Influence of Serum Supplemented Cell Culture Medium on Colloidal Stability of Polymer Coated Iron Oxide and Polystyrene Nanoparticles With Impact on Cell Interactions *In Vitro*

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When nanoparticles interact with cells, the possible cellular responses to the particles depend on an array of parameters, in both particle and biological aspects. On the one hand, the physicochemical properties of the particles (e.g., material, size, shape, and surface charge) are known to play a key role in particle-cell interactions. On the other hand, it has been shown that prior to coming into contact with cells, nanoparticle interaction with the surrounding biological fluid may lead to a change of the initial particle properties. For example, the colloidal behavior of nanoparticles is strongly influenced by the density and viscosity of the surrounding media in both *in vitro* and *in vivo* systems.

In this study, we demonstrate how the surface charge and composition of different nanoparticles can impact upon their physicochemical characteristics, such as their colloidal stability, within a representative biological fluid and how the change of these parameters can significantly influence the subsequent cellular interaction *in vitro*. Therefore, we compared charged polymer coated superparamagnetic iron oxide nanoparticles to polystyrene nanoparticles of different surface charges. Particles of lower colloidal stability, namely positively charged superparamagnetic iron oxide nanoparticles, and the polystyrene nanoparticles, showed a higher cell-penetration *in vitro* than the colloidally stable particles.

I. Introduction

WITH the rapidly progressing development of nano-based materials and applications in the past decade [1], the interactions of nanoparticles (NPs) with biological systems has gained increased attention within the field of nanoscience [2]–[4].

Cellular responses to NPs depend on an array of different intrinsic properties of the particle in question (e.g., material, size, shape, and surface charge) [5]–[9]. The particle's surface charge (i.e., the functional surface groups) plays a key role in particle-cell interaction [10], [11] by direct interaction with the outer cell membrane [12]. In addition, the surface charge of NPs strongly impacts upon their colloidal behaviour [13].

Before particles interact with cells, their intrinsic physicochemical properties may change due to interaction with the proteins (e.g., blood serum proteins) and electrolytes present within the surrounding biological fluid [14], [15]. This possible change of the initial properties of the particle can lead to the aggregation of NPs, which may cause unexpected results in the assessment of *in vitro* and *in vivo* experiments [16], [17]. The possible higher sedimentation velocity of NP-aggregates within *in vitro* cultures for example can promote a higher concentration of NPs on the outer cell membrane, resulting in a mistaken observation of increased cell uptake [18], [19], since cell uptake is directly linked with the concentration of present particles [20]. Therefore, it is of highest importance to first understand how NPs interact with their surrounding biological media, before investigating and interpreting responses and effects of NPs at a cellular or *in vivo* level.

The use of superparamagnetic iron oxide nanoparticles (SPIONs) in both biomedical research and clinical applications has increased significantly in the last decade [21], [22] as they represent useful tools for magnetic separation purposes (e.g., rapid DNA sequencing) [23] and can be used as contrast enhancers in the field of magnetic resonance imaging [24]–[27]. Polystyrene NPs have demonstrated a low cytotoxicity profile and are widely used as biocompatible model drug carrier systems for *in vitro* and *in vivo* experiments [28]–[30].

The aim of the paper is to determine how different surface charges (i.e., different functional surface groups) and different particle composition (i.e., iron oxide versus polystyrene) may impact upon the intrinsic physicochemical properties of these NPs, such as their colloidal stability, within a representative biological fluid (i.e., fetal bovine serum supplemented cell culture medium) and how this may affect the subsequent cellular uptake *in vitro*.

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II. Experiment

A. Chemicals and Reagents

All chemicals were purchased from Sigma Aldrich, Switzerland unless otherwise stated and were of analytical reagent grade and used without further purification. Water refers in all synthesis steps to ultrapure deionized water of 18 $\Omega\mathrm{S}/\mathrm{cm}$ (Millipore AG, Switzerland). The serum was EU approved fetal bovine serum (FBS) originating from a singular batch (reference n°10270106, lot n°41G8582K, Invitrogen, Switzerland).

