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Impact of silica nanoparticle surface chemistry on protein corona formation and consequential interactions with biological cells

Andréa Kurtz-Chalot [a,b,c], Christian Villiers [d,e], Jérémie Pourchez [a,b,c], Delphine Boudard [b,c,f,g], Matteo Martini [h], Patrice N. Marche [d,e], Michèle Cottier [b,c,f,g], Valérie Forest [a,b,c,*]

[a] Ecole Nationale Supérieure des Mines de Saint-Etienne, CIS-EMSE, SAINBIOSE, F-42023 Saint Etienne, France
[b] INSERM, U1059, F-42023 Saint Etienne, France
[c] Université de Lyon, F-69000 Lyon, France
[d] Centre de Recherche INSERM U1209, F-38700 La Tronche, France
[e] Université Grenoble-Alpes, Institut Albert Bonniot, F-38700 La Tronche, France
[f] Université Jean Monnet, F-42023 Saint-Etienne, France
[g] CHU de Saint-Etienne, F-42055 Saint-Etienne, France
[h] Institut Lumière Matière, UMR5603 CNRS, Université Claude Bernard Lyon 1, F-69622 Villeurbanne, France

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ABSTRACT

Nanoparticles (NP) physico-chemical features greatly influence NP/cell interactions. NP surface functionalization is often used to improve NP biocompatibility or to enhance cellular uptake. But in biological media, the formation of a protein corona adds a level of complexity. The aim of this study was to investigate *in vitro* the influence of NP surface functionalization on their cellular uptake and the biological response induced. 50 nm fluorescent silica NP were functionalized either with amine or carboxylic groups, in presence or in absence of polyethylene glycol (PEG). NP were incubated with macrophages, cellular uptake and cellular response were assessed in terms of cytotoxicity, pro-inflammatory response and oxidative stress. The NP protein corona was also characterized by protein mass spectroscopy. Results showed that NP uptake was enhanced in absence of PEG, while NP adsorption at the cell membrane was fostered by an initial positively charged NP surface. NP toxicity was not correlated with NP uptake. NP surface functionalization also influenced the formation of the protein corona as the profile of protein binding differed among the NP types.

1. Introduction

Nanoparticles (NP) represent promising tools for biomedical applications as they can be used as therapeutic and/or diagnostic agents. Their surface can be modified to foster NP/cell interactions and thus enhance their subsequent cellular uptake to reach their intracellular targets [1]. Indeed, NP/cell interactions strongly depend on the NP physico-chemical features and 8 parameters seem to have a crucial importance as defined by ISO/TS 80004-2:2015: agglomeration/aggregation state, composition, size, shape, solubility/dispersibility, specific surface area, density of surface groups and surface chemistry [2,3]. In particular surface charge is recognized to play a major role and it is commonly acknowledged that positively charged NP are more internalized by cells than neutral or negatively charged NP [1,4–10]. A correlation between the amount of positive charges and cellular uptake has even been observed [8]. Consequently, NP surface functionalization by modifying surface charge is an efficient and easy way to drive cellular uptake [5–7,11]. The modulation of the NP physico-chemical features is important not only for the enhancement of cellular uptake but also for the induced biological response (and the potential cytotoxicity) [3,8,12,13]. It is thus crucial to define accurate physico-chemical parameters to take into consideration to manufacture NP with a "safer by design" approach [14]. For example, Nabeshi et al., reported that the reduction of cell proliferation of macrophages was more important when cells were incubated with unmodified silica NP than with the same NP functionalized with amine or hydroxyl groups [15]. Therefore, one easy way to increase NP biocompatibility and enhance their cellular uptake is through surface functionalization. In this context, amorphous silica is a particularly interesting material because the chemistry of the silanes allows surface modification of silica NP with chemical functional groups like specific antibodies (targeting to cancer cells and drug delivery) or fluorescent labels (tumor labeling) [16,17].

But an additional level of complexity should be considered when NP are in contact with biological fluids. Indeed, in these conditions NP are surrounded by a wide variety of biomolecules, especially proteins,

* Corresponding author at: Ecole Nationale Supérieure des Mines de Saint-Etienne, Center for Biomedical and Healthcare Engineering, 158 Cours Fauriel, CS 62362, 42023 Saint-Etienne Cedex 2, France.
E-mail address: vforest@emse.fr (V. Forest).

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which rapidly adsorb at their surface and entirely cover it. This so-called protein corona modifies the original NP physico-chemical features and constitutes the first contact of the nanomaterial with the cells and may influence the biological responses [18–26]. Various strategies may be used to decrease protein adsorption at the NP surface, one consists of the grafting of linear chains of hydrophilic polymers, such as polyethylene glycol (PEG) at NP surface. This process allows reducing protein adsorption by blocking protein-binding sites and creating a steric hindrance [27]. Moreover, the relative density of each type of adsorbed proteins is known to depend on PEG grafting density [28]. The formation of the protein corona is a dynamic process that depends, among others, on nature of the environment and on the NP physico-chemical properties and especially surface charge [18,20,23,29]. It has been shown that increasing the surface charge of NP resulted in a protein adsorption increase in general and of negatively charged serum proteins in particular [4,18,30]. Due to the discrepancy in the published results, the relationship between protein adsorption on NP and the decrease of the cellular internalization of NP remains to be established. Indeed, some studies show that protein adsorption on NP decreases their cellular uptake while other tend to demonstrate the opposite [18,19,25,29–32]. Furthermore, it is difficult to draw firm conclusions from results dealing with different NP types, different functionalization groups, different cellular models, different biological assays, *etc.* To bring new insights to this issue we propose the present study which aim was to investigate *in vitro* the influence of the functionalization of NP on their cellular uptake and the subsequent biological response. For that purpose, fluorescent silica-based NP were functionalized with chemical groups of different charges in presence or not of a steric hindrance generated by PEG chains. These NP were incubated with macrophages and the cellular uptake was assessed by fluorimetry. Cellular cytotoxicity, secretion of inflammatory factors and oxidative stress were also evaluated. A major asset of this work is that NP were synthesized in similar conditions and the biological activity was also assessed in the same experimental conditions, limiting variability and allowing reproducible and trustable results. In addition, a study on the influence of the NP surface functionalization on the formation of the protein corona was carried out.

