at 37 °C for 2 h at (0.85 cm/s) and (8.5 cm/s) to mimic the median cubital vein and arterial blood flow conditions, respectively.<sup>16,17</sup>

**Nanoparticle recovery** following incubation in either PBS or 10 % v/v FBS in the absence and presence of flow, was performed by three centrifugation-resuspension cycles to remove unbound proteins from bulk incubation media. Nanoparticle samples were subjected to three centrifugation-resuspension cycles in a pre-chilled centrifuge (4°C).

### Sample Analysis

#### Dynamic Light Scattering (DLS) and Zeta Potential Measurements

A Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK) equipped with a 633 nm Helium-Neon laser was used for all DLS measurements. Nanoparticle size and polydispersity index (PDI) were measured prior to and following recovery from incubation media (PBS control or 10 % v/v FBS, following 2 and 24 h incubation) in non-invasive backscatter mode (173°). All measurements were performed using three independent biological replicates, with five technical replicates for each biological replicate.

Electrophoretic Light Scattering was performed to measure changes in the  $\zeta$ -potential of the nanoparticles following recovery from incubation media, and the Smoluchowski approximation was

used for data processing. All size and zeta potential measurements were performed at 25 °C with 120 s equilibration time. The mean ( $\pm$ standard error) for each sample was determined from five independent technical replicates.

#### 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 (control particles and particles recovered following incubation in protein-containing medium) and treatment medium (*in situ* analysis). 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. 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 ( $\sim 25$  °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. Data were analysed using the NTA software (v3.4.0.0.3). Three biological replicates and five technical replicates were performed for each sample.

**Protein Quantification** was performed following a centrifugation-resuspension protocol for the isolation of incubated nanoparticles from incubation media. Isolated samples were treated with Laemmli buffer and detergent overnight at ambient temperature (25 °C) under gentle agitation to elute proteins, followed by centrifugation to recover the proteins. The total protein content was quantified using the 660-nm protein assay (ThermoFisher, Rockford, USA) as per the manufacturer's instruction, and absorbance measurements were performed at 660 nm using a FlexStation III Microplate Reader (Molecular Devices, UK).

#### SDS-PAGE

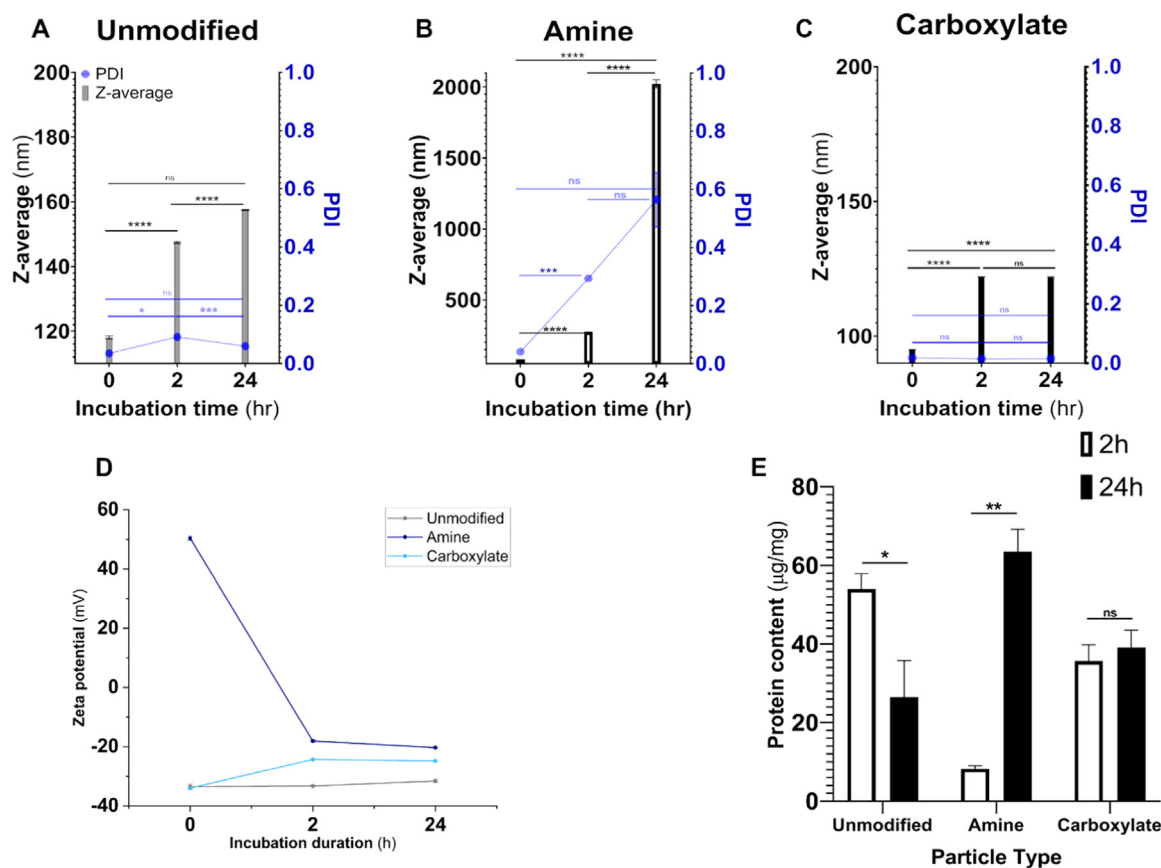
The composition and relative abundance of proteins eluted from the polystyrene latex nanoparticles was analysed with SDS-PAGE. In brief, following quantification of the total protein content eluted from isolated polystyrene nanoparticles, a sample containing 20  $\mu\text{g}$  of protein was loaded onto each lane of a Mini-Protean 4–20 % Pre-cast gel. The Coomassie Stain was used to stain all gels as per manufacturer instructions. All gels were imaged using the Bio-RAD Gel Doc EZ imaging system.

