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Cytotoxicity of magnetite-loaded polylactide, polylactide/glycolide particles and solid lipid nanoparticles

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Abstract

Particles from polylactide (PLA), polylactide/glycolide (PLA/GA) and solid lipid nanoparticles (SLN) were produced and loaded with different amounts of magnetite. The in vitro cytotoxicity was determined to assess their toxicological acceptance as intravenous formulation for magnetic resonance imaging and as potential carrier for drug targeting. Viability determinations were performed in suspensions of human granulocytes using the dimethylthiazolyl-diphenyltetrazolium (MTT) test. Particle internalization by the granulocytes was followed using luminol enhanced chemiluminescence (CL). The effective concentrations to reduce the viability to 50% (ED 50%) were 0.38% and 0.30% for high and low molecular weight PLA, 0.15% for PLA/GA. The mechanism of toxicity is the intracellular uptake, with the highest toxicity being observed for the faster degrading polymers, low molecular weight PLA and PLA/GA. The solid lipid nanoparticles proved to be the least cytotoxic preparation with an effective concentration (ED 50%) above 10%.

Keywords: Cytotoxicity; Magnetite; PLA particles; PLA/GA particles; Solid lipid nanoparticles; SLN

1. Introduction

Intravenously injected magnetite dispersions improve the contrast in magnetic resonance imaging (MRI) (Niendorf et al., 1986). Macromolecules such as carboxydextran or chondroitin sulfate (Pfefferer, 1993) are used to stabilize these dispersions. Alternatively, the magnetite can be incorporated into microparticles (Morris, 1981). Magnetite-loaded particles sufficiently low in size can also be used as intravenously injectable carriers for drug targeting. The particles containing superparamagnetic magnetite (Fe_3O_4) can be lo-

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Table 1
Results of particle size characterisation using photon correlation spectroscopy (PCS diameter (nm), polydispersity index (PI)) and laser diffraction particle size analysis (VD = volume distribution, ND = number distribution) of PLA, PLA/GA and SLN particles (lw/hw — low/high molecular weight of polymer, percentage in system code gives magnetite load calculated in % of lipid matrix)

System	Content of magnetite (%) (m/m)	PCS (nm)	PI	Laser diffraction particle size analysis					
				VD(μm)			ND (μm)		
				D10	D50	D90	D10	D50	D90
PLA-lw-0%	0	768	0,54	0,8	1,8	3,3	0,2	0,4	1,2
PLA-lw-10%	10	694	0,45	1,0	7,4	13,0	0,2	0,3	0,6
PLA-lw-30%	30	558	0,37	0,7	6,9	13,0	0,2	0,3	0,6
PLA-lw-50%	50	636	0,41	0,5	6,5	13,0	0,2	0,3	0,6
PLA-hw-0%	0	890	0,36	0,7	1,8	4,6	0,2	0,4	0,9
PLA-hw-30%	30	678	0,31	0,6	1,5	4,2	0,2	0,4	0,9
PLA/GA-0%	0	456	0,40	0,3	0,6	1,0	0,2	0,3	0,6
PLA/GA-10%	10	536	0,36	0,8	2,7	14,0	0,2	0,4	0,9
PLA/GA-30%	30	506	0,36	0,7	2,0	4,6	0,2	0,4	0,9
PLA/GA-50%	50	488	0,31	0,4	0,9	4,1	0,2	0,3	0,9
SLN-0%	0	657	0,35	0,2	0,6	2,3	0,1	0,2	0,3
SLN-10%	10	440	0,21	0,6	1,3	2,8	0,3	0,5	1,1
SLN-10%	10	440	0,21	0,6	1,3	2,8	0,3	0,5	1,1

calized in a certain region of the body by applying an external magnetic field. In general, bovine serum albumin is used to produce magnetic drug carriers (Widder et al., 1980). However, bovine serum albumin particles possess the major disadvantage of a possible immune response. A further application of antibody-linked magnetic particles is cell separation, e.g. in bone marrow transplants to remove lymphocytes (Koegler et al., 1990) causing the host versus graft reaction. Magnetic particles linked to antibodies can also be used to specifically isolate a desired population (Miltenyi et al., 1990). The cells are recovered after isolating the particle-antibody-cell conjugate with an electromagnetic field. Commercial products for cell separation are, amongst others, Dynabeads, magnetite-loaded polystyrene particles. A major disadvantage of the Dynabeads and other similar commercial products is the lack of in vivo biodegradation. This prohibits their use, e.g. in bone marrow cell purification, because unavoidable particle contamination of the transplant will introduce non-biodegradable particles into the body.

