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Research paper

Surface-modified biodegradable albumin nano- and microspheres. II: effect of surface charges on in vitro phagocytosis and biodistribution in rats

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Abstract

The surface charges on biodegradable albumin nanoparticles were introduced by covalent coupling different primary amines to examine their influence on phagocytosis by macrophages under in vitro conditions. Albumin particles with a zeta potential close to zero showed a reduced phagocytic uptake in comparison with charged particles, especially nanoparticles with a positive zeta potential. The phagocytic uptake in the present study was examined using an established cell culture model based on primary mouse peritoneal macrophages and a human hematopoietic monocytic cell line (U-937) treated with phorbol-12-myristic-13-acetate to induce cell differentiation. The influence of opsonins on in vitro phagocytosis experiments was characterized using carriers pre-treated with human serum. In the presence of human serum the phagocytic activity of U-937 cells was found to be similar to primary mouse macrophages without serum. In contrast to peritoneal macrophages, U-937 cells showed no phagocytic activity in the absence of serum. In particular, only the C3b- complement deposition on the particle surface seems to promote the phagocytic process. The in vivo distribution of albumin carriers in rats was investigated using magnetic resonance imaging (MRI). No differences in blood circulation times and organ accumulation between different nanoparticle preparations with positive, neutral and negative surface charges could be observed in rats, suggesting that the in vivo fate of albumin nanoparticles is significantly influenced by factors not reflected in the in vitro cell culture models.

Keywords: Phagocytosis; Surface charge; Albumin-spheres; Opsonization; Human macrophage cell line (U-937)

1. Introduction

Nano- and microparticles have been suggested for the selective delivery of drugs to specific target sites, vaccine administration and as diagnostic agents [1]. Following i.v. administration the in vivo distribution of particulate drug carriers depends on their particle size and surface properties such as surface charge and surface hydrophobicity. The influence of these physico-chemical characteristics on the uptake of particles by the mononuclear phagocyte system (MPS) comprising mainly the macrophages of the liver and the spleen has been described by Douglas et al. [2]. The MPS has a major role in removing foreign materials from the blood circulation. Advances in prolonged circulation time, selective drug deposition and reduced MPS uptake have been achieved by modification of the surface characteristics of particles determining the interaction with the MPS [3]. So far, selective targeting to the specific organs has rarely been achieved.

A potential therapeutic application necessitates biodegradability and biocompatibility of the nanoparticulate carrier system [4] and surface modifications which are stable under in vivo conditions [5]. A chemical modification of nanoparticle surfaces by covalent attachment of function groups has not been studied so far. Therefore, glutaralde-

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hyde cross-linked albumin micro- and nanoparticles which are known to have a favourable biocompatibility profile for the use in humans, were prepared by using an emulsion technique according to Roser et al. [6]. The surface of the spheres was modified by quenching residual aldehyde functions with several primary amines.

The phagocytic uptake of colloidal drug carrier systems, the major obstacle to the efficient delivery to target sites can be quantified in vitro by studying the uptake by phagocytic active cells [7]. To successfully mimic phagocytosis with an in vitro cell culture system, the selection of the cell culture model is critical. Many authors use primary cultures of mouse peritoneal macrophages as a standard cell culture model [8]. Since inter-individual differences between various preparations affect the reproducibility of the results, a permanent cell culture with characteristic properties over all generations is more suitable for comparative experiments. The in vivo phagocytosis is influenced by adsorption of serum components to the surface of particles [9]. To examine the relationship between surface properties, opsonization and phagocytosis under in vivo conditions, phagocytic experiments with a human cell line in cell culture medium supplemented with human serum albumin and human opsonizing serum factors were conducted.

The following experiments were carried out to evaluate the effect of particle size and surface charge on in vitro interaction of chemically modified, biodegradable albumin micro- and nanoparticles with the mononuclear phagocyte system. The biological behaviour of the particles is discussed in terms of the in vitro cell interaction and the in vivo distribution in the rat model. We used two types of cells, primary peritoneal mouse macrophages and U-937 cells, a monocyte cell line with macrophage-like attributes, derived from the pleural effusions of a patient with histocytic lymphoma. Moreover, we investigated the influence of surface modifications on in vivo distribution and blood circulation times in rats after intravenous administration. The uptake of particles in vivo has been determined by several analytical methods such as gamma scintigraphy, autoradiography and liquid scintillation counting [1]. To avoid techniques requiring radiolabelled drugs or polymers, we prepared albumin nanoparticles containing magnetite (Fe_3O_4), a technique known from the external magnetic guidance of nanospheres [10]. The magnetizable material can be detected by magnetic resonance imaging (MRI), a non-invasive imaging technique [11,12].

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate (mixed isomers), bovine serum albumin (BSA), fraction V, aqueous glutaraldehyde solution (50%, w/w), glycine, *N*,*N*-dimethyl-1,3-diaminopropane, (carboxymethyl) trimethylammoniumchloride hydrazide (Girard's reagent T) and phorbol-12-myristate-13-acetate were purchased from Fluka AG (Buchs, CH). Butylamine and ethanolamine were obtained from Aldrich Chemie (Steinheim, Germany) and human fibronectin (Fn), human immunoglobulin G (IgG) and human serum albumin (HSA) were obtained from Sigma (Deisenhofen, Germany). All chemicals and substances of analytical grade were used as received.

Bacto NIH Thioglycolat Broth was obtained as dry powder from Difco Lab (Detroit, MI) and prepared under sterile conditions according to the instructions of the manufacturer.