#### Statistical Analysis

Unless otherwise stated, all experimental conditions were performed using a minimum of three biological replicates, and a one-way analysis of variation (ANOVA) was performed to compare the impact of incubation parameters on nanoparticle properties. A Shapiro-Wilks normality test was performed on all datasets, and for non-normal data the Kruskal-Wallis test was used to compare nanoparticle samples subjected to flow conditions versus non-incubated baseline conditions (Control). All statistical analyses were performed in Prism (v. 8.0.1).

## Results

**Nanoparticle analysis following treatment with protein containing media and a comparison of centrifugation-resuspension effects versus *in situ* measurements with PTA** Multiparametric measurement of changes occurring in polystyrene latex nanoparticles following treatment in protein-containing media (10 % v/v, corresponding to 3.9 mg/mL total protein concentration) was carried out following incubations up to 24 h at 37 °C. Global patterns of



**Figure 2.** Differential trends in nanoparticle physicochemical parameters following incubation with protein containing media and isolation using centrifugation-wash. Mean ( $\pm$  standard error) z-average and polydispersity index (PDI) measured by DLS (A–C), and corresponding mean ( $\pm$  standard error) zeta potential for polystyrene latex nanoparticles (unmodified, amine, and carboxylate) nanoparticles (D) and changes in mean ( $\pm$  standard error) measured protein concentration at 2- and 24-hour incubations (E) for nanoparticles recovered from media containing 10 % v/v FBS (3.9 mg/mL total protein concentration) following a 2- and 24-hour incubation ( $n=3$ ). All nanoparticles were recovered using the centrifugation-resuspension approach ( $n=3$ ). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ , ns: non-significant, following a Kruskal–Wallis for (A–C) and a single factor ANOVA for E.

nanoparticle parameter changes were tracked to map changes as a function of incubation time and nanoparticle surface chemistry.

Measured baseline characteristics for each nanoparticle were in the manufacturer specified size range (100 nm diameter,  $PDI \leq 0.1$ ). The z-average significantly increased from baseline (0 h) to 24 h treatment for amine-, carboxylate- modified and untreated polystyrene latex nanoparticles with 126 %, 36.2 % and 28.7 % increases in nanoparticle diameters measured by DLS (Fig. 2 A–C), respectively. The extent of measured 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 to a lesser extent 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. PTA was used as an orthogonal higher resolution particle sizing technique for measuring changes in particle size following exposure to protein-containing media (Supplementary information, Table S1).