To sum up, there are many applications for magnetic particles. By producing polylactide and polylactide/glycolide particles loaded with magnetite, the disadvantages of immunogenicity and lacking in vivo biodegradability were avoided. As alternative carrier magnetite was incorporated into solid lipid nanoparticles (SLN) produced by high pressure homogenisation (Müller and Lucks, 1991; Maaβen et al., 1993; Müller et al., 1993; Schwarz et al., 1994, Müller et al., 1995). In contrast to other methods of preparation (Morris, 1981; Speiser, 1990), high pressure homogenisation of lipids leads to a product with a low number of particles > 5 μm being acceptable for i.v. injection by the regulatory authorities (Schwarz et al., 1994). The SLN possess a sufficiently low content of large particles, therefore avoiding capillary blockade. To assess their injectability with regard to toxicological acceptance, the in vitro cytotoxicity of the three kinds of magnetite-loaded particles was investigated in suspensions of human granulocytes.

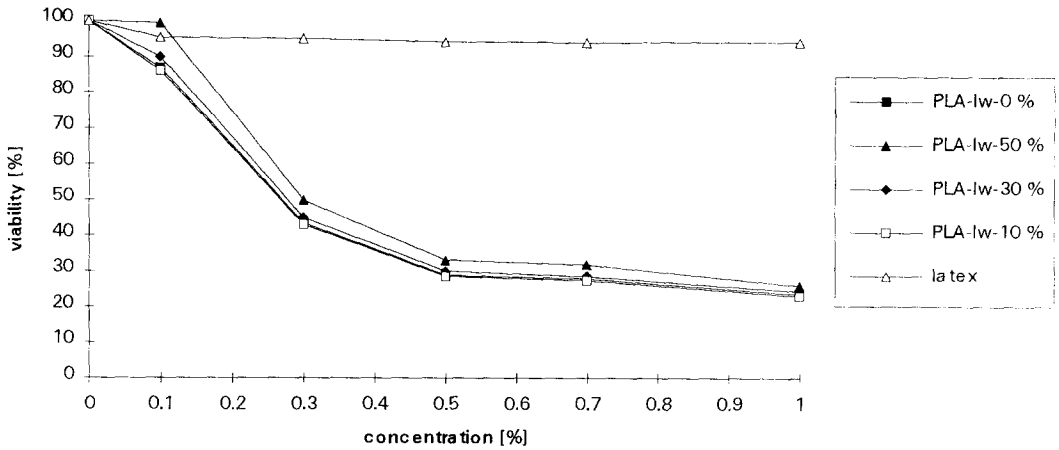

Fig. 1. Viability of human granulocytes after incubation with increasing concentrations of low molecular weight polylactide particles without magnetite (PLA-lw-0%) and with increasing magnetite content (10%, 30%, 50%) compared to non-cytotoxic 498 nm polystyrene particles (latex) (Curves of PLA-lw-0% and PLA-lw-10% superimpose).

2. Materials and methods

Commercial polylactides (PLA) Resomer R 104 and Resomer R 208 (molecular weights 2000, 152 000, inherent viscosities < 1 and 2.2 dl/g, respectively) and polylactide/glycolide (PLA/ GA) Resomer RG 504 (50% lactic acid (LA), 50% glycolic acid (GA), molecular weight 13 000 inherent viscosity 0,5 dl/g) were purchased from Boehringer Ingelheim (Ingelheim, Germany). Synperonic PE/ F68 (Poloxamer 188) was a gift from ICI Surfactants (Middlesbrough, Great Britain). Ferro(III)chloride and ferro(II)chloride (both p.a. quality) were obtained from Merck (Darmstadt, Germany). NH_3 was purchased from Sigma (Deisenhofen, Germany). All chemicals were used as received. Compritol (glycerolbehenate) was a gift from Gattefosse (Weil, Germany).

Polystyrene particles (2.5% w/w aqueous dispersion) with nominal diameters of 480 nm were purchased from Polysciences (Eppelheim, Germany). The diameter determined by PCS was 498 nm, the polydispersity index was 0.09. The cell culture reagents were obtained from Flow Laboratories (Meckenheim, Germany) or from Sigma (cf. for details of the materials) (Rudt, 1992). Luminol was obtained from Merck; dimethylthiazolyl-diphenyltetrazolium (MTT) was obtained from Sigma.

Magnetite was prepared by precipitation of the ferrum (II) and (III) salts with ammonium modifying a method described by (De Cuyper and Joniau, 1990):

$$2FeCl_3 \times 6H_2O + FeCl_2 \times 4H_2O + 8NH_3 \rightarrow$$

$$Fe_3O_4 + 8NH_4Cl + 6H_2O$$

The precipation was performed in water at 70°–80°C which reduces the oxygen content of the precipitation medium. This minimizes the oxydation of superparamagnetic Fe_3O_4 to ferromagnetic Fe_2O_3. The use of high analytical grade ferrum (III) salts is necessary for quantitative reaction to magnetite. Thus, the contamination of ferrum (III) chloride by ferrum (II) salt should be minimized. The precipitate was washed three times with hot water to remove the ammonium chloride and dried over phosphor pentoxide under vacuum. After removal of the water (3 days), magnetite aggregates with a size of 50–100 μm were obtained. The aggregates were easy to deaggregate. The particle size of the magnetite itself was identified being 90 nm by redispersing the aggregates in water using sonication. The magnetite size was therefore higher than in commercial ferrofluids, being in the range of 20–30 nm.