All reagents for cell culture media were purchased from GIBCO (Basel, Switzerland). The medium consisted of 209.25 ml RPMI 1640 supplemented with 5.0 ml L-glutamine (4 mM), 6.25 ml HEPES (25 mM), 4.5 ml NaOH (solution 7.5% w/w) and 25 ml heat-inactivated fetal calf serum (10% v/v). Additionally, 1 ml of penicillin/streptomycin solution (10 000 IU/ml) per 100 ml medium was added to the culture medium for primary mouse macrophages. The incubation medium consisted of 100 ml RPMI 1640, 2.5 ml HEPES (25 mM), 2.2 ml NaOH (solution 7.5% w/w) preserved with penicillin/streptomycin solution (10 000 IU/ml) and 1 ml anti-PPLO. 100 ml RPMI 1640 preserved with 1 ml penicillin– streptomycin solution (10 000 IU/ml) was used as washing medium.

2.2. Methods

2.2.1. Preparation of surface modified albumin nanoparticles

Glutaraldehyde cross-linked albumin nanospheres were prepared as described previously [6] and characterized by photon correlation spectroscopy for particle size determination and scanning electron microscopy. Bovine serum albumin (BSA) was labelled with fluorescein isothiocyanate (FITC) according to the procedure described in Ref. [13]. Approximately 2% of the lysine-residues of native albumin were conjugated with FITC (albumin/FITC molar ratio: ca. 1). Three millilitres of FITC–BSA solution (25% w/v) were used for each particle preparation. To prevent enzymatic or hydrolytic degradation during uptake experiments, the glutaraldehyde concentration for cross-linking was relatively high with a ratio of ca. 15 for aldehyde/lysine (μmol).

The modification of the particle surface was initiated by adding 75 mM butylamine (AP-B), ethanolamine (AP-E) or *N*,*N*-dimethyl-1,3-diamino-propane (AP-N) before washing and isolation of particles to quench monovalently bound aldehyde groups of glutaraldeyde. Stirring was continued at room temperature for 6 h. The modified particles were purified and isolated as described previously for unmodified albumin spheres [13]. The covalent coupling of glycine (AP-G) and trimethylammoniumacethydrazide (AP-T) followed the isolation and washing of the unmodified glutaraldehyde cross-linked albumin nanoparticles. The particles were resuspended in 75 ml glycine solution (1 M in phosphate buffered solution pH 7.5, 100 mM) and subsequently

stirred for 24 h at room temperature. For coupling of trimethylammoniumacethydrazide, spheres were mixed in 20 ml 99% ethanol with 100 ml 75 mM amine solution in ethanol/acetic acid/water (8:1:1). Particles were purified in PPS (3 × 150 ml) and distilled water (1 × 150 ml) for glycine modification and 3 × 150 ml distilled water for trimethylammoniumacethydrazide modification. The spheres were harvested by centrifugation at 35 000 g for 20 min at 4°C. Finally, all preparations were dehydrated in acetone and isolated as described by Roser et al. [6,13].

For MRI experiments the preparation started with a solution of 750 mg BSA in 3 g Ferrofluid EMG 507 suspension (240 mg Fe_3O_4) (Ferrofluidics, Nürtingen, Germany) and 0.2 ml water. To remove excess Fe_3O_4 particles were washed several times with water and finally with acetone/water (9:1) as described above. The quantification of the Fe content was performed by atom absorption spectroscopy. Nanoparticles (8–12 mg) were heated in 5 ml of 96% (w/w) sulfuric acid and 65% (w/w) nitric acid (1:9 v/v). After cooling to room temperature, 1 ml 30% (w/w) hydrogen peroxide was added and heated again. This process was repeated until a clear solution had been obtained which was mixed with 5 ml 30% acidic acid and diluted up to 50 ml with distilled water. Fe solutions 20, 40 and 60 μg/ml in the same acid/water solution were used as standards for the calibration curve. Measurements were carried out using a Perkin–Elmer 5000 spectrometer at 248.3 nm using a mixture of 96% sulfuric acid, 30% acidic acid and water (1:10:89 v/v) to calibrate the instrument to zero.

2.2.2. Zeta potential measurement

The electrophoretic mobility was determined by Laser Doppler Anemometry with a Malvern Zetasizer IIc (Malvern Instruments, Malvern, UK). All experiments were performed at 25°C and 100–2000 Hz. The cell voltage for measurements in double distilled water was set at 100 mV, that in buffer solution (1 mM and 5 mM phosphate buffer pH 7.2 and phosphate buffered NaCl solution pH 7.2) was set at 20 mV. The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation. The results are expressed as mean values of five samples.

2.2.3. In vitro studies with U-937 cells

All experiments were carried out at 37°C in an atmosphere of 10% CO_2 and 95% relative humidity, unless stated otherwise. U-937 cells were cultured at approximately 1×10^5 to 1×10^6 cells/ml in culture medium. Six to eight hours prior to the experiments the medium was changed and the cell viability was determined to be approximately 95% by acridine orange/ethidium bromide staining [7]. One hundred microlitres U-937 cell suspension (1.5×10^5 cells/ml culture medium) per chamber were seeded on Lab–Tek Tissue Culture Chamber Slides (Miles Scientific, Naperville, IL) in the presence of 10 μl PMA solution (80 nM in incubation medium) and grown at 37°C for 72 h. The medium was aspirated and the cells were washed with 200 μl incubation medium. For phagocytic experiments, cells were treated with a mixture of 100 μl incubation medium, 100 μl particle suspension (2×10^7 particles/ml in incubation medium) and, for serum dependent experiments, with 20 μl human serum. Opsonization was carried out by incubation of coated particles with 0.1 mg/ml Fn, IgG or HSA at 37°C for 1 h. Human serum obtained and pooled from venous blood of healthy adult human donors was stored at −70°C and thawed as required. The complement was inactivated by storage at 56°C for 1 h. PMA-stimulated cells were incubated with various particle preparations and washed with incubation medium to remove the excess and the non-adherent particles. For microscopic observations the macrophages were fixed with 1.5% (v/v) glutaraldehyde in phosphate buffered saline (PBS).