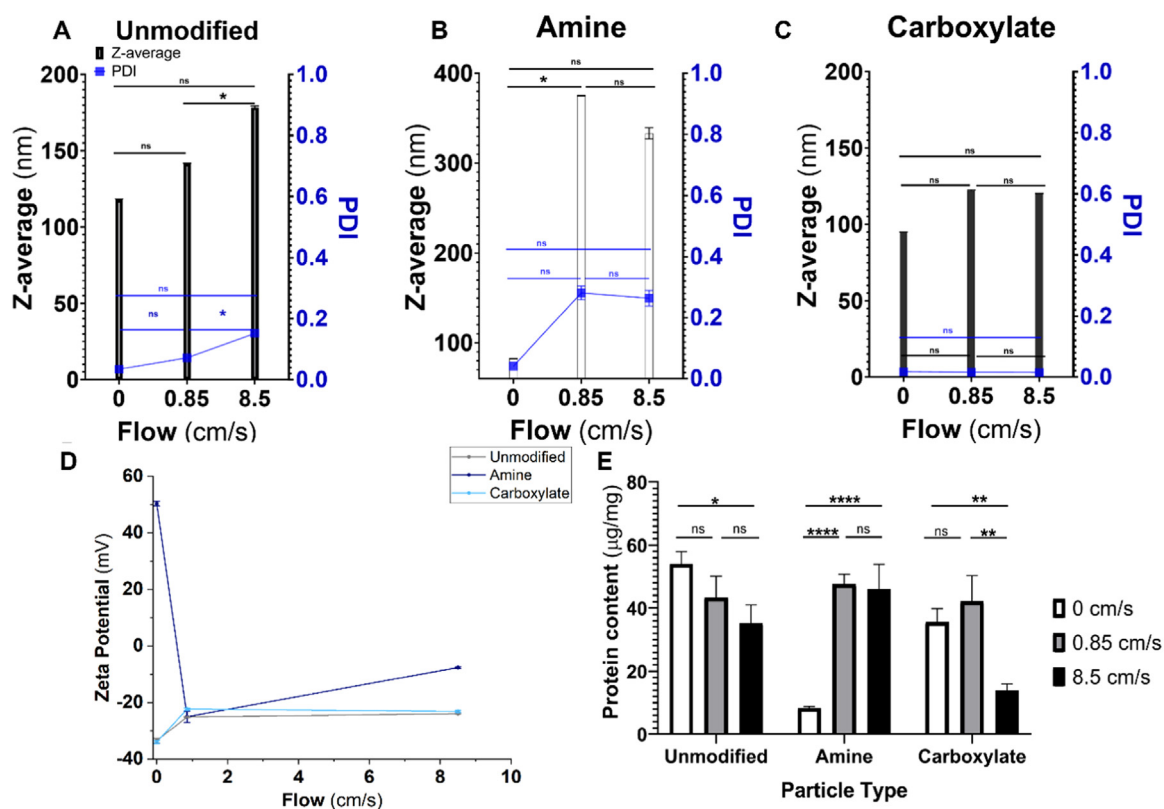
Nanoparticle surface charge plays an important role in nanoparticle-protein interactions, with zeta potential changes observed following incubation with protein-containing media for all samples. The most significant change in zeta potential was observed in the amine-modified nanoparticles (+50.4 at baseline to -18.1 mV at 2 h, and -20.3 mV at 24 h). A small change in zeta potential was observed for

unmodified nanoparticles from -33.5 mV to -31.6 mV following 24-hours. The zeta potential of negatively charged carboxylate-modified nanoparticles changed from -34.0 mV at baseline, to -24.3 mV at 2 h with no further change in zeta potential at 24 h (-24.8 mV) (Fig. 2D). These results overall indicate that nanoparticle-protein interactions occur to a lesser extent with the negatively-charged polystyrene latex nanoparticles.

