Superparamagnetic iron oxide nanoparticles for multiple biomedical applications

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ABSTRACT

Superparamagnetic nanoparticles (SPION) were prepared by alkaline co-precipitation of ferric and ferrous chlorides in aqueous solution. The obtained particles were mixed at various ratios with different polymer solutions to obtain either SPION coated with polyvinyl alcohol (Mowiol® 3-83), (PVA-SPION) or SPION coated with PVA modified by functional groups (amino-SPION, carboxy-SPION, and thiol-SPION). Studying human melanoma cells by theses different functionalized SPION preparations, only the amino-PVA SPION demonstrated the capacity to be up-taken by, and not beeing cytotoxic to these cells. This uptake by melanoma cells was dependent on the amino-PVA to iron oxide ratio, as an active mechanism, and all cells in a culture internalized these SPION. Depending on the size and of the surface charge of the coated and derivatized particles, the up-take rate can be optimized. Best results are shown with a positive surface charge and a hydrodynamic size of 50 to 80 nm. Static as well as dynamic magnetic forces increase significantly the up-take rate. It could also shown that these particles are useful tools in transfection as alternative to the viral vectors. Interestingly is to note, that the same particles generate heat, which can be used e.g. for local hyperthermic treatment of tumor cells. Therefore, a multitasking including transport, transfection, heating and imaging with these superparamagnetic particles will be possible. Applications like magnetically target drug delivery, magnetofection and hyperthermic treatment will be discussed in detail.

Keywords: drug delivery, transfection, nanoparticle, supeparamagnetic, iron oxide

1 INTODUCTION

The application of the different forms of iron oxides for radiological diagnostic procedures has gained wide acceptance in clinical practice but therapeutic applications are still under investigation and development. Such applications are exploiting two major advantages of magnetic iron oxides: their low toxicity to human beings and the possibility to exploit their outstanding magnetic properties, potentially allowing using the high magnetization of superparamagnetic iron oxide nanoparticles (SPION) to target drugs to specific sites like tumor area or inflamed zones through external static magnetic fields. The use of SPION in drug targeting and delivery, non-viral transfection or as multifunctional particles which allows also imaging and heating (e.g. hyperthermia) requires to deliver these entities inside the desired cells. Therefore the objectives of our present approach were to develop, characterize and optimize biocompatible functionalized SPION, which could be further derivatized with drugs, antibodies and or DNA's, and to determine the ability of these particles to achieve cell uptake by specified cells like human cancer cells and synovial cells respectively applying a static field to maintain particles at the targeted place or, for the first time, an alternate magnetic field which allows the movement of the particles to enhance the up-take rate.

2 EXPERIMENTAL

Iron oxide nanoparticles are prepared by alkaline co-precipitation of ferric and ferrous chlorides in aqueous solution (step 1). After cleaning (step 2), a thermochemical treatment is then applied according to [1] (step 3). Eventually a dialysis step is carried out (step 4) and the ferrofluid is centrifuged (step 5). 30 ml of concentrated NH_3aq (25%) is prepared in a 50 ml beaker and added to the solution while stirring vigorously. It is important to realise this operation quickly and in one shot. The stirring (about 1000 rpm depending on the magnet dimensions) should be strong enough to create a vortex that largely cut the recipient bottom. A dark precipitate forms immediately and the suspension is kept under high stirring for 3 minutes. The black precipitate is then allowed to sediment on a powerful permanent magnet for 5 minutes. The supernatant is discarded using a 20 ml syringe. The powerful magnet is then removed and 250 ml of H_2O is added to the slurry and stirred at low speed for 1 minute so that to loosen the precipitate from the bottom of the beaker. The stirring bar is removed by attracting it with an external magnet. For the thermochemical treatment a 40 ml of the 2 M HNO_3 solution is mixed with a 60 ml of the $Fe(NO_3)_3$ solution. The thus obtained solution is used to collect as much solid