B. PVA Coated SPIONs

Superparamagnetic iron ixide nanoparticles (SPIONs) were prepared by alkaline coprecipitation of ferric and ferrous chlorides in aqueous solution as described previously [31]–[33] and characterized thoroughly by X-ray diffraction (XRD), surface area measurements (Brunner, Emmet, and Teller (BET) method) and magnetic measurements as previously described by Chastellain *et al.* [31]. The uncoated SPIONs were subsequently coated with polyvinyl alcohol (PVA), vinylalcohol/vinylamine copolymer or carboxylated PVA (i.e., PVA with randomly distributed carboxylic acid groups) in order to obtain different charged surfaces [11]. The PVA had an average molecular weight (MW) of 14 000 g/mol and a hydrolysis degree of 83% and was supplied by Omya AG, Switzerland (Mowiol 3-83). Vinylalcohol/Vinylamine copolymer, with an average MW of 80 000–140 000 was supplied by Erkol S.A, Spain (M12) and carboxyl-modified PVA was supplied by Kuraray Specialties Europe GmbH, Germany (KL506). Polymer solutions (10% w/v Mowiol 3-83, 2% w/v M12, and 6% w/v KL506) were added to the NP suspensions in a v/v ratio of one and the pH of the final suspensions was adjusted to seven using a 5% aqueous ammonia solution. The particles will be referred to as OH-PVA-SPIONs (i.e., regular PVA-SPIONs) and NH_2-PVA-SPIONs and COOH-PVA-SPIONs (i.e., functionalized PVA-SPIONs) in this work.

C. PS-NPs

Hydroxyl-, amino-, and carboxyl-modified polystyrene nanoparticles (PS-NPs) were purchased from Merck (Merck, Switzerland). Detailed characterization of the purchased nanoparticles has been carried out in-house by photon correlation spectroscopy (PCS). The particles will be referred to as OH-PS-NPs, NH_2-PS-NPs and COOH-PS-NPs in this work.

D. Iron Quantification

Uncoated SPIONs were quantified by (i) titration with potassium permanganate, as previously described by Skoog [34]. Polymer coated particles were then quantified using the Prussian blue colorimetric assay (H_2O served as the negative control) by dissolving the particles in 6 N HCl (Honeywell Burdick, and Jackson) (50 μL/well of a 96-well plate) for one hour. Subsequently, 50 μL of a freshly prepared 5% $K_4[Fe(CN)_6]$ (Merck, Switzerland) solution was added to each well. The absorbance was then determined at a wavelength of 690 nm using a multi-label plate reader ($Victor^3$, PerkinElmer, Switzerland) $(n = 3)$. The standard curve was established using previously analyzed uncoated SPIONs.

E. PS Quantification

Polystyrene shows an absorbance maximum at 259 nm when dissolved in an organic solvent. In addition, polystyrene solutions are also fluorescent. To determine the concentration of the NP suspension, the amount of PS present in the uncoated PS-NP suspension was measured following the method described by Muller and Schuber [35]. Briefly, PS-NPs were dissolved in Dimethoxyethane (DME, Sigma Aldrich, Switzerland) and fluorescence was measured after 10 min at 335 nm in a range of 2–10 μg (H_2O served as the negative control) in a 96-well quartz plate (TECAN, Switzerland). Each experiment was performed in triplicate $(n = 3)$.

F. Particle Characterization by Photon Correlation Spectroscopy (PCS)

The physicochemical parameters of the different uncoated and coated NPs, specifically the size and zeta potential, were assessed via light-scattering measurements at 90° by PCS equipped with a BI-9000AT digital autocorrelator (Brookhaven Instruments Cooperation, LABORCHEMIE GmbH, Austria). The CONTIN method was used for data processing. The NP suspensions were diluted in 20 mM borate buffer (pH 7.5) to perform the measurements. Viscosity, refractive index and dielectric constant of pure water were used to characterize the solvent.