Total protein content measured was observed to be the highest following 24 h incubation for amine-modified, carboxylate-modified and unmodified nanoparticles in descending order of detected protein content. There was no statistically significant difference in protein content measured for 2 h and 24 h incubations for carboxylate-modified nanoparticles with only unmodified (half protein content at 24 h versus 2 h) and amine-modified (8-fold increase in protein content at 24 h versus 2 h) nanoparticles showing a significant change in protein content following 24-hr incubation (Fig. 2E).

#### Measurement of Nanoparticle Properties Following Incubation Under Shear Flow

Unmodified, amine-modified, and carboxylate-modified polystyrene latex nanoparticles were incubated for 2 h and subjected to shear flow conditions of 0.85 cm/s (mimicking median cubital vein blood flow) and 8.5 cm/s (mimicking arterial blood flow) and recirculated using a previously described method.<sup>18</sup>



**Figure 3.** Trends in nanoparticle physicochemical parameters after incubation with protein containing media under different flow conditions and centrifugation-wash recovery. Trends in nanoparticle Z-average and PDI as measured by DLS (A–C), and changes in zeta potential change occurring for polystyrene latex nanoparticles (unmodified, amine, and carboxylate) nanoparticles (D) and changes in protein concentration as a function of incubation time (E) for nanoparticles recovered from media containing 10 % v/v FBS (3.9 mg/mL total protein concentration) following a 2 h incubation under 0 (static), 0.85 and 8.5 cm/sec shear flow. All nanoparticles were recovered using the centrifugation-resuspension approach (n=3). \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001, ns: not significant following a Kruskal–Wallis for (A–C) and a single factor ANOVA for E.

A significant increase in nanoparticle size was observed for all particles studied following incubation in protein media under physiological shear flow conditions (median cubital vein) with a 31% increase in mean diameter observed for unmodified particles (versus baseline), 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) (Fig. 3A–C, Table S2).