To differentiate between firm attachment of the particles to the cell membranes and uptake into the cells, all experiments were also done at 4°C. Usually, no significant phagocytic activity was observed at this temperature [14]. For control we carried out experiments using the phagocytosis inhibitors sodium azide and cytochalasin B as described by Roser [13].

For determination of the percentage of phagocytic cells and the phagocytic index (PI, the mean number of particles ingested per cell) in three Lab–Tek chambers 100 U-937 cells were viewed as described by Vetvicka et al. [15]. All assays were performed in duplicate.

2.2.4. In vitro studies with mouse macrophages

Male C 57 BL/6J mice obtained from BRL (Fuellinsdorf, Switzerland) weighing approximately 25 g, were injected intraperitoneally with 1 ml thioglycolate medium. After 72 h mice were sacrificed and the peritoneal cells were harvested with a peritoneal lavage using 10 ml of ice-cold washing medium. The peritoneal exudate was diluted with culture medium and cell viability determined by trypan blue exclusion [16]. The number of macrophages was judged by characteristic cell morphology [8,17].

The cell number was adjusted to 7×10^6 cells/ml with culture medium and 100 μl per Lab–Tek chamber were seeded. After 3 h cells were rinsed in washing medium, 100 μl culture medium were added and incubated for 21 h. Before experiments, mouse macrophages were characterized using a 1-Naphthylesterase Diagnostik Kit (Sigma, Deisenhofen, Germany) [8,18].

All experiments were carried out in analogy to the studies with activated U-937 cells.

2.2.5. In vivo studies

Albumin nanoparticles were suspended in NaCl solution (0.9% w/w) to obtain a final concentration of 0.2 and 1.2 mg/doses and injected in the femoral vein of pentobarbital narcotized male Wistar rats (300 g, BRL, Füllinsdorf, Switzerland). The measurement was recorded on a Biospee

BMT 47/15 spectrometer (Brucker, Karlsruhe, Germany) equipped with a tesla magnet (15 cm horizontal drilling) and an Alderman-Grant resonator (radio frequency transmitter and receiver). The following experimental conditions were employed: a field-of-view of 60 mm, an echo spacing of 20 ms and a recycle delay of 500 ms. The intensity of the signal from regions of interest was measured and repeated twice. Upon starting the injection, the first picture of liver cross-section was taken and five to six repetitions were done at intervals of 2–3 min.

3. Results and discussion

It has been suggested that particulate carriers interact with blood components after intravenous injection. The adsorption of plasma proteins onto the surface of colloidal carriers influences the distribution in the body by affecting the uptake into the mononuclear phagocytic systems MPS. Avoidance of the MPS is a pre-requisite for targeting colloidal carriers to other organs and tissues than liver and spleen. With these considerations in mind, our aim in this study was to modify the surface properties of particulate carriers by covalent linkage of primary amines to modify the surface properties of albumin particles by different charge densities, thus influencing the phagocytic uptake into macrophages. In in vitro experiments the FITC-labelled nanoparticles were visualized by combined phase and epi-fluorescence microscopy after phagocytosis, and the average number of particles ingested and the phagocytic index were determined microscopically (Fig. 1). Tables 1 and 2 give an overview of the albumin carriers used in these experiments, the different primary amines for surface modification and the surface charge of the modified particles determined by zeta potential measurement. As expected, the zeta potential decreases with increasing ionic strength of suspension medium but varied significantly when measuring different surface modified particles. However, in water, positively charged Girard T loaded particles showed a slightly negative zeta potential in phosphate buffered solutions. Performing the modification in acetic/ethanol solution, residuals of acetic acid were adsorbed onto the surface of the particles. In phosphate buffered solutions the positively charged amine residues could be neutralized and the acid led to a reversion of the zeta potential measured. Particles are reproducibly obtained with good yields (70–80%) and a narrow size distribution. No significant difference in particle size before and after surface modification could be detected. Since all preparations had comparable average diameters and polydispersity coefficients, the influence of size on phagocytosis could be neglected. The chemical surface modification is stable in fresh human serum and in aqueous solutions of different pH as described by Roser et al. [6].

Usually U-937 cells are rounded, small, non-adherent cells with a low phagocytic activity [19,20]. When U-937 cells are treated with PMA they differentiate into macrophage-like cells. PMA induces the phagocytic activity, the attachment to surfaces and the differentiation of the immature cells to activated cells with varied cell shape morphology [21]. The differentiation into a macrophage-like phenotype was dependent on PMA incubation time. In our experiments maximal differentiation was attained after 72 h in the presence of 80 nM PMA.