G. Cell Culture

Human cervix carcinoma cells (HeLa cells) were purchased from HPA Culture Collections UK and cultured in at 75 cm^2 cell culture flask at 37°C and 5% CO_2. Dulbecco's Modified Eagle Medium 1× (DMEM phenol red free, Invitrogen, Switzerland) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin (Invitrogen, Switzerland) was used throughout all the experiments. At 24 h prior to experimentation cells were detached using Trypsin-EDTA (Invitrogen, Switzerland) and their viability was determined via Trypan blue exclusion. In a 48 well-plate (Costar, Corning Incorporated, USA), HeLa cells were seeded at a density of 2.5×10^4 cells per well and were cultured at 37°C and 5% CO_2 for 24 h prior to NP exposure.

H. NP Exposure

Prior to NP exposure cells were washed with PBS. The investigated NPs were added at a concentration of 100 $\mu g/mL$ of iron or PS in 10% FBS supplemented cell culture medium and incubated for 1, 6, and 24 h at 37°C and 5% CO_2. Supplemented cell culture medium, in the absence of particles, acted as the negative control.

I. Cell Uptake Determination by Cellular Iron Quantification

The ability of each PVA-SPION type to enter HeLa cells after 1, 6, and 24 h of exposure at 100 μg iron/mL was determined using the Prussian Blue assay as previously described. Briefly, after exposure to PVA-SPIONs the cell layer was dissolved in 6 N HCl (100 μL/well of a 48-well plate) for 1 h, then 50 μL/well of a 5% aqueous solution of $K_4[Fe(CN)_6]$ (Merck, Switzerland) was added and the absorbance was measured at 690 nm using a multilabel plate reader ($Victor^3$, PerkinElmer, Switzerland) after 10 min. A standard curve of the differently coated SPIONs was recorded to quantify the mass of cell internalized iron. The iron content in cells not exposed to SPIONs was always below the detection limit of 1 ppm. Each experiment was performed in triplicate $(n = 3)$.

J. Lactate Dehydrogenase (LDH) Release

A Cytotoxicity Detection Kit (Roche Applied Science, Germany) was used to quantify the level of cytotoxicity exerted upon the HeLa cells after exposure to PVA-SPIONs. This calorimetric assay is based on the measurement of LDH activity, an enzyme released from the cytosol of damaged cells into the supernatant. Hela cells were cultured as described above. COOH-PVA-SPIONs, NH_2-PVA-SPIONs and OH-PVA-SPIONs were exposed to HeLa cells in 10% FBS supplemented cell culture

media at 100 μg/mL iron for 24 h at 37°C and 5% CO_2. The negative control was 10% FBS-supplemented cell culture media alone. The detergent Triton X-100 acted as the positive control at a concentration of 0.2% in PBS. Each exposure was repeated a total of four times (n = 4). Supernatants were measured in triplicate at 490 nm (with a reference of 630 nm) using a multi-plate spectrometer (Benchmark Plus, Bio-Rad, Switzerland). Total extracellular LDH was then measured according to the manufacturer's manual.

K. Cell Uptake Determination by Fluorescence Spectroscopy

HeLa cell uptake of the different PS-NPs was determined using the method described above. Briefly, after exposure of 100 μg PS/mL, for 1, 6, and 24 h, the cell layer was mechanically destroyed by repeated pipetting (after the addition of 100 μL water/well in a 48-well plate). Then an equal volume of Dimethoxyethane (DME, Sigma Aldrich, Switzerland) was added and fluorescence was measured after 10 min at 335 nm. Each experiment was performed in triplicate (n = 3).

L. Turbidity Measurements by UV/Vis-Spectroscopy

Turbidity measurements of all NPs were carried out by mixing the suspensions (100 μg/mL) with the cell culture medium in the presence of 10% FBS. After rapid homogenization by gently pipetting the suspension up and down, the turbidity was measured at 480 nm (PVA-SPIONs) and 556 nm (PS-NPs) over 24 h in special optical glass precision cells from Hellma Analytics (Hellma GmbH & Company KG). All results were normalized against supplemented cell culture medium alone and each experiment was performed in triplicates (n = 3).

