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ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 68 (2008) 129–137
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Effect of cell media on polymer coated superparamagnetic iron oxide nanoparticles (SPIONs): Colloidal stability, cytotoxicity, and cellular uptake studies

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Received 15 November 2006; accepted in revised form 2 February 2007
Available online 13 July 2007

Abstract

The influence of the composition of the polymer coated polyvinyl alcohol (PVA), vinyl alcohol/vinyl amine copolymer (A-PVA) and polyethylenimine (PEI) coated superparamagnetic iron oxide nanoparticles (SPIONs) on the colloidal stability, cytotoxicity and cellular uptake of these particles in different cell media is reported in this paper. Although all examined polymer coated SPIONs were stable in water and PBS buffer these colloidal systems had different stabilities in DMEM or RPMI media without and supplemented with fetal calf serum (FCS). We found that A-PVA coating onto the surface of the SPIONs decreased the cytotoxicity of the polymer compared to the same concentration of A-PVA alone. As well, polyplexes of PEI-SPIONs with DNA in concentration used for transfection experiments showed no cytotoxicity compared to PEI and PEI-SPIONs. Our data show that the choice of medium largely influences the uptake of these particles by HeLa cells. The optimal medium is different for the different examined polymer coated SPIONs and it should be determined in each case, individually.

Keywords: Superparamagnetism; Iron oxide; Nanoparticles; PVA; PEI; Colloidal stability; Cytotoxicity

1. Introduction

The synthesis and characterization of nanoparticles have been a focus of intensive research for more than 10 years since they play an important role in electronics, catalysis, biology and medicine. Nanoparticles that can be biochemically functionalized are potential medical device systems that can be used in many different biological and medical fields of application. Beside the unique physical properties induced by surface or quantum effects, the size of the primary particles is with 2–30 nm comparable to the size of biological building blocks and allows investigation of the cellular functioning or direct interaction with biological targets. Well described examples are inorganic photoluminescent particles (quantum dots) as markers or nanosized superparamagnetic iron oxide particles. Superparamagnetic particles with a diameter of around 10 nm have been used for many years for e.g. non-viral gene delivery, as MRI contrast agents, or for typical separation applications [1]. Those applications are relatively well established on the market, whereas targeted particle delivery is still in the development stage. It has been shown that magnetic particles are physiologically well tolerated and that the surface

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doi:10.1016/j.ejpb.2007.02.024

of the particles is responsible for the biocompatibility and stability to the reticulo-endothelial system [2]. On the other hand, concerns have been raised about nanoparticle-derived adverse health effects and scientists currently aim at developing a rational, science-based approach to nanotoxicology. Recent investigations were focused on relatively simple cytotoxic tests as readily available prescreening methods, on the effect of inhaled or instilled ambient nanoparticles that can induce oxidative stress or pulmonary inflammation, or on possible consequences of particle related dysfunction of the cardio-vascular system [3]. A key question is the assessment of nanoparticles' potential toxicity due to their nanosize nature whereas the type of particle does not seem to play an important role [4,5]. The surface properties of the particles are particularly investigated in more detail since the surface seems to be the determining factor for cell uptake and cytotoxicity.

Many applications and investigations use nanoparticles in colloidal suspensions. By tailoring interactions between colloidal particles, one can design stable fluids, gels, or colloidal crystals. Long range, attractive van der Waals forces are ubiquitous and must be balanced by Coulombic, steric, or other repulsive interactions to engineer the desired degree of colloidal stability [6]. The colloidal behaviour of nanoparticles in different cell media or body fluids is almost never considered or related to particle–cell interactions. Williams et al. investigated the impact of silica, silica/iron oxide, and gold nanoparticles on the growth and activity of *Escherichia coli* and correlated the results with dynamic light scattering experiments that were performed in growth media [7]. It has been shown that submicron polymeric particles coagulate in the cell medium, whereas colloidal stability in aqueous solution was ensured for several weeks [8].

The aim of this work is to show in detail the influence of the composition of the polymer coating and surface charge of nanoparticles on the colloidal stability of these particles in different cell media. Furthermore, we tried to establish correlations between cytotoxicity or uptake rates and agglomeration behaviour of the particles. This correlation in particular is very important for the design of simplified toxicity tests and for the further development of such particles for *in vivo* applications.

2. Materials and methods

2.1. Materials

All chemicals were of analytical reagent grade and were used without further purification. Polyvinyl alcohol PVA (Mowiol® 3-83) with an average molecular weight (MW) of 14,000 g/mol and a hydrolysis degree of 83% was supplied by courtesy of CLARIANT. Vinyl alcohol/vinyl amine copolymer M12, with an average MW of 80,000–140,000 was supplied by courtesy of ERKOL.