At 4°C incubation temperature PMA-stimulated U-937 cells expressed low levels of phagocytic activity. Only few cells ingested a maximum of three nanoparticles per cell independent of serum addition. No time-dependent increase could be observed. Fig. 2 shows the phagocytic index (the mean number of particles ingested per cell) of different albumin particle preparations after incubation with U-937 cells at 37°C with serum. The values obtained at 60 min were significantly different. We found a good correlation between stimulation of macrophages and the zeta potential measured by electrophoretic mobility. The lower the phagocytic activity of U-937, the lower the surface charge of particles. An increase in phagocytic response was observed using particles with a high surface potential, especially a high positive net charge (Fig. 3). These results can be reproduced with different particle preparations and are not influenced by FITC- labelling [13].

Incubation of the particles with activated mouse peritoneal macrophages in serum-free medium led to comparable results: the lower the surface charge, the lower the phagocytosis. A higher negative or positive surface charge of albumin particles significantly increased the uptake by cells. For glycine-coated particles with the highest negative charge of all preparations, mouse macrophages showed higher phagocytotic capacity than U-937 cells (Fig. 3). A likely explanation for this behaviour could be the opsonization of albumin carriers by serum components. Studies with mouse macrophages were carried out in serum-free medium, the incubation with U-937 took place in medium supplemented with serum i.e. with opsonized particles. For uptake experiments with U-937 cells the addition of serum was necessary because of their ability to ingest only opsonized particles [22]. Experiments done without serum resulted in 8% maximum phagocytic cells after 90 min and PI $< 0.6 \pm 0.3$.

Immunglobulin G, IgG, complement factor C3b, and fibronectin, Fn, have been identified as opsonizing serum factors [9,23]. On the other hand, human serum albumin, HSA, is known to decrease the phagocytic response [24]. Likewise selected particle preparations coated with IgG, Fn and HSA showed only slight phagocytosis when treated with U-937 cells in serum-free medium (3–5% phagocytic cells, PI $<$ 0,03). The results for surface modified particles incubated with U-937 cells in serum supplemented medium are shown in Table 3. The phagocytosis of particles modified with glycine was not significantly influenced by the three proteins tested. In contrast, the covalent binding of butylamine and *N*,*N*-dimethyl-1,3-diamino-propane influ-

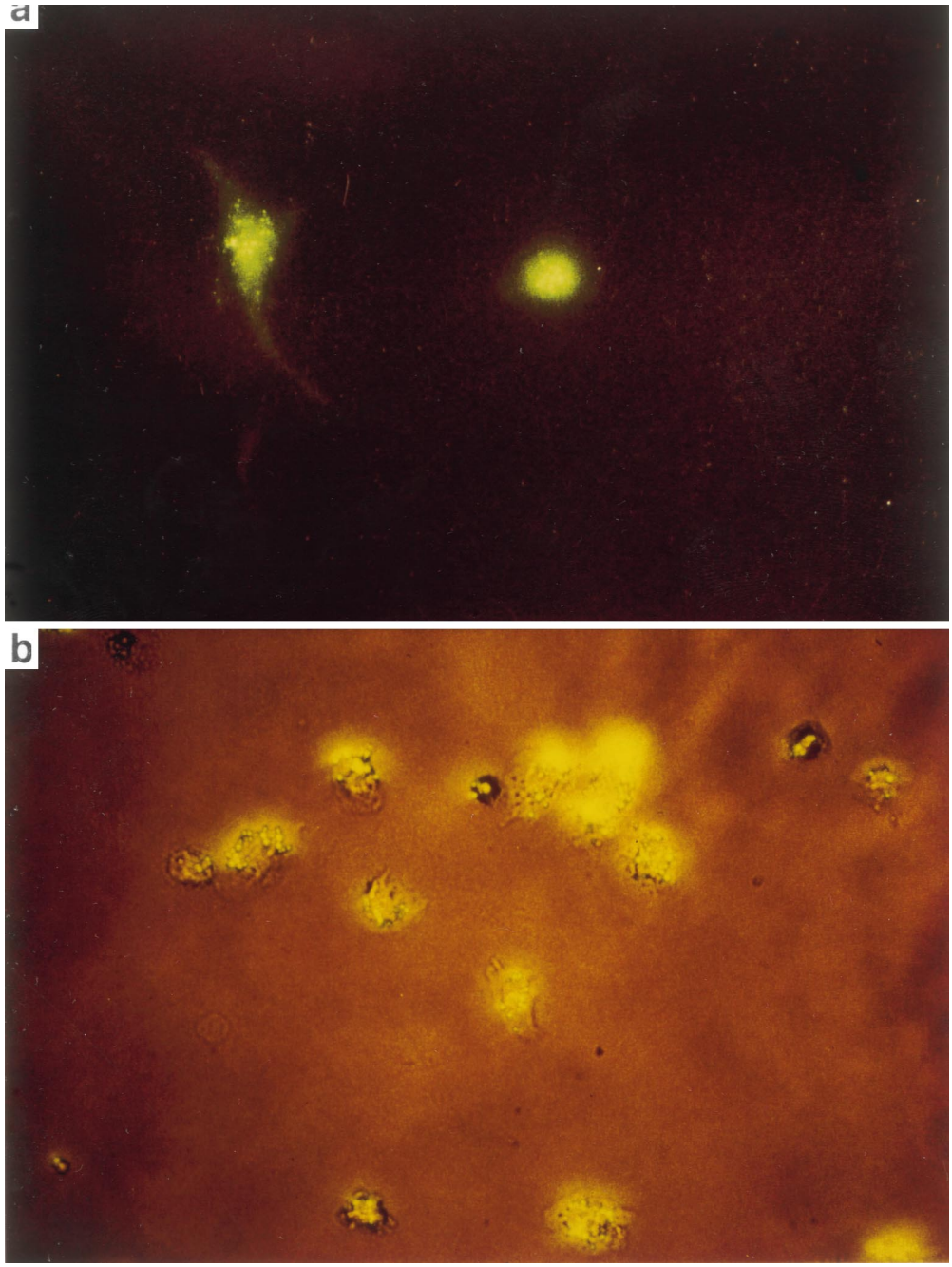

Fig. 1. Micrographs of stimulated U-937 cells after the phagocytic uptake of FITC labelled albumin nanoparticles (average diameter 550 nm), magnification: 1.25 × 40, fluorescence microscopy combination of transmitted bright light and epifluorescence microscopy.