III. Results and Discussion

A. Characterization of Nanoparticles

After synthesis, the uncoated SPIONs showed a mean diameter (d50) of 11.9 ± 3.7 nm as obtained by dynamic light scattering (DLS) and a positive zeta potential of 34.6 ± 1.6 mV (pH = 2). Subsequent polymer coating led to an increased d50 of 34.9 ± 7.2 nm and zeta potentials of -5.9 ± 0.9 mV, 29.4 ± 1.3 mV and 1.5 ± 2.3 mV (pH = 7) for COOH-PVA-SPIONs, NH_2-PVA-SPIONs and OH-PVA-SPIONs, respectively.

Hydroxyl-, amino- and carboxyl-modified polystyrene nanoparticles (PS-NPs) were purchased from Merck (Merck, Switzerland). DLS showed a d50 of 63.9 ± 7.0 nm, 83.3 ± 8.0 nm and 76.67 ± 4.6 nm and a zeta potential of -2.1 ± 3.9 mV, 8.8 ± 1.4 mV and 2.6 ± 4.1 mV (pH = 7) for COOH-PS-NPs, NH_2-PS-NPs and OH-PS-NPs, respectively.

Both particle types, i.e., PS-NPs and SPIONs, showed comparable sizes whereas their zeta potentials varied according to their functional surface groups (Table I).

All NPs were stable in ultra-pure water and phosphate buffer over months (data not shown).

B. Nanoparticle-Cell Interactions

To study the importance of surface charges and consequently the impact of functional surface groups and composition of the NPs on the subsequent cellular interaction, HeLa cells were exposed in cell culture medium supplemented with 10% FBS to 100 μg/mL of iron (for the different PVA coated iron oxide nanoparticles) or 100 μg/mL of PS (in case of the PS-NPs) for 1, 6, and 24 h. All data is presented in quantities of mass of iron/mL suspension (instead of polymer coated SPIONs) to allow comparison with the common literature. The uptake of the particles was measured by quantification of the intracellular iron and PS content by assessing the Prussian Blue reaction for SPIONs (Fig. 1) and the described fluorescence assay for PS-NPs (Fig. 2). The reliability of the Prussian blue reaction for the quantification of intracellular iron *in vitro* has been reported previously [36]. The intracellular iron content of cells not exposed to any of the studied PVA-SPIONs was constantly below the detection limit of one ppm. The fluorescence assay was adapted from Muller and Schuber, who developed a sensitive method for the quantification of polystyrene latex beads by measuring the intrinsic fluorescence of polystyrene dissolved in organic solvents [35].

Figs. 1 and 2 show that cell uptake of NH_2-functionalized PVA-SPIONs (i.e., positively charged PVA-SPIONs) by HeLa cells was higher than the cell uptake of OH- or COOH-PVA-SPIONs (i.e., neutral and negatively charged PVA-SPIONs). Furthermore, cell uptake of NH_2-PVA-SPIONs increased over

TABLE I
Mean Particle Size and Zeta Potential (pH = 7) for PVA-SPIONs and PS-NPs

	COOH-PVA-SPIONs	OH-PVA-SPIONs	NH_2-PVA-SPIONs	COOH-PS-NPs	OH-PS-NPs	NH_2-PS-NPs
Size, d50 [nm]	38.1 ±2.5	28.3 ±2.1	39.1 ±2.2	63.9 ±7.0	76.7 ±4.6	83.3 ±8.0
Zeta potential [mV]	-5.9 ±0.9	1.5 ±0.3	29.4 ±1.5	-2.1 ±3.9	2.6 ±4.1	8.8 ±1.4

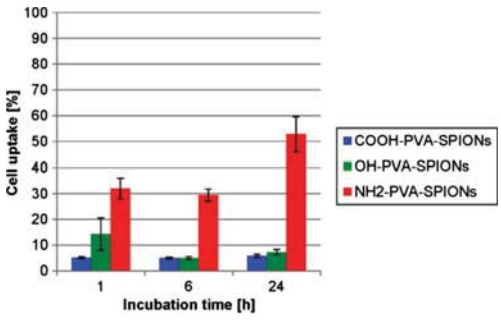

Fig. 1. Cell uptake of the different investigated PVA-SPIONs. The amount of cell uptake is shown as % of total added iron as a function of incubation time after NP exposure at a concentration of 100 μg iron/mL to HeLa cells in 10% FBS supplemented cell culture medium (n = 3, Error bars = STDEV).

