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Protocols

Influence of dynamic flow environment on nanoparticle-protein corona: From protein patterns to uptake in cancer cells

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ABSTRACT

The fast growing use of nanoparticles (NPs) in biotechnology and biomedicine raises concerns about human health and the environment. When introduced in physiological milieus, NPs adsorb biomolecules (especially proteins) forming the so-called protein corona (PC). As it is the PC that mostly interacts with biological systems, it represents a major element of the NPs' biological identity with impact on nanotoxicology, nanosafety and targeted delivery of nanomedicines. To date, NP-protein interactions have been largely investigated *in vitro*, but this condition is far from mimicking the dynamic nature of physiological environments. Here we investigate the effect of shear stress on PC by exposing lipid NPs with different surface chemistry (either unmodified and PEGylated) to circulating fetal bovine serum (FBS). PC formed upon *in vitro* incubation was used as a reference. We demonstrate that PC is significantly influenced by exposure to dynamic flow and that changes in PC composition are dependent on both exposure time and NP's surface chemistry.

Notably, alterations induced by dynamic flow affected cellular uptake of lipid NPs in both human cervical cancer (HeLa) and human breast adenocarcinoma (MCF7) cell lines.

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1. Introduction

Recent years have seen rapid progresses in nanoparticle (NP) biotechnology, propelled by many promising applications in biomedicine [1–3]. When designed for biomedical applications, such as drug delivery, pristine NPs come into contact with biological environments where they adsorb biomolecules forming the so-called protein corona (PC) [4–11]. PC is the bio-interface of NPs that is 'seen' by living organisms, while the primary particle surface remains buried and largely inaccessible [12–14]. Proteins involved in physiological [15,16] as well as toxicological relevant processes in the blood system, such as complement activation and coagulation, are main components of the coronas of various NPs. Thus, PC is now believed to regulate nanotoxicology [17,18], macrophage recognition [19–22] and opsonization [23] of NPs. Furthermore, as the interaction between NPs and target cells is (at least in part) controlled by the 'corona proteins', PC is emerging as an important cofactor for targeted drug delivery [11,17,24–29]. In particular, understanding correlations between PC 'fingerprints' (PCFs) and cellular uptake is critical for clinical translation of NPs [30–34]. To date, almost the totality of these explorations has been carried out *in vitro*, while few studies on the PC have been performed *in vivo* [35,36], mainly because of the difficulty of recovering NPs after administration. Unfortunately, *in vitro* incubation is far from accurately representing the dynamic nature of physiological environments. When injected in the human body, NPs experience speeds from a few micrometers s^{-1} (in the peripheral blood vessels) up to $\approx 60\,cm\,s^{-1}$ (in the aorta). A circulating flow could affect NP-protein interactions by creating shear stress on NPs and providing an incessant source of biomolecules [37,38]. To address this issue lipid NPs [39] with different surface chemistry (either unmodified and PEGylated) were injected into fetal bovine serum (FBS) propelled by a peristaltic pump furnished with silicon

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tubes (FBS speed = 50 cm s^{-1}), while *in vitro* incubation (i.e. exposure to static FBS) was used as reference (Supplementary Fig. S1). FBS was chosen because of its similar viscosity to human serum, while allowing for accurate and reproducible characterization of PC. Protein coronas have been thoroughly compared in terms of quantitative composition and impact on NP properties by dynamic light scattering, micro-electrophoresis and nano-liquid chromatography tandem mass spectrometry (nanoLC–MS/MS). Here we show that PC is influenced by exposure to dynamic flow and that alterations are dependent on both incubation time and NP's surface chemistry. This observation could be potentially relevant in cancer therapy where few NPs have made it to clinical trials and even less are in clinical practice. An emerging strategy is to functionalize NPs to promote the adsorption of plasma components that are naturally targeted to the desired cells [40,41]. Thus, we asked ourselves whether changes in PC due to dynamic flow could also affect NP uptake by cancer cells. To test this suggestion, we used two commonly used cell lines, namely human cervical cancer (HeLa) and human breast adenocarcinoma (MCF7) cell lines. Notably, we demonstrate that changes in PC induced by dynamic flow could affect cellular uptake of lipid NPs in HeLa and MCF7 cancer cell lines.