Polymer solutions were prepared by dissolving the powders in water followed by rapidly heating the solutions for 15 min (Mowiol® 3-83) to 4 h (M12) at 90 °C, and filtering the hot solutions over paper filters (Schleicher & Schuell AG). Ultra-pure deionized water (Seralpur delta UV/UF setting, 0.055 μS/cm) was used in all synthesis steps. D-9527 Sigma cellulose membrane dialysis tubing with a molecular weight cut-off at 12,000 was used for dialysis.

2.2. Iron oxide nanoparticles

Superparamagnetic iron oxide nanoparticles (SPIONs) were prepared by alkaline co-precipitation of ferric and ferrous chlorides in aqueous solution as described elsewhere [9–11]. The obtained brown suspension was dialyzed against 0.01 M nitric acid for two days, and stored at 4 °C.

2.3. Polymer coated particles

In order to obtain SPIONs coated with either polyvinyl alcohol (Mowiol® 3-83) or with a mixture of polyvinyl alcohol (Mowiol® 3-83) and vinyl alcohol/vinyl amine copolymer (M12, vinyl alcohol/vinyl alcohol copolymer mass ratio = 45) the nanoparticle dispersion was mixed at various ratios with the different polymer solutions. The products will be referred to as PVA-SPION and A-PVA-SPION in this work.

For PEI coating the iron oxide nanoparticles were mixed at a PEI:Fe mass ratio of two ($R = 2$) with 25 kDa polyethylenimine (Aldrich). The samples will be referred to as PEI-SPION in this work. DNA-PEI-SPIONs were prepared at a N/P ratio (ratio of nitrogen-containing groups of the polymer to phosphate groups of the nucleic acid) of 7.5, assuming the DNA was entirely complexed. The nitrogen content of PEI has been measured and calculated according to Harpe et al. [12], and the phosphate content was calculated from the size of the plasmid (4.7 kb).

The iron content of the suspensions was determined by redox-titration, essentially as described [13].

2.4. Transmission electron microscopy

Transmission electron microscopy (TEM) was performed using a Phillips CM-20 microscope operating at 200 kV. For sample preparation, dilute drops of suspensions were allowed to dry slowly on carbon-coated copper grids.

2.5. Colloidal stability

The colloidal stability of coated particles in different environments was investigated by turbidity measurements. Therefore, the particle dispersions were mixed with commonly used cell media such as RPMI and DMEM, both in presence and absence of 10% fetal calf serum (FCS) thereby setting the iron concentration to 100 μg/ml. After rapid homogenization, the turbidity was measured by light absorption at a wavelength of 500 nm as a function of time (t) [14].

2.6. Particle size

Light scattering measurements were carried out at 90° on a photon correlation spectrometer (PCS) from Brookhaven equipped with a BI-9000AT digital autocorrelator. The CONTIN method was used for data processing. The concentration of iron oxide nanoparticles was set to 100 μg iron/ml for all measurements. The theoretical refractive index of 2.42 of magnetite [15] was used to calculate the number weighted distribution from the raw intensity weighted data. Viscosity, refractive index and dielectric constant of pure water were used to characterize the solvent.

2.7. Cell culture and treatments

HeLa (human cervix carcinoma cells) cells were grown in RPMI medium (Gibco-BRL), supplemented with 10% fetal calf serum (FCS; Promochem) and 1% penicillin/streptomycin. One day prior to experiments, the cells were detached in trypsin–EDTA (Gibco-BRL) and grown in complete medium in 48-well plates (Costar) at $\sim 10^4$ cells per well. On the day of experiment, cells were washed with PBS and medium was changed. The dilutions of PVA-SPION, A-PVA-SPION, PEI-SPION and DNA-PEI-SPION were added for the concentration, time and temperature indicated. After 22 h of incubation, the MTT assay was performed to determine cell viability.

2.8. Evaluation of cell viability

MTT reduction was used to quantify metabolically active cells. Briefly, medium was removed and cells were exposed to 1 mg/ml MTT (3,4,5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium, Sigma in PBS for 2 h at 37 °C. After incubation, the cells were examined under an inverted microscope to ascertain the density of violet spots corresponding to active mitochondria in order to exclude a potential mitochondrial toxicity of the compounds. The supernatant was then removed and the precipitated formazan was dissolved in isopropanol and quantified at 560 nm in a multiwell plate reader (Saphire^2, Tecan). Each experiment was repeated in triplicate wells at least three times. Means and standard deviations were calculated.