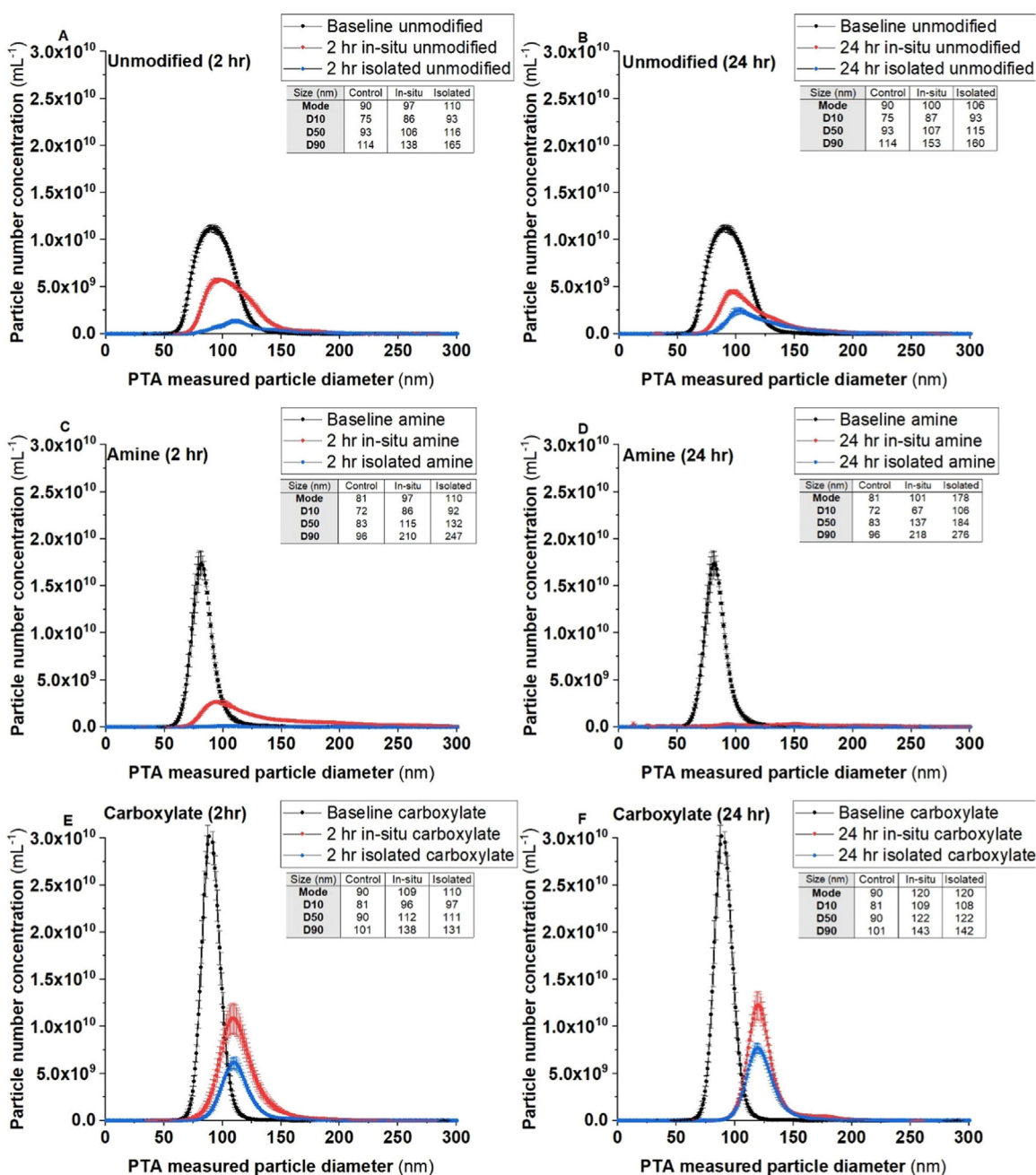
The results suggest a change in particle-protein interactions when incubated under shear flow conditions with overall trends in size analysis showing significant changes occurring in particle size across nanoparticle surface chemistries in response to different shear flow rates. This is further supported by the shift in zeta potential from  $(-25 \pm 2 \text{ mV})$  at 0.85 cm/s to  $(-7.6 \pm 0.3 \text{ mV})$  at 8.5 cm/s for amine-modified nanoparticles which indicates a change in the protein corona composition (Fig. 3D). The observed shifts in zeta potential for amine-modified nanoparticles were consistent with changes in measured adsorbed protein content, where the most significant increase in protein concentration was observed between static and 0.85 cm/s flow conditions (8-fold increase), with no significant change in measured protein content observed between 0.85 and 8.5 cm/s flow conditions. In the case of unmodified and carboxylate-modified nanoparticles only small changes were observed between static and 0.85 cm/s flow conditions, with a reduction in protein concentration observed at higher shear flow rate conditions (i.e., 8.5 cm/s).

#### The Impact of the Centrifugation-Resuspension Recovery Process on Nanoparticle Size as Measured by Particle Tracking Analysis

##### Time-Based Incubations

Unmodified, amine-modified, and carboxylate-modified polystyrene latex nanoparticles were incubated at 37 °C for 2 and 24 h in incubation media, and isolated using the centrifugation-resuspension method. In parallel, additional samples were incubated with treatment media for 2 and 24 h and analysed with PTA without recovering the nanoparticles (*in situ*) from protein incubation medium (Fig. 4).

For all polystyrene latex nanoparticle samples, the *in-situ* analysis of particle size distribution showed a reduction in nanoparticle concentration across all sample types following 2 h and 24 h incubation in protein media at 37 °C. Consistent with this observation, 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. Amine-modified polystyrene nanoparticle size was the most susceptible to effects of the particle recovery protocol (Fig. 4). This is likely caused by



**Figure 4.** Nanoparticle recovery using centrifugation-resuspension protocols alters nanoparticle concentration and particle size distribution. PTA particle size distributions for A) unmodified (2 h), B) unmodified (24 h), C) amine- (2 h), D) amine- (24 h), E) carboxylate- (2 h), and F) carboxylate-modified (24 hour) polystyrene latex nanoparticles measured following incubation with treatment medium (mean  $\pm$  standard error,  $n=3$ ). The size distributions represented in each graph are control measurements (black), *in-situ* measurement (red) and nanoparticles isolated using the centrifugation-resuspension protocol (blue). (For interpretation of the references to color in this Fig. legend, the reader is referred to the web version of this article.)