enced the phagocytic uptake significantly. Albumin particles coated with Fn and IgG strongly activated the uptake by U-937 cells, whereas particles pre-treated with HSA showed a rather weak phagocytotic uptake. IgG caused the most distinct effect.

In conclusion, interactions between the modified particle surface and opsonizing- or phagocytosis-inhibiting serum components influenced the extent of phagocytosis. As U-937 cells phagocytosed particles coupled to primary amines only in the presence of fresh human serum, it could be expected that the covalent attached surface becomes further coated with additional serum components.

Serum complement is one of the most important components of the opsonin system of the body. Complement C3b, a strong promotor of phagocytosis, is generated from complement C3 [25] in vivo by activation of the complement system and can be inactivated by heating [9]. As shown in Table 3, coating particles with various concentrations of heat-inactivated human serum has only a very small effect on phagocytosis. Differences between various carriers could

Table 1

Mean diameter and polydispersity coefficient of surface modified albumin nanoparticles used in in vitro phagocytosis experiments, measured by photon correlation spectroscopy ($n = 5$)

Albumin particles	Primary amine	Mean diameter ± SD (nm)	
		Polydispersity coefficient ± SD (%)	
AP	–	545.5 ± 10.5	38.2 ± 2.2
AP-B	Butylamine	577.2 ± 13.5	38.7 ± 2.4
AP-E	Ethanolamine	569.4 ± 10.0	38.2 ± 3.1
AP-G	Glycine	562.3 ± 29.8	36.9 ± 3.8
AP-T	Girard's reagent T	516.4 ± 5.5	35.4 ± 3.3
AP-N	*N*,*N*-dimethyl-1,3-diamino-propane	605.7 ± 26.8	37.2 ± 3.3

not be observed. Obviously U-937 cells are able to ingest spheres only in the presence of intact complement. So not only the physico-chemical characteristics of the particles, including size distribution and surface modification, significantly trigger the in vitro interaction with macrophage-like cells but also the coating with blood components.

In comparison, we evaluated the influence of surface modifications on body distribution behaviour of albumin nanospheres after intravenous injection in rats. We used magnetic resonance imaging (MRI) as an non-invasive, alternative technique to radioactive methods, such as gamma scintigraphy to visualize the kinetic energy and extent of accumulation of surface-modified albumin nanospheres in various organs in rats. The time- and concentration-dependent registration of proton signals allows the determine the accumulation of paramagnetically labelled particles in specific organs of interest. We measured the kinetic energy of carrier accumulation in the liver as the major organ of the MPS. In contrast to gamma scintigraphy, no radiolabelling of carriers was necessary. To increase the MRI contrast the nanospheres were labelled by loading them with ca. 26–27% (w/w) of a paramagnetic Fe_3O_4 suspension [26], followed by surface modification of the albumin nanoparticles using butylamine, glycine and N,N-dimethyl-1,3-diamino-propane to generate hydrophobic, negatively and positively charged particles, respectively. To allow direct comparison of the signal

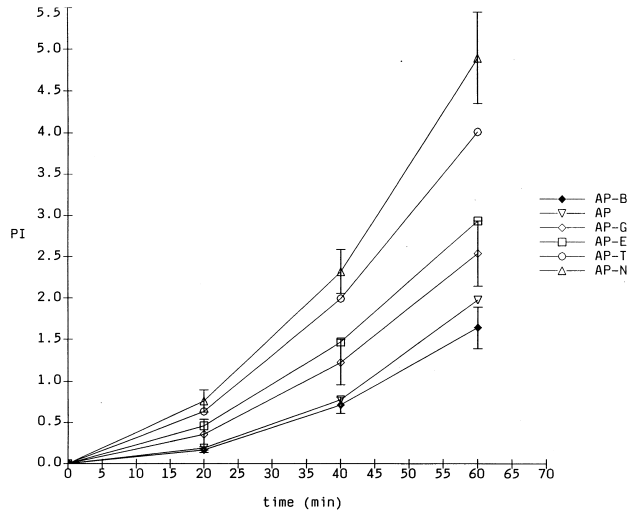

Fig. 2. Effect of incubation time on the phagocytic index of surface-modified albumin nanospheres incubated with activated U-937 cells in the presence of human serum ($n = 3 \times 300$).

intensities, depending only on concentration of carriers accumulated in liver, all nanospheres were of similar size, i.e. ca. 500 nm and had comparable loadings of ca. 27% (w/w) Fe_3O_4 as determined by atom absorption spectroscopy. Table 4 gives an overview of the characteristics of the nanoparticles. For the experiments, only Fn and IgG uncoated particles were used to study the effects of surface charge on biodistribution without the influence of pre-coated opsonins. The results should only be influenced by the opsonization of the spheres with substances occurring naturally in the body to determine whether the effect of in vitro opsonization could be reproduced with the in vivo complement system.