as possible the solid remaining in the centrifugation probes. After about 20 minutes boiling the black suspension turns brown and after 1 hour the suspension is pored into a 500 ml beaker and put on the powerful magnet for 5 minutes. As much as possible yellow supernatant is discarded using the syringe to get highly viscous brown slurry. The suspension is then pored into dialysis tubing and dialysed against HNO_3 0.01 M for 48 hours at room temperature. The obtained particles were mixed at various ratios with different polymer solutions to obtain either SPION coated with polyvinyl alcohol (Mowiol® 3-83), (PVA-SPION) or SPION coated with PVA modified by functional groups (amino-SPION, carboxy-SPION, and thiol-SPION). Additionally, the particles were coated with silica or poly(ethylenimine) (PEI).

The preparation and characterization of the melanoma cell lines from human surgical samples (primary or metastatic tumors) was performed by D Rimoldi, Ludwig Institute for Cancer Research, Lausanne Branch [2]. They were maintained in RPMI medium (Gibco-BRL) containing 10% FCS and antibiotics (both from Gibco-BRL). Three days prior to experiments, the cells were detached in trypsin-EDTA (Gibco-BRL) and grown in complete medium in 48-well plates (Costar). On the day of experiment, medium was changed to fresh complete medium, and either PVA, functionalized PVA, PVA-SPION or functionalized PVA-SPION solutions were added for the concentration, time and temperature indicated. At the end of the experiment, either the MTT test was performed for the two last hours to determine cell viability (cf below) or the cell layers were washed twice in saline, or when indicated for 5 min at room temperature in 0.15 M Na-acetate buffer pH 5.2 to remove cell surface nanoparticles, and cellular iron content was quantified. Further experimental details are descript elsewhere [3].

3 RESULTS

3.1 Particle properties

The median of the hydrodynamic size distribution as a function of the pH is shown in Figure 1 for amino-SPION with a polymer/iron ratio of 13.8 and 18.4. Samples with lower polymer/iron mass ratios were colloidally unstable at pH>5 and were therefore not investigated. By gradually increasing the pH from 2.1 to 10, the median of the size distribution shifted from 19 nm to 33 nm (r = 13.8) and from 19 nm to 54 nm (r = 18.4), respectively. This information suggested that by increasing pH the polymer layer swelled from 5 nm up to 12 nm (r = 13.4) and from 5 nm to 22 nm (r = 18.4), respectively. The origin of this swelling can be explained by a change in the conformation of the polymer chains or by further adsorption of residual polymer. Based on these results, the median of the size distribution in function of the polymer/iron oxide ratio at the physiological pH of 7.4 could be estimated (Table 1). These estimated values will be used for evaluating the mechanisms of cell uptake.

Polymer /iron oxide ratio r	Median of the hydrodynamic size distribution [nm]
0	14
5	19
10	24
15	32
20	55

Table 1: Estimated values of the median of the hydro-dynamic size distribution of amino-SPION as function of the polymer/iron oxide ratio at physiological pH.

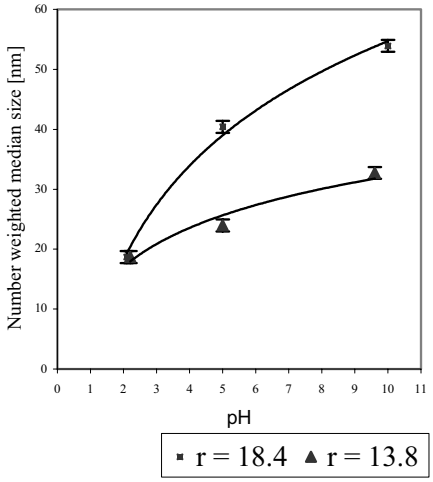

Figure 1: PCS hydrodynamic number weighted size (median) of amino-SPION at different pH for a polymer/iron mass ratio r of 13.8 and 18.4. The curve progression follows a logarithmic law, as indicated by the solid line.