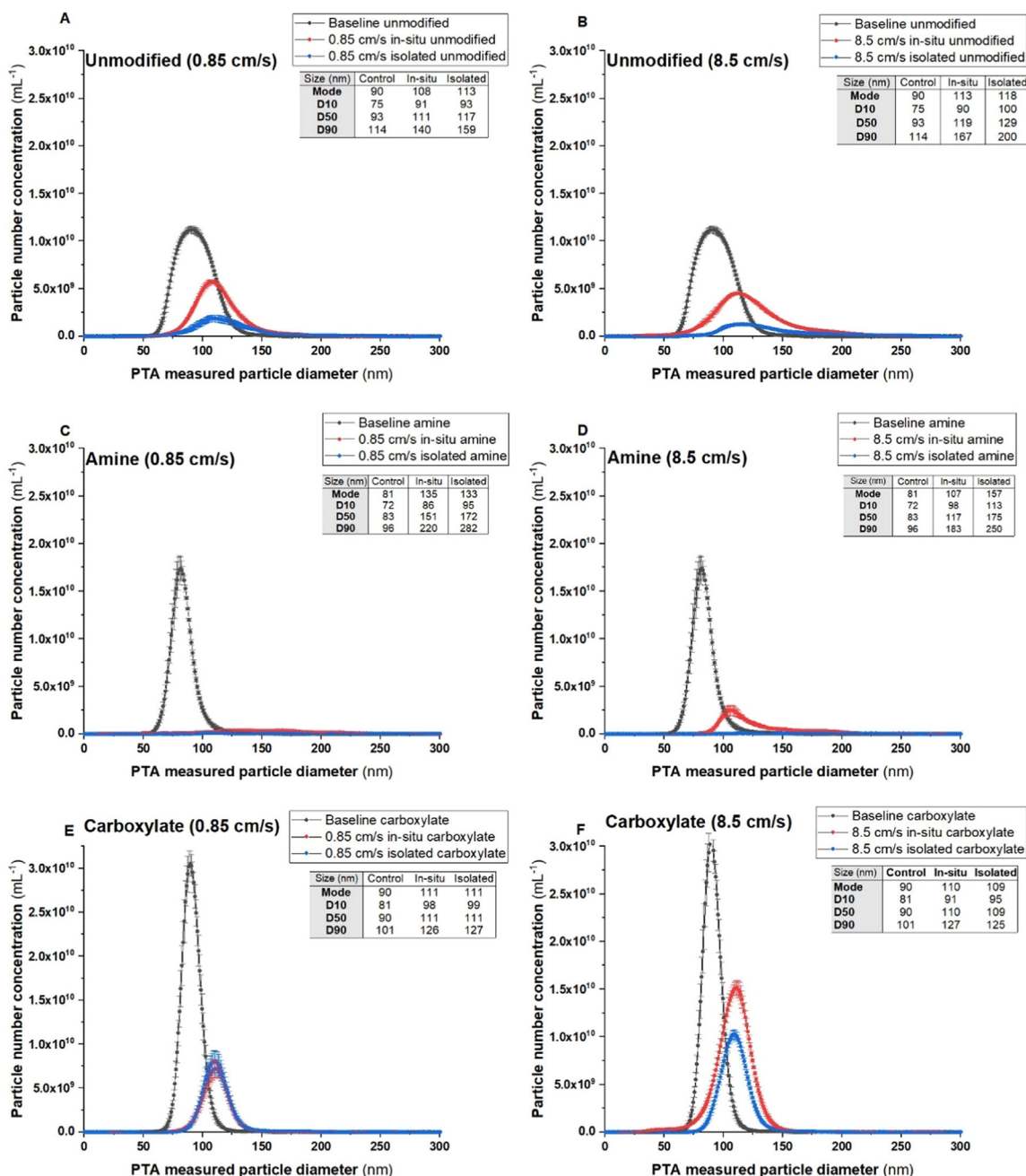
agglomerates formed from nanoparticle-protein and protein-protein interactions which cannot be measured using PTA or may have precipitated.