When administered intravenously in the femoral vein of rats, the highest number of particles in liver was found 2–3 min after injection. Signal intensity was strongly influenced by the injected dose of the nanoparticles. The Fe_3O_4 containing albumin nanospheres caused a decrease of the signal intensity in a non-linear fashion, therefore, a calibration curve in a range from 0.1 to 2.0 mg particles per rat was generated (data not shown). The liver was defined as region of interest and the animals were positioned to allow a cross-

Table 2

Zeta potential of surface modified albumin particles measured by Laser Doppler Anemometry in distilled water and phosphate buffered solutions with increasing ionic strength ($n = 5$).

Albumin particles	Zeta potential in water ± SD (mV)	Zeta potential in 1 mM PP[a] ± SD (mV)	Zeta potential in 5mM PP[a] ± SD (mV)	Zeta potential in PPS[b] ± SD (mV)
AP	–25.31 ± 0.80	–3.35 ± 0.96	–0.40 ± 0.67	–0.46 ± 0.44
AP-B	–19.26 ± 1.06	–3.16 ± 0.64	–0.05 ± 0.90	0.18 ± 0.64
AP-E	–18.83 ± 1.80	–4.48 ± 0.33	–1.33 ± 0.57	0.16 ± 0.90
AP-G	–30.45 ± 0.91	–7.79 ± 0.76	–4.55 ± 0.84	–1.95 ± 1.17
AP-T	4.21 ± 1.10	–8.05 ± 0.42	–1.86 ± 0.65	0.22 ± 0.57
AP-N	18.49 ± 0.92	–0.52 ± 0.72	0.53 ± 0.82	1.15 ± 0.93

[a]Phosphate buffer.
[b]Phosphate buffered sodium chloride solution.

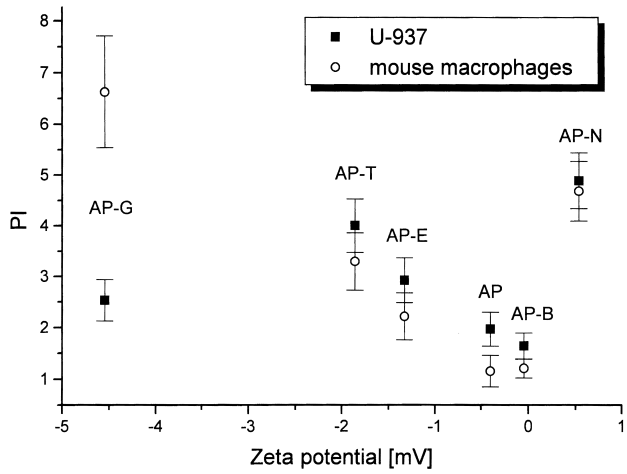

Fig. 3. Correlation between phagocytic index and zeta potential (phosphate buffer, 5 mM, pH 7.2) of surface modified albumin nanoparticles using U-937 cells (incubation time 60 min, presence of human serum) and mouse macrophages (incubation time 60 min, without serum).

sectional view. The signal intensity in the region of interest was recorded twice before injecting the nanospheres. After application of the specified dose in 0.6 ml saline and flushing the cannula with 0.2 ml saline, the change in signal intensity was recorded at 2, 4, 6, 8 and 10 min. The contrast was maximal after 4 min and remained unchanged afterwards, suggesting a very rapid clearance of the albumin nanospheres by the liver, similar to other colloidal carriers such as liposomes [27]. The results of the accumulation of surface modified albumin nanospheres in the liver are shown in Fig. 4. The relative signal intensity of liver was recorded for the three albumin particle preparations after injection of the two different concentrations into three rats each. Carriers coated with the three amines showed no significant difference in liver accumulation, blood circulation time and organ preference in contrast to in vitro phagocytosis experiments. A quantitation of the extent of liver accumulation in absolute terms requires further investigation, but, in comparative studies with ^{125}I-labelled butylamine-coated albumin nanospheres, we obtained an accumulation of 81% of the administered dose in the liver after 15 min (Fig. 5). These results point to the fact that electrostatic interactions between particles and phagocytic cells may be more important under in vitro conditions. We cannot rule out the possibility that interaction between plasma proteins and particles are different in stagnant cell cultures, leading to differences in coating of the particle surface with opsonins. Obviously, there are significant differences regarding the kinetic energy of surface modified albumin nanospheres in the circulating blood stream and under in vitro conditions. These differences seem to be more dominant and/or the charge density was insufficient to cause a significant change of the initial distribution pattern of this colloidal carrier system under in vivo conditions.

Table 3

Phagocytic index and percentage of U-937 cells having effectively taken up surface-modified albumin nanoparticles after incubation with Fn, IgG and increasing concentrations of heat-inactivated human serum ($n = 3 \times 300$), values compared to uncoated particles

	Percentage of phagocytic cells ± SD		
	AP-B	AP-G	AP-N
HSA	78 ± 10.1	102 ± 9.4	96 ± 5.1
Fn	140 ± 14.1	107 ± 6.8	110 ± 6.0
IgG	122 ± 9.9	103 ± 7.6	107 ± 6.2
HI-Serum[a] 10%	5 ± 0.8	5 ± 2.8	5 ± 2.8
HI-Serum[a] 20%	7 ± 2.1	n.d.	3 ± 2.0
HI-Serum[a] 50%	4 ± 3.5	n.d.	2 ± 1.5
	Phagocytic index		
HSA	65 ± 25.3	104 ± 11.0	77 ± 15.0
Fn	124.4 ± 20.1	119 ± 15.3	123 ± 8.22
IgG	142 ± 22.8	113 ± 14.8	132 ± 17.1
HI-Serum[a] 10%	0.06 ± 0.032	0.07 ± 0.043	0.05 ± 0.036
HI-Serum[a] 20%	0.13 ± 0.030	n.d.	0.05 ± 0.053
HI-Serum[a] 50%	0.04 ± 0.035	n.d.	0.02 ± 0.026

[a]Heat-inactivated human serum.
n.d., not determined.