3.2 Particle up-take by melanoma cells (in-vitro studies)

Using the Prussian Blue reaction, the cellular iron content was measured in 4 different human melanoma cell lines (Me191, Me300, Me275, Me237 cells) exposed to these nanoparticles. The iron content was always below detection limits in cells not exposed to nanoparticles, and was very low in cells exposed to PVA-SPION or exposed to carboxy-SPION or thiol-SPION (Figure 2). A very low increase in

iron content could be detected only after 24 h of continuous exposure. However, in cells exposed to amino-SPION (Figure 2), the cellular iron content was high after 24 h of continuous exposure, and was dependent on the amount of added nanoparticles. Thus the cells took up efficiently and rapidly only amino-SPION.

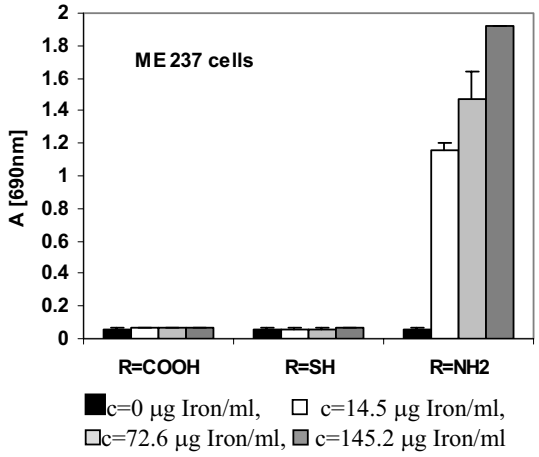

Figure 2: Cellular uptake of the various functionalized PVA-SPIONs by Me237 human melanoma cells. The cells were exposed for 24 h to the nanoparticles, then the cell iron content was determined using the Prussian Blue reaction. Results are shown as ±SD.

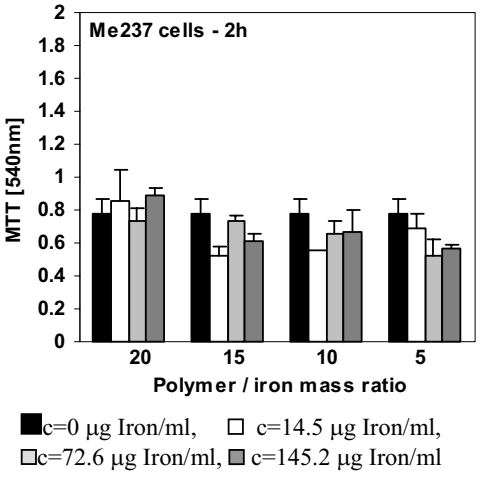

Figure 3: Cytotoxicity of the various polymer/iron mass ratios for Me237 human melanoma cells. The cells were exposed for either 2 h or 24 h, then cell viability was determined using the MTT assay. Results are shown as ±SD.

PVA-SPION or carboxy-SPION or thiol-SPION were not cytotoxic for melanoma cells (results not shown). After 2h exposure, no cytotoxicity of amino-SPION was observed at any polymer/iron ratio (Figure 3, 2h), while after 24h exposure no cytotoxicity was observed for $5 \leq r \leq 15$ ratios, whereas some cytotoxicity was observed only for high polymer concentrations ($r = 20$). The observation that the presence of amino groups improves cell uptake of the SPION was not surprising, since the preferential uptake of cationic liposomes has been widely used in the field of basic molecular biology, mainly for transfection purposes. The exact mechanism, and the role of the vinyl alcohol/vinyl amine copolymer in this phenomenon is more difficult to understand. The influence of the median hydrodynamic size distribution on the uptake of amino-SPION by Me237 and Me275 human melanoma cells was estimated. A very clear maximum in uptake of amino-SPION was observed for particles with a median diameter between 24 nm and 29 nm for Me237 cells, whereas a less clear behavior was found for Me275 cells. For these latter cells, a first maximal uptake was observed around 24 nm as for Me237 cells, but at 29 nm a secondary maximum was observed. The reasons for this behavior are presently unknown, but these results indicate that each cell type has a specific particle size for optimal uptake.
These experiments allowed us to define an optimal amino-polymer to iron ratio, with excellent shelf stability (over 3 month) and reproducible preparation (results not shown) to be used in cellular models for further investigations.