2.9. Iron determination

The cell layer was dissolved in 6 N HCl (125 μl/well of a 48-well plate) for 1 h, then 125 μl of a 5% solution of $K_4[Fe(CN)_6]$ (Merck) in H_2O was added and the absorbance was read after 10 min at 690 nm in a multiwell plate reader (Saphire^2, Tecan). A standard curve using the differently coated iron oxide nanoparticles was recorded in the same conditions to quantify the amount of cell-bound iron. Each experiment was repeated in triplicate wells at least three times. Means and standard deviations were calculated. It is important to note that the different particles were incubated in the corresponding media for 1 h prior to cell exposure.

3. Results

3.1. Transmission electron microscopy

Transmission electron microscopy (TEM) was carried out with individual and coated particles (Fig. 1). Primary uncoated SPIONs showed ellipsoidal iron oxide particles with an average size of 9 nm (Fig. 1a). Two perpendicular diameters were measured for 100 particles, and average axe lengths of 10 ± 2 nm and 8 ± 2 nm were determined. An aspect ratio of 1.2 ± 0.2 was determined, corresponding to the results obtained by other groups [15]. PVA-coated iron oxide particles (PVA-SPION) were also observed by means of transmission electron microscopy (Fig. 1b). Although the polymer was responsible for image blurring (due to film formation) it seemed that the particle spatial distribution was more homogeneous and that the minimum distance between the particles was larger compared to the uncoated particles for equivalent concentrations. Iron oxide particles embedded in a PEI polymer matrix (PEI-SPION) are shown in Fig. 1c. Each nanoparticle was associated with more than one strand of the PEI and, likewise, each strand of PEI attached to more than one nanoparticle, resulting in a bridging aggregation. An average bead size of 27 ± 12 nm was obtained for sample $R = 2$ (PEI:Fe mass ratio of 2) using 25 kDa PEI by measuring 360 beads by transmission electron microscopy. Comparable PEI iron oxide nanobeads using higher molecular weight (800 kDa) PEI showed an average size of 200 nm [16].

3.2. Turbidity measurements and photon correlation spectroscopy (PCS)

The agglomeration of the different polymer coated nanoparticles (PVA-SPION, A-PVA-SPION, and PEI-SPION) was examined by turbidity measurement and photon correlation spectroscopy (PCS). Uncoated SPIONs agglomerated in PBS and biological fluids immediately (data not shown). All investigated polymer coated SPIONs were absolutely stable in water and in PBS for months and over a pH range of 3–11 without showing agglomeration. A-PVA-SPIONs and PVA-SPIONs showed no important agglomeration in none of the investigated media after 30 min as measured by PCS. However, a different behaviour of the various examined polymer coated nanoparticles with similar primary size in different media with respect to their colloidal stability and hence their size was observed after 1 h. PVA-SPIONs showed high colloidal stability in FCS supplemented media with respect to their size (Fig. 2a, PVA-SPIONs). In both FCS supplemented media, we could not observe an increase in size within five days (maximum time period observed) and the dispersions remained transparent. Agglomeration of the particles in

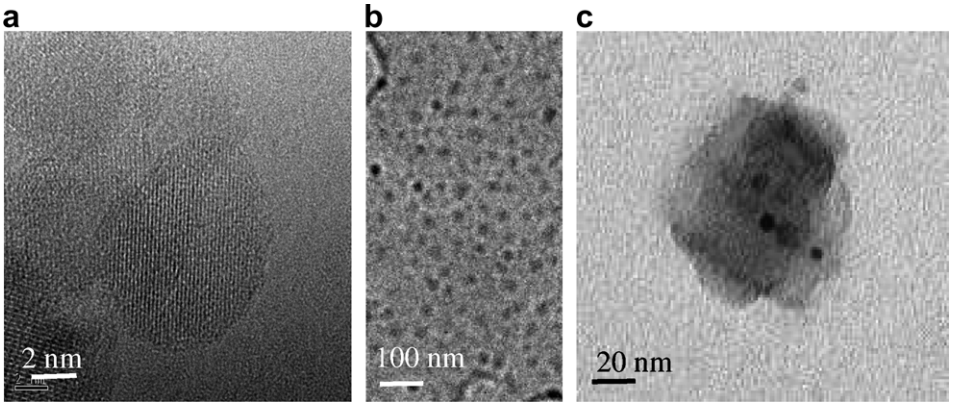

Fig. 1. (a) High resolution transmission electron micrograph showing slightly faceted crystalline particles in the 10 nm range. (b) Bright field TEM picture showing a homogeneous spatial distribution of the PVA coated iron oxide particles. (c) Bright field transmission electron micrograph showing one PEI-SPION bead.