Table 4

Mean diameter ($n = 5$), polydispersity coefficient ($n = 5$) and Fe content ($n = 2$) of albumin nanoparticles used for in vivo MRI studies

Albumin particle	Mean diameter ± SD (nm)	Polydispersity coefficient ± SD (%)	Fe_3O_4 content ± SD (% w/w)
AP-B	542.0 ± 21.2	36.7 ± 0.6	26.7 ± 0.10
AP-G	480.0 ± 16.8	33.5 ± 3.5	28.8 ± 0.09
AP-N	555.6 ± 16.1	39.7 ± 3.1	27.9 ± 0.39

4. Conclusions

The in vitro phagocytosis of biodegradable, surface-mod-

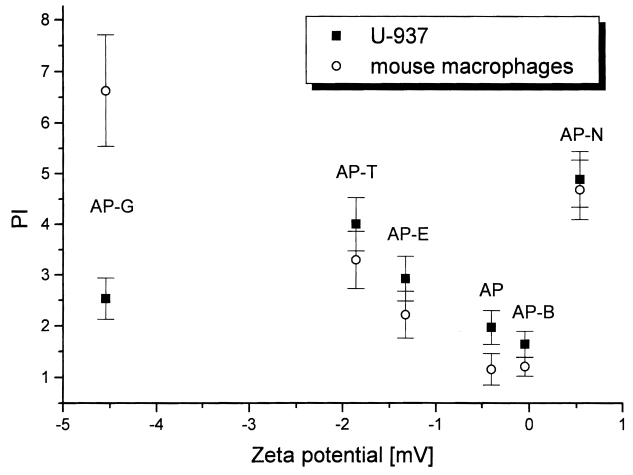

Fig. 4. Relative intensity of NMR signals of liver after injection of 0.2 and 1.2 mg surface-modified albumin nanoparticles in the femoral vein of rats ($n = 2$).

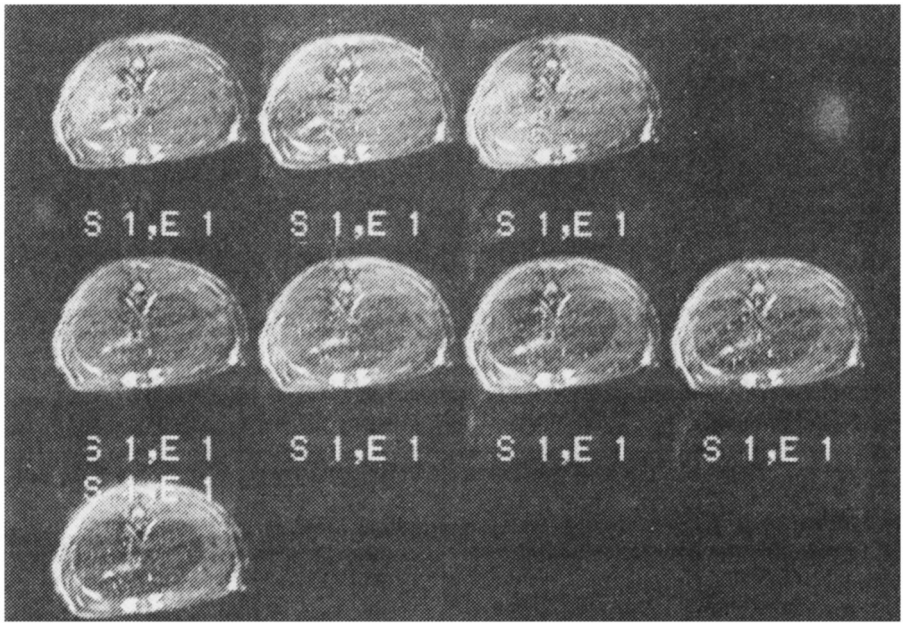

Fig. 5. Signal intensity of the liver before and after accumulation of modified albumin nanoparticles measured by MRI. Top: cross-section of the liver before injection of particles. Bottom: (left to right) 2, 4, 6, 8 and 10 min after injection.

ified albumin nanoparticles which was performed by covalent coupling of butylamine, glycine and Girard's T reagent, affected the zeta potential significantly. We confirm the results of Tabata et al. [28], who obtained comparable uptake in mouse peritoneal macrophages using modified cellulose particles in serum free medium.

Both freshly isolated mouse macrophages and the human hematopoietic macrophage-like cell line U-937 are useful models for the study of phagocytosis of colloidal carrier systems. Since opsonization of these particles seems to influence the body distribution, experiments should be done in the presence of species-specific serum. The U-937 cell line showed phagocytosis only in the presence of intact complement, highlighting the importance of opsonizing serum factors. This feature prevented a characterization of other opsonins such as fibronectin and IgG in the U-937 cell line.

MRI is a new, non-invasive method to test the kinetic features of particle distribution patterns in vivo without radioactive labelling. The resolution is sufficient for determination of organ distribution, in our case the liver, but for mass balance experiments other techniques seem to be more suitable. In the rat model no differences in liver accumulation between various surface- modified particulate systems using differently charged ligands could be observed. This aspect needs further investigation.