3.3 Particle uptake by synovial membrane (in-vivo experiments)

Chronic aseptic inflammatory diseases of the joint such as rheumatoid arthritis or osteoarthritis require long-time therapy of patients with analgesic, anti-inflammatory, immune-modulating or chondroprotective drugs to release pain and modulate the degree of disease symptomatically [4]. Prolonged medication periods are commonly associated with negative side effects of the drugs, such as nephrotoxicity, hepatitis or gastrointestinal ulceration leading to forced discontinuation of the medication [5]. In the last years several attempts were made to find new successful therapy strategies reducing the unwanted side effects while at the same time leading to optimal well being of the patients [6-8]. If possible, replacement of systemic drug administration through intraarticular injections is the most effective route to treat joint diseases [9]. However, the efficiency of the injected drug may be limited through a relatively short drug persistence or failure to maintain adequate drug concentrations in the joint cavity [10-12]. Amino_PVA-SPIONs were further derivatized by covalent coupling of a fluorescent dye to the nanoparticles via the polymer. For this work we have chosen Cy 3.5: derivatized CyDye™ (NHS ester; Eurogentec) that carries one reactive group on each dye molecule for accurate labelling of amine

groups. The absorption maximum of this dye is at 581 nm, the maximal emission at 596 nm. The final dispersion contains 0.9mg iron/ml dispersion and approx. 3 dye molecules /particle. A total of 14 Swiss Alpine sheep between 2-4 years of age was used for application of PVA coated and functionalized SPIONs into the stifle and carpometaphalangeal joints. Eight additional sheep without SPIONs as well as without magnets and slaughtered for other reasons than infectious or systemic disease served as controls.

Intraarticular and periarticular injection as well as recovery from anesthesia was uneventful for all animals. They were weight bearing immediately after surgery. Some degree of pain was noticed which disappeared after magnet removal at 12 hours. Sheep injected with the plain PVA coated SPIONs showed no clinical signs of joint inflammation or lameness after injection and magnet removal. Only sheep injected with the Cy3.5-amino-PVA coated particles revealed mild clinical signs of inflammation. At 72 hours heat at the joint area could be detected and all sheep examined were mildly painful on palpation. Slight joint effusion could be seen in only one sheep at 72 hours after injection. Joint effusion, heat and pain were considerably less at 120 hours. Control animals were normal without any lameness and inflammation. Evaluation of the score of particle localisation within the intima (most inner layer in synovial membrane) revealed that in combination with a magnet higher scores could be reached than without a magnet. Scores were generally increasing over time. A peak could be detected at 120 hours with two exceptions at 3 hours. In the carpal joint total scores were higher than in the stifle joints.

In the fibrous connective tissue underlying the intima the scores of particle localisation were lower with a magnet than without a magnet. There, particle distribution had a tendency to be focused more locally with a magnet in place. In the adipose connective tissue results were comparable to those of the fibrous tissue, whereas there was a tendency that total scores where higher with a magnet than without a magnet.

3.4 Hyperthermia

Magnetic nanoparticles were embedded in a silica matrix using a in-situ preparation of iron oxide nanoparticles in a silica precursor followed by thermal treatment at 500 °C for 24 h. Details of the synthesis as well as of the power loss measurements are published elsewhere [13]. The power losses obtained applying a AC magnetic field of 6 mT and 140 kHz was 7.7, W/g iron oxide. This value is high enough to heat a tumor of 4 cm diameter at tempeature higher than 42 °C

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