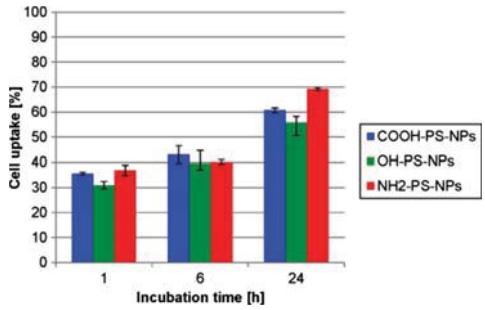

Fig. 2. Cell uptake of the different investigated PS-NPs. The amount of cell uptake is shown as % of total added PS as a function of incubation time after NP exposure at a concentration of 100 μg PS/mL HeLa cells in 10% FBS supplemented cell culture medium (n = 3, Error bars = STDEV).

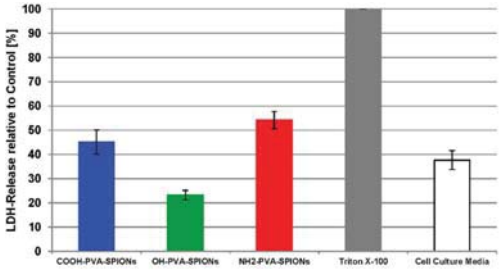

Fig. 3. Release of lactate dehydrogenase (LDH) in % relative to Triton X-100. COOH-PVA-SPIONs, OH-PVA-SPIONs and NH_2-PVA-SPIONs were exposed to HeLa cells at 100 μg/mL iron in 10% FBS supplemented cell culture media for 24 h. Triton X-100 acted as the positive control at a concentration of 0.2% in PBS. The negative control was 10% FBS supplemented cell culture media only (n = 4; Error Bars = SEM).

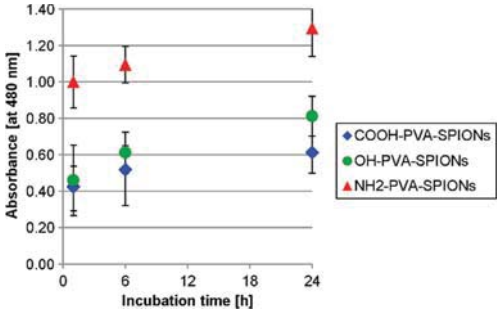

Fig. 4. Stability of all investigated PVA-SPIONs. The stability of NPs is presented as UV/Vis absorbance at 480 nm as a function of incubation time after NP incubation of 100 μg iron/mL in 10% FBS supplemented cell culture medium (n = 3, Error bars = STDEV).

time, whereas cell uptake stayed constantly low for OH- and COOH-PVA-SPIONs. Compared to this, a relatively high cell uptake was observed for all PS-NP types within the first hour and the internalization of these NPs increased equally over time.

It has been previously reported that uncoated iron oxide NPs could have toxic effects on cells [37]. However, the outer polymer coating layer covering the SPIONs has a greater influence on the cytotoxicity of polymer coated NPs than the core SPIONs themselves [38], [39]. In previous studies, we have already investigated the cytotoxicity of PVA-SPIONs using the MTT assay [40]. No cytotoxicity in HeLa cells was observed for the PVA-SPIONs over a 24 h period up to 100 μg iron/mL using the LDH assay and measuring the release of the cytosolic enzyme lactate dehydrogenase (Fig. 3)

C. Turbidity Measurements by UV/Vis-Spectroscopy

The colloidal behavior of NPs suspended in biological fluids i.e., the size of potential aggregates, impacts significantly upon any cellular and biological effects both *in vitro* and *in vivo* [14], [15], [41], [42].

In this study, the agglomeration behavior of the NPs was estimated by turbidity measurements (Figs. 3 and 4) which, in contrast to the exact determination of particle sizes, is straightforward and easily reproducible [43].