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References

[1] E. Allemann, R. Gurny, E. Doelker, Drug-loaded nanoparticles – preparation methods and drug targeting issues, Eur. J. Biopharm. 39 (5) (1993) 173–191.
[2] S.J. Douglas, S.S. Davies, L. Illum, Nanoparticles in drug delivery, CRC Crit. Rev. Ther. Drug Carrier System 3 (3) (1986) 233–261.
[3] J.W.B. Bradfield, The reticuloendothelial system and blood clearance, in: S.S. Davies, L. Illum, J.G. McVie, E. Tomlinson (Eds.), Microspheres and Drug Therapy. Elsevier, Amsterdam, 1984, pp. 25–37.
[4] E. Tomlinson, Microsphere delivery system for drug targeting and controlled release, Int. J. Pharm. Tech. Prod. Manuf. 4 (3) (1983) 49–57.
[5] S.J. Douglas, S.S. Davies, L. Illum, Biodistribution of poly(butyl 2-cyanoacyrlate)-nanoparticles in rabbits, Int. J. Pharm. 34 (1986) 145–152.
[6] M. Roser, T. Kissel, Surface modified biodegradable albumin-nano- and microspheres. I: Preparation and characterization, Eur. J. Pharm. Biopharm. 39 (1) (1993) 8–12.
[7] R. Absolom, Basic methods for the study of phagocytosis, in: G. di Sabato, J. Everse (Eds.), Methods in Enzymology, Vol. 132, Academic Press, Orlando, FL, 1986, pp. 95–180.
[8] O. Adams, Macrophages, in: W.B. Jacoby (Ed.), Methods in Enzymology, Vol. LVIII, Academic Press, New York, 1979, pp. 494–507.
[9] D.A. Absolom, Opsonin and dysopsonins: an overview, in: G. di Sabato, J. Everse (Eds.), Methods in Enzymology, Vol. 132, Academic Press, Orlando, FL, 1986, pp. 281–318.
[10] Hassan, J.M. Gallo, Targeting anticancer drugs to the brain. I. Enhanced brain delivery of oxantrazole following administration in magnetic cationic microspheres, J. Drug. Targeting 1 (1993) 7–14.
[11] W.G. Bradley, T.H. Newton, L. Crooks, Physical principles of nuclear magnetic resonance, in: T. Newton, D.G. Potts (Eds.), Modern Neuroradiology, Vol. 2, Clavadel Press, San Anselmo, CA, 1983, pp. 15–62.
[12] M. Rudin, In vivo nuclear magnetic resonance in pharmaceutical research, Triangle, 26 (1987) 101–116.
[13] M. Roser, Herstellung und Charakterisierung von Albumin-Partikeln, Ph.D. Thesis, University of Basel, Basel, 1990.

[14] S.C. Silverstein, R.M. Steinmann, Z.A. Cohn, Endocytosis, Ann. Rev. Biochem. 46 (1977) 669–722.
[15] V. Vetvicka, L. Fornusek, H. Tlaskalova-Hogenova, B. Rihova, Changes in some properties and functions of plasma membranes of macrophages from young, adult and aged mice, Dev. Comp. Immunol. 6 (4) (1982) 755–764.
[16] P.O. Seglen, Preparation of isolated rat liver cells, in: D.M. Prescott (Ed.), Methods in Cell Biology, Vol. XII, Academic Press, New York, 1976, pp. 30–78.
[17] M.E. Fedorko, J.G. Hirsch, Structure of monocytes and macrophages, Semin. Hematol. 7 (1970) 109–124.
[18] L.T. Yam, C.Y. Li, W.H. Crosby, Cytochemical identification of monocytes and granulocytes, Am. J. Clin. Pathol. 55 (1971) 183–190.
[19] C. Sundstroem, K. Nilsson, Establishment and characterisation of a human histiocytic lymphoma cell line (U-937), Int. J. Cancer 17 (1976) 565–577.
[20] P. Harris, P. Ralph, Human leukemic models of myelomonocytic development: a review of the HL-60 and U-937 cell lines, J. Leukocyte Biol. 37 (1985) 407–422.
[21] J.O. Minta, L. Pambrun, In vitro induction of cytologic and functional differentiation of the immature human monocyte like cell line U-937 with phorbol myristate acetate, Am J. Pathol. 119 (1985) 111–126.
[22] B.E. Lehnert, C. Tech, Quantitative evaluation of opsonin-independent phagocytosis by alveolar macrophages in monolayer using polystyrene microspheres, J. Immunol. Methods 78 (1985) 337–344.
[23] F.M. Griffin, Opsonization, in: N. K. Day, R.A. Good (Eds.), Comprehensive Immunology, Vol. 2, Plenum Press, New York, 1977, pp. 85–113.
[24] Y. Tabata, Y. Ikada, Phagocytosis of polymer microspheres by macrophages, Adv. Polmer Sci. 94 (1990) 107–141.
[25] R. Keller, Immunologie und Immunopathologie, Thieme, Stuttgart, 1987.
[26] R.C. Brasch, Contrast enhancement in NMR imaging, in: T. Newton, D.G. Potts (Eds.), Modern Neuroradiology, Clavadel Press, San Anselmo, CA, 1983.
[27] C.J. Van Oss, C.F. Gillman, A.W. Neuman, Phagocytic engulfment and cell adhesives, Dekker, New York, 1975.
[28] Y. Tabata, Y. Ikada, Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophages, Biomaterials 9 (1988) 356–362.