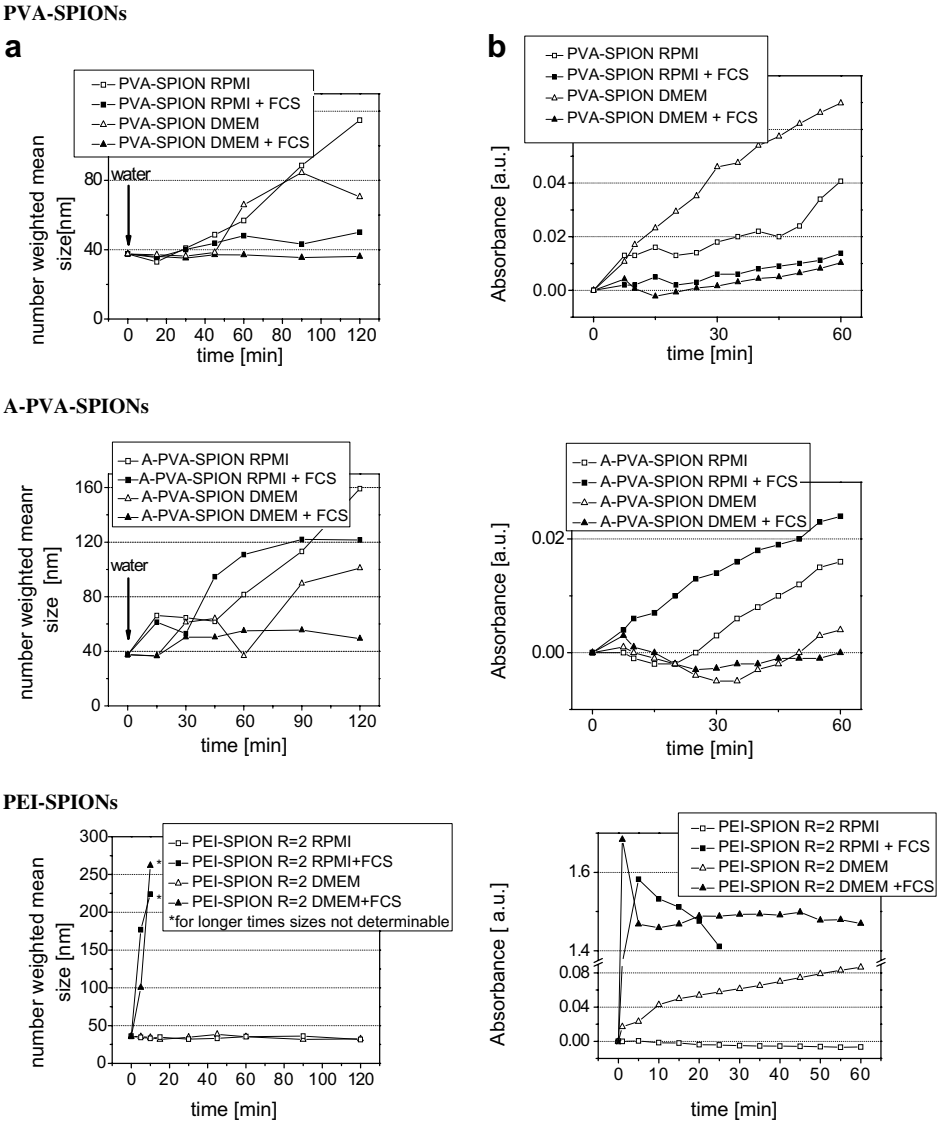

Fig. 2. Stability of PVA-SPIONs, A-PVA-SPIONs, and PEI-SPIONs in different cell media (RPMI, RPMI+FCS, DMEM, and DMEM+FCS) monitored by (a) PCS measurements and (b) UV measurements. The error is not indicated to simplify matters.

DMEM and RPMI without FCS was fast and agglomerate mean sizes of over 80 nm were observed after 120 min. The turbidity measurements were in good agreement with these observations. The agglomeration rate in media without FCS was higher than in FCS supplemented media (Fig. 2b; PVA-SPIONs). A-PVA-SPION nanoparticles were relatively stable in DMEM with and without FCS and comparatively less stable in RPMI with and without FCS as obtained from turbidity measurements over 1 h (Fig. 2a; A-PVA-SPIONs). This was confirmed by PCS measurements (Fig. 2b; A-PVA-SPIONs). The size of the A-PVA-SPIONs in DMEM with and without FCS was not increasing significantly within the first hour and measured sizes around 40 nm were obtained. Agglomeration of the A-PVA-SPIONs was observed in DMEM after 1 h and the diameter increased to 110 nm after 2 h, whereas the particles in DMEM supplemented with FCS kept their initial size up to nine days (maximum time observed).