Fig. 3 shows that compared to the OH- and COOH-PVA-SPIONs only the NH_2-functionalized PVA-SPIONs tend to aggregate in serum-supplemented cell culture medium and that aggregation progresses over time. Fig. 4 shows that in the tested biological fluid all three investigated PS-NP aggregated within the first hour and aggregation remained constant over a 24 h period. All investigated NPs were stable in water for more than a

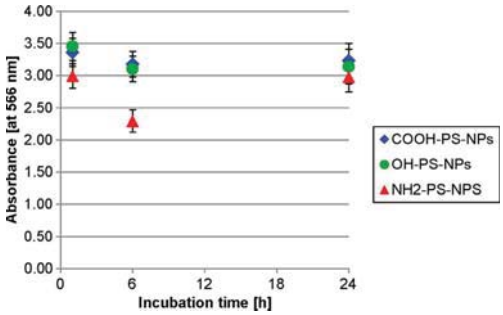

Fig. 5. Stability of all investigated PS-NPs. The stability of NPs is presented as UV/Vis absorbance at 566 nm as a function of incubation time after NP incubation of 100 μg PS/mL in 10% FBS supplemented cell culture medium (n = 3, Error bars = STDEV).

month at pH 2 and pH 7 without showing any signs of agglomeration (data not shown).

The observation that colloidally less stable NPs showed a higher cell uptake compared to colloidally stable NPs could be due to the possibility that larger NPs or aggregates elicit a higher sedimentation velocity within *in vitro* cultures and thus lead to a higher particle concentration on the cell surface, resulting in a misrepresentative increased cell uptake [18], [19], since cell uptake is directly linked to the concentration of present particles [20].

The preferred uptake of aggregated NPs might also be explained by the specific uptake mechanism of HeLa cells [44]. Although epithelial cells are known to have a rather low phagocytic rate compared to other cells (i.e., phagocytic cells like macrophages), Stone *et al.* showed that epithelial cells can use this active mechanism for uptake of NPs [45]. Investigating the impact of size of receptor-targeting transferrin-coated gold NP aggregates on their cellular uptake and subsequent biological impact on epithelial cells (i.e., HeLa and A549 cells) and carcinoma cells (i.e., MDA-MB-435 cells), Albanese and Chan showed that, depending on the type of cell, an array of cellular responses to NP aggregates are possible [46]. It is well recognized that the cellular responses to materials at the nanoscale generally depend on the cell type, a phenomenon which is usually described as cell "Vision" [47].

IV. Conclusion

In this study, we compared the impact of different surface charges (i.e., different functional surface groups) and different particle compositions (i.e., iron oxide versus polystyrene) on colloidal stability and subsequent cellular interaction *in vitro*. After exposure to HeLa cells, in the presence of serum-supplemented cell culture medium, cell uptake of positively charged PVA-SPIONs (i.e., PVA-SPIONs bearing NH_2-groups on the surface) was significantly faster and higher compared to both neutral and negatively charged PVA-SPIONs (i.e., PVA-SPIONs bearing OH- and COOH-groups on the surface). PS-NPs showed a comparably high cell uptake independent of their surface charge. Turbidity measurements showed that positively charged PVA-SPIONs in turn showed a lower colloidal stability than neutral and negatively charged PVA-SPIONs. For PS-NPs, a generally low colloidal stability was observed. Here we showed that cell uptake can be independent of NP-composition (i.e., core and/or coating material), and that it strongly depends on the physicochemical changes that NPs

may undergo in biological fluids. The possible alteration of the physicochemical characteristics of the NPs in turn impacts upon their colloidal stability and consequently on their cellular uptake.

In-depth light scattering and stereological investigations will reveal the size and polydispersity of the occurring aggregates in supplemented cell culture medium and inside the cells. This will help understand the impact of aggregation on subsequent cellular uptake in a quantitative way. Cho *et al.*, studied the sedimentation/diffusion velocity ratio, with respect to enhanced cellular uptake, and defined a critical ratio of three [19]. However, due to the comparably low density of the NPs used in this study, sedimentation cannot be the only reason to explain the much-increased cellular uptake. Consequently, colloidal properties should be investigated in more detail to better correlate cellular and material data.

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