#### Incubation Under Shear Flow

Polystyrene latex nanoparticles were incubated for 2 h under shear flow mimicking the median cubital venous (0.85 cm/s) and arterial (8.5 cm/s) blood flow rates, and nanoparticles were isolated by centrifugation-resuspension. In parallel, samples were treated

under the same conditions and directly analyzed with PTA in the protein medium (*in situ* analysis, Fig. 4).

*In-situ* analysis of particle size following incubation at 37 °C for 2 h under shear flow (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 peak broadening across all particles examined. Particles incubated in PBS in the absence of protein did not show this effect (supplementary data). There was a further decrease in particle



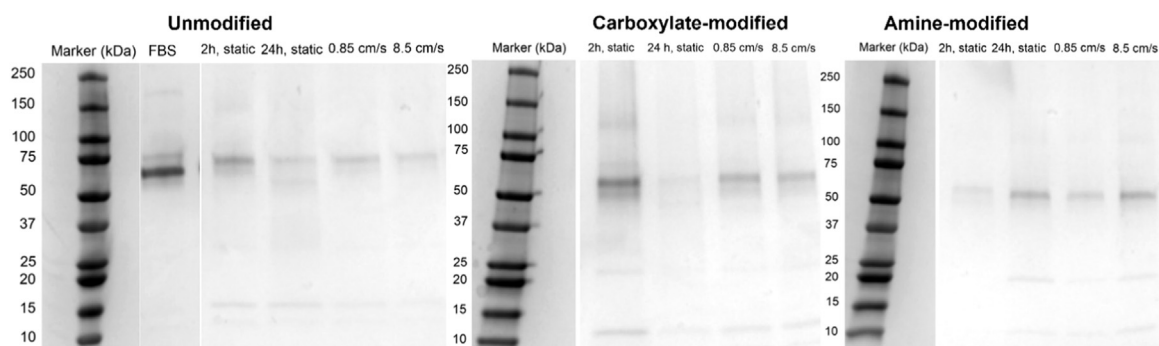
**Figure 5.** The centrifugation-resuspension isolation protocols alters the nanoparticle concentration and particle size distribution. With A) unmodified (0.85 cm/s), B) unmodified (8.5 cm/s), C) amine- (0.85 cm/s), D) amine- (8.5 cm/s), E) carboxylate- (0.85 cm/s), and F) carboxylate-modified (8.5 cm/s) polystyrene latex nanoparticles measured after 2 h incubation with 10 % v/v FBS at 0.85 cm/s and 8.5 cm/s, (mean and standard error,  $n=3$ ). 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 nanoparticles isolated by centrifugation-resuspension (blue). (For interpretation of the references to color in this Fig. legend, the reader is referred to the web version of this article.)

concentration detected by PTA measurements across all polystyrene latex nanoparticles following recovery by centrifugation-resuspension.

PTA analysis for samples at (8.5 cm/s) (Fig. 5) showed a shift to larger sizes in comparison to samples incubated at 0.85 cm/s and 2 h static (Fig. 4) conditions across all particle types. 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 h 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 h) 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.



**Figure 6.** Protein isolated from polystyrene latex nanoparticles after 2 and 24 h incubations under static (0 cm/s) and shear flow (0.85 and 8.5 cm/s) conditions. Corresponding SDS-PAGE gel images. 20  $\mu$ g of protein was loaded per lane and all gels were stained with QC Colloidal Coomassie Stain and imaged. Marker: molecular weight marker (Kaleidoscope ladder); FBS: foetal bovine serum

#### Analysis of Compositional Changes in Surface-Adsorbed Proteins in Response to Sample Incubation and Isolation Conditions

For a visual 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 h) 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 from the nanoparticles prior to loading onto SDS-PAGE gels (Fig. 6).

Multiple protein bands were detected for each sample type, predominantly occurring at 60 kDa, which was likely bovine serum albumin.<sup>19,20</sup> Bands corresponding to lower molecular weight species were also observed at 12 and 25 kDa.