In summary, we could not observe that the presence of serum in the medium induced agglomeration of the PVA-SPIONs and A-PVA-SPIONs. It seems that the combination of cell media and serum determines the colloidal stability. The agglomeration behaviour of PEI-SPIONs in these four cell media differed considerably from the results described above (Fig. 2a and b; PEI-SPIONs). PEI-SPION beads agglomerated immediately in the presence of fetal calf serum whereas they remained stable in all media without FCS and no change in the turbidity and bead size could be observed within 2 h. However, the particles interacted differently with the media without FCS. In DMEM, high turbidity and larger particle sizes (70 nm) of PEI-SPIONs were observed after 24 h, whereas in RPMI the initial particle size remained unchanged during 2 days.

3.3. Biological evaluations

We assessed cytotoxicity of PVA-, A-PVA-, PEI-, and DNA-PEI-SPIONs after 24 h exposure to HeLa cells in RPMI and DMEM, both in presence and absence of 10% FCS. Cytotoxic effects of the polymer coated SPIONS were always compared to cytotoxic effects of the pure polymer solutions (Fig. 3). Four different iron concentrations (20, 40, 80, and 120 μg/ml) were assayed and the polymer/iron ratio was maintained in all tests. The results are shown relative to the cell viability in DMEM+FCS, since optimal growth of mammalian cells in tissue culture is routinely obtained with medium supplemented with whole serum. The culturing of HeLa cells was reported in the literature with different media, such as RPMI 1640 [17,18] and DMEM [19] all supplemented with 10% serum. PVA-SPIONs, as well as PVA alone, showed no influence on cell viability of HeLa cells at the incubated concentrations ranging from 200 to 1200 μg/ml (results not shown). In all investigated cases, A-PVA-coated SPIONs were far less toxic compared to the equal amount of the copolymer in solution. Note that A-PVA is actually a mixture of polyvinyl alcohol (Mowiol® 3-83) and vinyl alcohol/vinyl amine

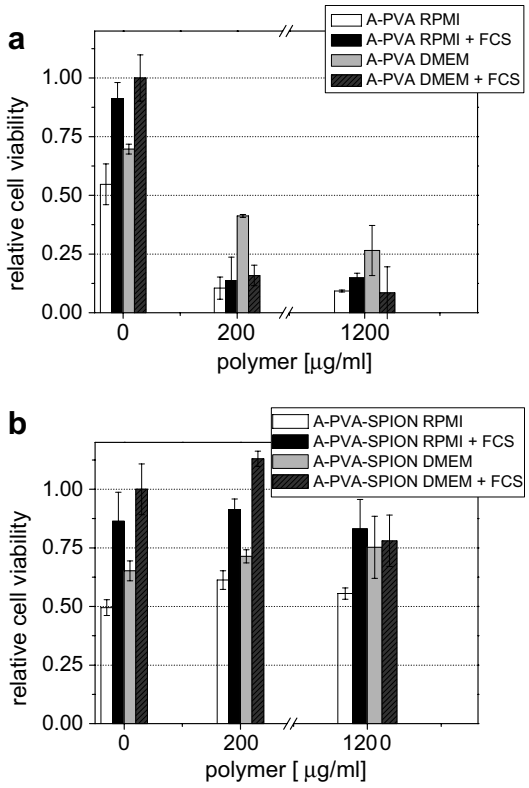

Fig. 3. (a) Relative cell viability of HeLa cells exposed to differently concentrated (a) aqueous solutions of A-PVA in different cell media (RPMI, RPMI+FCS, DMEM, and DMEM+FCS) and (b) dispersions of A-PVA-SPIONs in different cell media (RPMI, RPMI+FCS, DMEM, and DMEM+FCS).

copolymer (M12, vinyl alcohol/vinyl alcohol copolymer) at a mass ratio of 45. Low concentration of A-PVA-SPIONs (20 μg Fe/ml) had no influence on cell viability in any of the media, whereas high concentrations (120 μg Fe/ml) showed only pronounced cytotoxicity in DMEM containing FCS. Fig. 4 shows the influence of PEI-SPIONs and DNA-PEI-SPIONs on cell viability at 24 h exposure to HeLa cells in RPMI and DMEM, both in presence and absence of 10% FCS. PEI has been found to be a versatile polymeric vector for gene delivery that tightly condenses plasmid DNA [20]. Although the ability to deliver DNA to the nucleus enhances transgene expression, the presence of PEI in the nucleus may interfere with transcriptional and translational processes and induce cell death [21,22]. Efforts have been undertaken to increase transfection efficiency but at the same time decrease adverse effects of PEI on cell viability [23]. Therefore, comparatively lower polymer concentrations were investigated here. The investigated concentration range as well as the used N/P ratio of 7.5 (molar ratio of nitrogen-containing groups of the polymer to phosphate groups of the nucleic acid) corresponded to the values needed for optimal magnetically enhanced non-viral transfection in HeLa cells [24]. PEI-SPIONs showed significant toxicity at concentrations around 500 ng PEI/ml dispersion. A relation between cell viability and medium in the presence or absence of