2. Materials and methods

2.1. Preparation of NPs

Organic solvents and Fetal Bovine Serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Ultrapure water (resistivity 18.2 MΩ cm) was achieved by an Arium water purification system (Sartorius, Florence, Italy). Cationic lipids 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and (3-[*N*-(*N*′,*N*′-dimetylaminoethane)-carbamoyl])-cholesterol (DC-Chol) and zwitterionic helper lipids dioleoylphosphatidylethanolamine (DOPE), DOPE-polyethyleneglycol (PEG) 2000 and dioleoylphosphatidylcholine (DOPC), were purchased from Avanti Polar Lipids (Alabaster, AL). All the lipids were used without further purification. Liposomes were prepared by dissolving appropriate amounts of lipids at φ = neutral lipid/total lipid (mol/mol) = 0.5, in accordance with standard protocols [39]. Lipid NPs were synthesized according to these molar ratios: DOTAP:DOPC:DC-Chol:DOPE (1:1:1:1) and DOTAP:DOPC:DC-Chol:DOPE-PEG (1:1:1:0.7:0.3). Lipid films were hydrated with phosphate saline buffer (PBS) to achieve a final lipid concentration of 1 mg/mL, then extruded 20 times through a 0.1 μm polycarbonate carbonate filter by the Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL) and stored at 4 °C.

2.2. Dynamic light scattering and zeta-potential measurements

Liposome/FBS complexes were diluted 1:100 with PBS 10 mM and size and zeta-potential measurements were carried out using a Zetasizer Nano ZS90 (Malvern, U.K.) at 25 °C. Results are given as mean ± standard deviation of five independent replicates.

2.3. Transmission electron microscopy (TEM)

Ten microliters of liposomal dispersion were deposited onto Formvar-coated grids, negatively stained using 1% uranyl acetate, washed with ultrapure water and air-dried. Measurements have been carried out with a Zeiss Libra 120, and image processing was performed with ImageJ software (National Institute Health Image; http://rsbweb.nih.gov/ij/).

In vitro incubation

Liposomes were mixed with clarified FBS (1:1 v/v) and were incubated for 5 or 90 min at 25 °C. For size and zeta-potential experiments 20 μL of mixed solution was diluted with 980 μL of a PBS:H_2O (1:80 v/v) solution, to obtain a final concentration of 10 ng/mL. For proteomics experiments the same procedure was followed, but the final volume of liposome:FBS dispersion was of 1.8 mL. According to previous findings [42], this is the minimum volume required for accurate protein identification and quantification by nanoLC–MS/MS.

2.4. Dynamic incubation

A peristaltic pump (Watson and Marlow, UK) furnished with silicon tubes (internal diameter 1.6 mm, total length 250 mm) was used to propel the liposome:FBS solution. According to the manufacturer's instructions, the rounds per minute (RPM) were adjusted to mimic the human abdominal aortic flow speed (≈ 50 cm s^{-1}). For size and zeta-potential experiments, liposome solution (1 mg/mL in PBS 10 mM) was mixed to freshly clarified FBS (1:1 v/v) and introduced inside the silicon tube by a micro-fine insulin syringe (Becton, Dickinson and Company, USA). The syringe was used as a stopper to avoid formation of air bubbles. After 5 and 90 min, 20 μL of the circulating solution was collected from the silicon tube using a Hamilton syringe and instantaneously transferred into the Zetasizer Nano ZS90 cuvette. For proteomics experiments, the large sampling volume of liposome:FBS (1.8 mL) was obtained by using a 820 mm long silicon tube.

2.5. Proteomics experiments

FBS was centrifuged at 14,000 rpm for 10 min at 25 °C. This centrifugation step allows eliminating free proteins and protein aggregates. As a result, further steps allow isolating liposome-protein complexes with no undesired protein contamination. MC unmodified and PEGylated liposomes were mixed with FBS (1:1 v/v) and were incubated at room temperature for 5 min and 90 min, either for static and dynamic incubation, as previously described. After incubation, liposome-protein complexes were centrifuged for 15 min at 14,000 rpm. Unbound proteins were removed washing pellets three times with 200 μL of PBS 10 mmol L^{-1}. The tubes were changed after each washing step to minimize contamination of plasma proteins bound to the tubes.

2.6. In solution digestion and desalting

In solution protein digestion was carried out as previously described [43,44]. Briefly, the protein pellets were resuspended in 50 μL of a denaturant buffer composed of 8 mol L^{-1} urea in 50 mmol L^{-1} NH_4HCO_3. Proteins were reduced, alkylated and digested with trypsin (1:20, w/w, enzyme to protein ratio). After overnight digestion at 37 °C, the reaction was quenched by the addition of trifluoroacetic acid (TFA). Digested samples were desalted using SPE C18 cartridges as already described and then were dried in a Speed-Vac SC 250 Express (Thermo S 164 avant, Holbrook, NY, U.S.A.). Each sample was reconstituted with 0.1% HCOOH aqueous solution and stored at −80 °C until nano-LC–MS/MS analysis.

2.7. NanoLC–MS/MS analysis

Peptides were separated using the Dionex Ul-timate 3000 coupled to a linear quadrupole ion-trap Orbitrap (LTQ Orbitrap XL) mass spectrometer (ThermoScientific, Bremen, Germany) equipped with a nanospray ion source. Samples were on-line preconcentrated on a μ-precolumn (Dionex, 300 μm i.d. × 5 mm Acclaim PepMap 100 C18, 5 μm particle size, 100 Å pore size),

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