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 chemistries examined, shear flow conditions at 0.85 cm/s led to an increase in the number of bands and their 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 h 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),<sup>21</sup> 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. The adsorption of proteins onto the particle surface leads to changes in the nanoparticle physicochemical parameters including size, zeta 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.<sup>22,23</sup>

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.<sup>23,24</sup> Conversely, there is also a lack of understanding of how the protein corona contributes to an altered biological fate in the context of changes occurring in nanoparticle physical parameters (i.e., size and charge) following exposure to protein containing media and subsequent protein 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.<sup>25</sup> 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.<sup>26</sup>

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. We selected polystyrene latex nanoparticles as a model system due to their chemical stability over the duration and range of conditions examined in this study, to exclude effects resulting from polymer degradation. The protein incubation conditions selected for this work were based on the typical protein serum concentrations used in *in vitro* cell culture experiments, which are normally used for the early *in vitro* evaluation of nanoparticle interactions with biological media. We selected PBS as the dispersant as opposed to cell culture media (e.g., DMEM, RPMI), to exclusively study the protein content of serum and its role in nanoparticle physicochemical properties, since cell culture media are known to contain other biomolecules.

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 h (Fig. 2). This trend correlates with previous findings where gold nanoparticles showed an increase in particle size following prolonged incubation within protein containing media.<sup>27</sup> 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.<sup>28</sup>

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-h, 24-h, 0.85 cm/s, and 8.5 cm/s). We show that amine-modified particles are positively charged before incubation 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.<sup>24</sup> 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.<sup>29</sup> Similar observations were made by Lundqvist et al.<sup>30</sup> which compared polystyrene latex nanoparticles with similar surface chemistry incubated in 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.<sup>31</sup> Our findings show that (Figs. 4, 5), 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.<sup>32</sup>

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. We also observed an apparent significant increase in the measured total protein concentration between 2 and 24 h incubation timepoints for amine-modified nanoparticles (see 3E). Such a significant increase in protein content may be attributable to the loss of colloid stability resulting from the presence of protein containing media. Moreover, the process of centrifugation-resuspension for the isolation of nanoparticles may enrich for agglomerated and precipitated particles that contain higher surface-adsorbed protein content. Overall, this process may lead to an overestimation of surface-adsorbed protein content at later timepoints.

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,<sup>33</sup> 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.

A limitation associated with the present analyses of particle size concentration changes is that metrology techniques such as PTA and DLS are unable to differentiate between particle types by composition (i.e., polymeric versus protein). However, techniques such as asymmetric flow field flow fractionation with inline light scattering and other detector modalities (e.g., fluorescence, UV, RI) can resolve different molecular weight species, allowing for specific intermediates (e.g., protein fraction, nanoparticle-protein complexes, agglomerates) to be studied.<sup>34</sup>

Most nanoparticle formulations are intended for parenteral administration and will result in administered nanoparticles experiencing shear flow conditions in the circulatory system.<sup>35,36</sup> 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-h incubation with end over end rotation 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 (Fig. 6).

A qualitative 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.<sup>18</sup> 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,<sup>37</sup> and spherical nucleic acids.<sup>38</sup> 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 performed with the same nanoparticles.<sup>30</sup>

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 for the analysis of the nanoparticle protein corona are disruptive in nature, where sample handling steps induce either particle dissociation or 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.

### Data Availability Statement

The datasets generated and used/or analysed are available from the corresponding authors upon request.

### Declaration of Competing Interest

The authors declare no conflicts of interest.

### CRedit authorship contribution statement

**Karim Daramy:** Investigation, Methodology, Writing – original draft, Visualization, Writing – review & editing, Funding acquisition. **Panida Punnabhun:** Methodology. **Muattaz Hussain:** Methodology, Writing – original draft. **Caterina Minelli:** Funding acquisition. **Yiwen Pei:** Funding acquisition. **Yvonne Perrie:** Funding acquisition. **Zahra Rattray:** Investigation, Methodology, Writing – original draft, Visualization, Writing – review & editing.

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### Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2023.12.021.

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