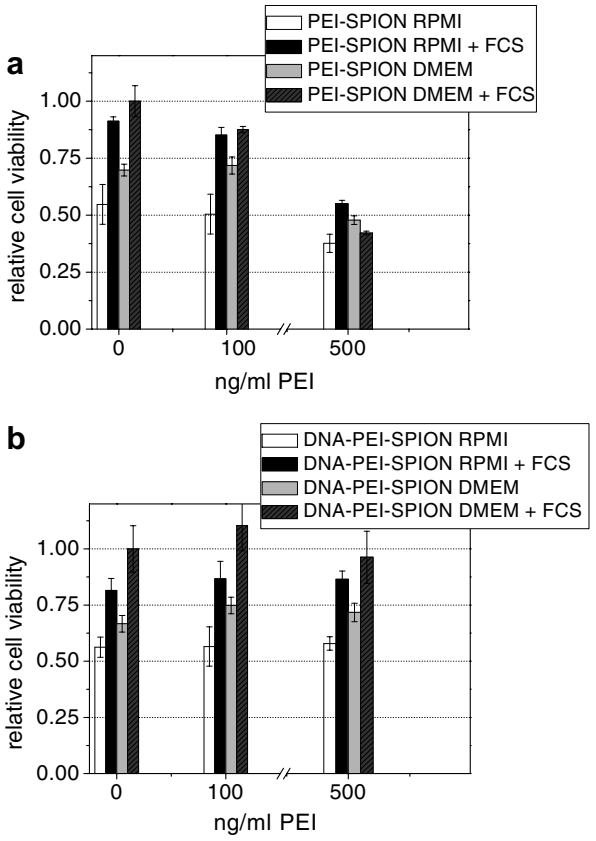

Fig. 4. (a) Relative cell viability of HeLa cells exposed to differently concentrated dispersions of (a) PEI-SPIONs in different cell media (RPMI, RPMI+FCS, DMEM, and DMEM+FCS) and (b) DNA-PEI-SPIONs in different cell media (RPMI, RPMI+FCS, DMEM, and DMEM+FCS).

serum was not observed. It is interesting to note that increasing concentrations of DNA-PEI-SPIONs had almost no effect on cell viability.

In a second test series we determined if HeLa cells could interact with PVA-, A-PVA-, PEI- or DNA-PEI-SPIONS. Using the Prussian Blue reaction, the cellular iron content was measured in DMEM and RPMI, both with and without FCS, at four different particle concentrations after 1, 3, and 24 h exposure to the differently coated nanoparticles. Earlier studies with dextran and silica coated iron oxide particles had confirmed the reliability of the Prussian blue reaction in cell cultures [25]. The detection limit of this reaction was reported to be around one ppm [26]. The iron content was always below detection limits in cells not exposed to nanoparticles.

The uptake of A-PVA- and PVA-coated SPIONs (120 μg Fe/ml) after 1 and 3 h in different cell media was investigated (Fig. 5). The cellular iron content increased over time and was dependent on the polymer used to coat these nanoparticles. Earlier studies have shown that uptake was also dependent on the amount of incubated particles [27]. A-PVA-SPIONs were better absorbed by cells than PVA-coated SPIONs. After 24 h of incubation, this effect becomes more obvious, when the amount of cell bound

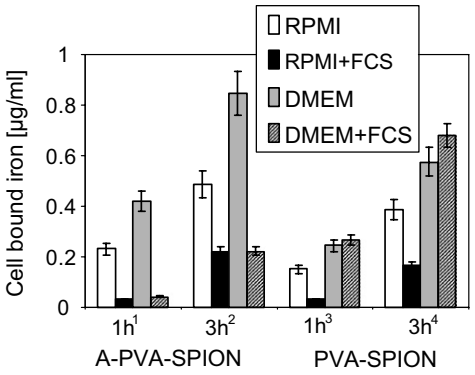

Fig. 5. Uptake of A-PVA- and PVA-coated SPIONs (120 μg Fe/ml) after 1 and 3 h in different cell media: RPMI, RPMI+FCS, DMEM, and DMEM+FCS.

iron increased up to 12 μg/ml in the case of A-PVA-SPIONs, whereas only a very low increase in iron content could be detected for PVA-SPIONs (data not shown). This observation was not surprising, since the preferential uptake of cationic agents has been widely used in the field of molecular biology, mainly for transfection purposes. The uptake of A-PVA-SPIONS after 1 and 3 h of exposure was strongly dependent on the cell medium, since uptake was only detected in the absence of serum. This effect is less pronounced for PVA-SPIONs, where merely a negative influence of RPMI with FCS can be detected.