2. Experimental

2.1. Nanoparticle synthesis

Silica-based NP were prepared according to a method developed by Martini et al. [33]. The water/oil (W/O) microemulsion procedure was used to produce homogeneous and reproducible core-shell samples. Indeed, reverse micelle (aqueous droplets sized ~10 nm) acts as template for the controlled-growth of core-shell structures. Quaternary W/O microemulsions were prepared by mixing Triton X-100 (surfactant), *n*-hexanol (co-surfactant) and cyclohexane (oil), followed by sequential additions of specific polar-like precursors. An inclusion of gold clusters at the center of each particle was obtained by the reduction of gold salt in presence of ligands and $NaBH_4$. Please note that although NP contained a gold core, we mainly considered them as made of silica as gold is tightly enclosed in the silica shell. Moreover, assays of stability allowed concluding to the stability of the NP as no dissolution of the silica shell was observed over time. The formation of polysiloxane matrix arose from the base-catalyzed hydrolysis and condensation of two silica precursors: 92%w TEOS (tetraethoxysilane) and 8%w dye conjugated-APTES ((3-aminopropyl)triethoxysilane). APTES conjugates ensured a covalent bonding of dyes (FITC) and their random distribution within NP. The colloidal stabilization was then achieved by addition of silane precursors with amine or carboxylic acid groups grafted determining the surface charge (Table 1). Thereafter, all solvents were eliminated by the addition of acetone followed by several cycles of vortexing and centrifuging. Unreacted dyes and precursors were removed by ultrafiltration using 300 kDa PES membranes. Particles were dispersed in aqueous solution (2 g/L) and stored at 4 °C.

2.2. Nanoparticle physico-chemical characterization

Scanning electron microscopy (SEM) images of NP were performed using an ESEM XL30-FEI microscope equipped with a thermal field emission gun (FEG). NP samples were prepared by depositing a drop of diluted colloidal solution onto a carbon grid (200 meshes), the solvent was allowed to evaporate at room temperature. Samples were observed under vacuum. Transmission electron microscopy (TEM) images were obtained using a Philips CM200 microscope at a 200 kV accelerating voltage. The size distribution of NP and their zeta potential (ζ) were determined using the nano Zetasizer apparatus (Malvern) based on dynamic light scattering measurement. Measures were performed both in distilled water and in cell culture medium DMEMc (Dulbecco's Modified Eagle's Medium, Invitrogen, Cergy Pontoise, France) supplemented with 10% of fetal calf serum (Invitrogen), 1% penicillin–streptomycin (penicillin 10,000 units/mL, streptomycin 10 mg/mL; Sigma-Aldrich, Saint-Quentin Fallavier, France).

2.3. Cell culture

RAW 264.7 cell line derived from mice peritoneal macrophages transformed by the Abelson murine leukemia virus and was provided by ATCC Cell Biology Collection (Promochem, LGC, Molsheim, France). Cells were cultured in DMEMc at 37 °C under a 5% carbon dioxide humidified atmosphere.

2.4. Nanoparticle/cell contacts

For cellular uptake and cytotoxicity assays, cells were seeded in 96-well-plates (100,000 cells in 200 μL of medium per well) and were allowed to adhere for 4 h. NP were diluted in cell culture medium to reach the following final concentrations: 75, 750, 4500 and 9000 NP/cell (corresponding to 5, 50, 300 and 600 μg NP/mL respectively). NP were added to cells and further incubated for 20 h. Note that for the study of the NP distribution, it seems more relevant to express the NP dose as a number per cell, it seems more explicit, whereas for the cytotoxicity assessments the more relevant unit was μg/mL to ease comparison with literature studies.

2.5. Cellular uptake assessment

Uptake of FITC-labeled NP was quantified using a fluorometer (Ex: 485 nm, Em: 538 nm, Fluoroskan Ascent, Thermolabsystems, France). The total fluorescence of NP was first measured in each well, then the fluorescence of NP in supernatant (1), adsorbed to cell membrane (2) and internalized by cells (3) were discriminated by a "trypan blue (TB) quenching" as previously described [34–38]. TB is known for its ability to "turn off" the green fluorescence emitted by FITC allowing the discrimination of internalized NP from those adhering to plasma membrane [39]. Control wells without NP were used to assess the autofluorescence of cells in culture medium.

2.6. Cytotoxicity assays

2.6.1. Cell membrane integrity

The cellular release in the supernatant of cytoplasmic lactate dehydrogenase (LDH) was assessed using the CytoTox-96™ Homogeneous Membrane Integrity Assay (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions. The optical density of the samples was determined using a microplate reader (Multiskan RC; Thermolabsystems, Helsinki, Finland) set to 450 nm. The activity of the released LDH was reported to that of control cells (incubated