4. Discussion

4.1. Turbidity measurements and photon correlation spectroscopy (PCS)

The study of particle aggregation is important since the aggregation of SPIONs reduces the superparamagnetic properties of the nanoparticles as magnetic interactions may be present between the particles during the agglomerate formation [28]. In addition, the size of the aggregates may influence the biological function of the particles, in *in vitro* [29,30] and in *in vivo* applications [31]. Although the exact determination of particle sizes by turbidity measurements is difficult [32], the agglomeration behaviour of the particles is straightforward, more sensitive and easily reproduced with turbidity measurements. This is advantageous as most biological laboratories do not have precise size measurement equipment but usually have access to UV/VIS spectrometers.

In our study we found that A-PVA-SPIONs and PVA-SPIONs showed no important agglomeration in none of the investigated media after 30 min. Therefore, biological evaluations can only neglect the influence of the media on colloidal stability when working in this time frame. At least in the case of HeLa cells, 30 min of incubation at the investigated concentrations is not sufficient to achieve considerable uptake. This can be overcome by actually exploiting the superparamagnetic properties of

the core iron oxide nanoparticles. In this case, uptake can be significantly enhanced in the presence of a magnetic field [24]. After 1 h of incubation significant differences between A-PVA-SPIONs and PVA-SPIONs were detected in depending on the medium. The high stability of PVA-SPIONs in FCS supplemented media could be explained by depletion stabilization caused by the serum proteins showing a similar size as the nanoparticles. Increased stability of A-PVA-SPIONs in FCS supplemented DMEM (after 1 h) is attributed to a similar mechanism proposing the additional adsorption of small negatively charged molecules at the surface of the particles leading to a neutral surface charge as observed with PVA-SPIONs. In the case of RPMI no such preferred adsorption could take place and the adsorption of FCS on the particles could be possible, since the surface of proteins is heterogeneous with regard to charged and hydrophobic domains [29]. If one or more FCS proteins are attached to the particles, no depletion stabilization is possible and agglomeration occurs. However, the amine content is low (compared to e.g. PEI) and this ''medium'' effect is overcome by the intrinsic particle properties after 2 h. The results between the two systems were comparable already after 2 h. Only DMEM with FCS offered long term colloidal stability for both systems. The medium and the surface of the particles have to be evaluated fundamentally using statistical methods to gain an insight into the impact and participation of both effects. The agglomeration behaviour of PEI-SPIONs was consistent with previous reports that showed that PEI interacts unclearly with negatively charged molecules in the serum as well as plasma proteins such as opsonins [33]. The difference in agglomeration behaviour in the different media is hard to explain. The two media RPMI 1640 and DMEM vary in their glucose content which is in RPMI 1640, 2000 mg/ml, and in DMEM, 4500 mg/ml. Apart from differences in glucose content the main differences lie in the presence/absence of e.g. sodium pyruvate pyridoxine, L-arginine, L-proline and L-histidine. However, these variations could not explain the different agglomeration behaviour.

4.2. Biological evaluations

It was previously reported that uncoated iron oxide nanoparticles could have toxic effects on cells [34]. However, the cytotoxicity of polymer coated nanoparticles is rather influenced by the outer polymer coating layer covering the SPIONs than by the iron oxide nanoparticles themselves [4,5]. We found that PVA-SPIONs were not cytotoxic for HeLa cells (results not shown). In general, A-PVA-coated SPIONs were far less toxic compared to the equal amount of the co-polymer in solution. This decrease in cytotoxicity might be due to the conformational change of the polymer when adsorbing to the surface of the nanoparticles. The interaction with the surface results from hydrogen bonding between polar functional groups of the polymer and hydroxylated and protonated surface sides of the oxide [35]. The configuration of the adsorbed polymer is difficult to determine [36], and is affected by the polymer concentration, molecular weight, pH, ionic strength, and surface charge. To study the coating mechanism, it is very helpful to work with particles that are uniform in size and shape. A number of such model systems have been studied in the last few years using inorganic particles as a core and various polymers as shells [37–39]. According to the common literature, an increased block character of the vinyl acetate units in the PVA is beneficial [40]. To our knowledge, real systems (i.e. particles that are not monodisperse in size and are coated with a commercial polymer) have not been studied in detail so far, especially not for particles in that size range.

The lower toxicity of the nanoparticle associated polymer indirectly suggested that the polymer is not desorbed from the surface of the particles in cell media; otherwise comparably lower cell viabilities would have been measured. The cationic and therefore toxic charge density might be diminished when the polymer is adsorbed on the surface. The different mitochondria activity of A-PVA-SPIONs in media was an artifact attributed to the different cell viabilities in the media and could not be related to the particles. As well, DNA-PEI-SPION complexes showed low cytotoxicity towards HeLa cells compared to PEI coated SPIONs alone. It seemed that the density of uncomplexed amino groups plays an important role regarding cytotoxicity. The cytotoxicity of PEI-SPIONs was overcome only when DNA was added. It has been postulated that during the spontaneous PEI-DNA complex formation PEI wraps DNA via electrostatic interactions, thereby diminishing the high cationic and toxic charge density of PEI to a magnitude that promotes DNA delivery but decreases the negative effects of PEI on viability [20]. The DNA-PEI-SPIONs used in this study were prepared at an N/P ratio of 7.5 with a PEI/SPION ratio of 2. At these concentrations and ratios, respectively, the zeta potential showed that there was only a slight net positive charge of around 12 mV [41]. In addition, the uptake of PVA-SPIONs and A-PVA-SPIONs was examined. We already found in previous studies that PVA-coated SPIONs are well tolerated by cells and that uptake was negligible in the absence of a magnetic field. On the other hand, A-PVA-SPIONs (not PVA-SPIONs) were well taken up by human melanoma tumor cells according to an active, saturable and energy-dependent mechanism [27]. In our study we found that the presence of serum strongly inhibited the uptake of A-PVA-SPIONs due to the previously mentioned adsorption of serum on the particle surface. This is in agreement with the results from other groups who showed that the presence of serum in the medium inhibits cellular uptake and binding of cationic carriers reduces their efficiency as nucleic acid carriers [42,43]. On the other hand, the uptake of PVA-SPIONs is facilitated in DMEM supplemented with FCS. Serum protein adsorption on the PVA surface is very low especially on polymers with hydrolysis degrees higher than 88% [44]. At the same time these

particles showed the best stability in DMEM supplemented with FCS.

Our experiments were not designed to formally prove cell internalization of SPIONs, to determine their location in the cellular compartments and/or to characterize the uptake pathways involved, and it is presently too premature to postulate the exact mechanisms involved, either endocytosis/phagocytosis or receptor-mediated or channel-mediated uptake. A logical next step involves the thorough investigation of the particles' surface after (a) incubation in cell media or biological fluids and (b) cell uptake to understand the processes that occur at the surface of the particles. According to our preliminary results it becomes obvious that each nanoparticle system has to be considered individually since cell uptake, and to certain extent cytotoxicity, was not only dependent on the particle size and surface, but also highly dependent on the cell lines (unpublished data). There is an undeniable influence of the medium and the presence/absence of serum. However, the correlation between the colloidal and biological data is premature and requires extensive and more sophisticated investigations. The impact of the medium on colloidal stability and consequently cell interaction competes with the particles' intrinsic properties (size and surface). Currently, we can say that agglomeration behaviour in different media cannot be correlated to cell uptake within the measured sizes of 40 to 130 nm and uptake times from 1 to 3 h. At current state-of-the-art, the stability of nanoparticle dispersions in cell media should have to be checked routinely.

5. Conclusion

The comparison of PVA-SPIONs with A-PVA-SPIONs showed that already minor modifications of the nanoparticles lead to an altered behaviour in stability, uptake, and toxicity. Therefore, the stability of the particle dispersions needs to be assessed after each surface modification such as fluorescent labelling or chemical coupling of drug molecules.

Although we answered some of the currently discussed questions on colloidal nanoparticles and their behaviour *in vitro*, more fundamental studies will have to be carried out in the future. Those must focus on colloidal stability and its influence on biological properties and will provide a profound base for future discussions on toxicity and potential application of nanoparticles in the field of biomedicine.

Acknowledgements

This work was supported by the Swiss National Science Foundation (SNF 200020-109490 and SFN 205321-111908) and the Competence Centre of Materials Science of the Swiss Federal Institute of Technology, Project PAPAMOD. We thank Prof. J. Hubbell for the access to cell culture facilities.

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