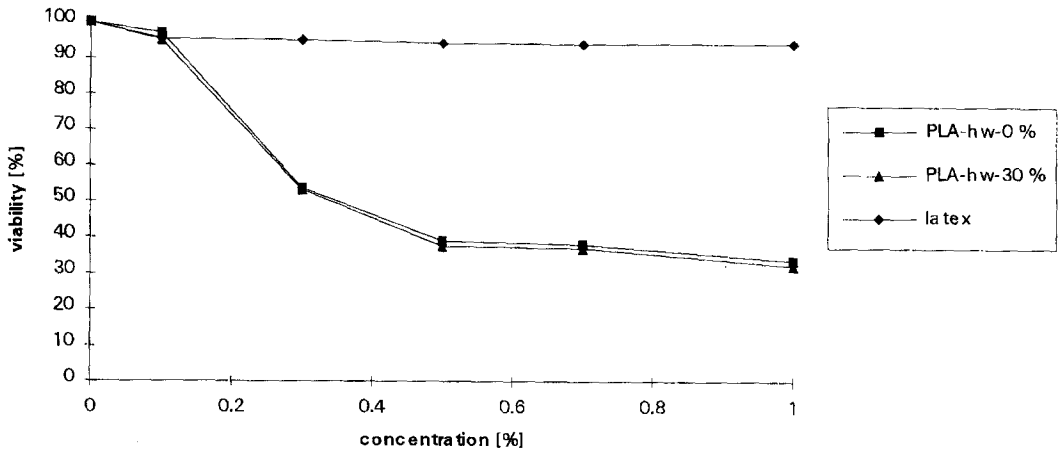

Fig. 2. Viability of human granulocytes after incubation with increasing concentrations of high molecular weight polylactide particles without magnetite (PLA-hw-0%) and with 30% magnetite content (30%) compared to non-cytotoxic 498-nm polystyrene particles (latex).

Magnetite incorporation in polyester polymers was performed by dispersing the magnetite aggregates in ethanol (weight ratio 1:5) by sonication. This ethanolic magnetite dispersion was incorporated in the polyester polymers slightly above the transition temperature (approx. 55°C) and then the temperature slowly increased under stirring to remove the ethanol.

Heating was continued under stirring to melt the polyester for improving dispersion of the magnetite in the polymer. After solidification of the magnetite-containing polymer, grinding was performed, the powder melted again under stirring to achieve homogenous dispersion of the magnetite in the polymer matrix. The grinding and melting process was repeated two times. Polyester particles were produced by high pressure homogenisation of the magnetite-containing polymer particles dispersed in an aqueous poloxamer 188 solution (Müller and Junis-Specht, 1995) Magnetite-free polyester particles were produced in identical manner.

Magnetite-loaded solid lipid nanoparticles were produced by adding the powdered lipid matrix (appr. 50 μm) under stirring to the ethanolic magnetite dispersion at room temperature. The obtained lipid-magnetite dispersion was sonicated and heated slowly under stirring to the melting point of the lipid to remove the ethanol and disperse the magnetite. After solidification, repeated grinding and melting was carried out as described for the polyester particles. Production of the lipid nanoparticles was performed by applying the high pressure homogenisation of solid lipids, as described previously (Müller and Lucks, 1991; Schwarz et al., 1994). Briefly, the powdered lipid was dispersed in aqueous 1.2% Poloxamer 188 solution and homogenized at 500 bar using 3 cycles. Magnetite-free solid lipid nanoparticles were produced by homogenising the magnetite-free lipid. The magnetite payload of the particles was verified by analysing their ferrum content (Specht, 1995).

Photon correlation spectroscopy (Malvern RR102 spectrometer, Malvern Instr., Malvern, UK) in connection with an ALV 1000 channel correlator (ALV Langen, Germany) was used to determine the mean diameter and the polydispersity index. Particle size distribution (measuring range: 0.1–35 μm) was analysed by laser diffractometry (Helos, Sympatek, Clausthal-Zellerfeld, Germany) (Table 1).

Human granulocytes were obtained by density centrifugation as decribed previously (Rudt and Müller, 1992). The cytoxicity was assessed by determining the viability using the MTT test on microtitre plates (Mosmann, 1983). The human granulocytes were dispersed in Dulbeccos phos-

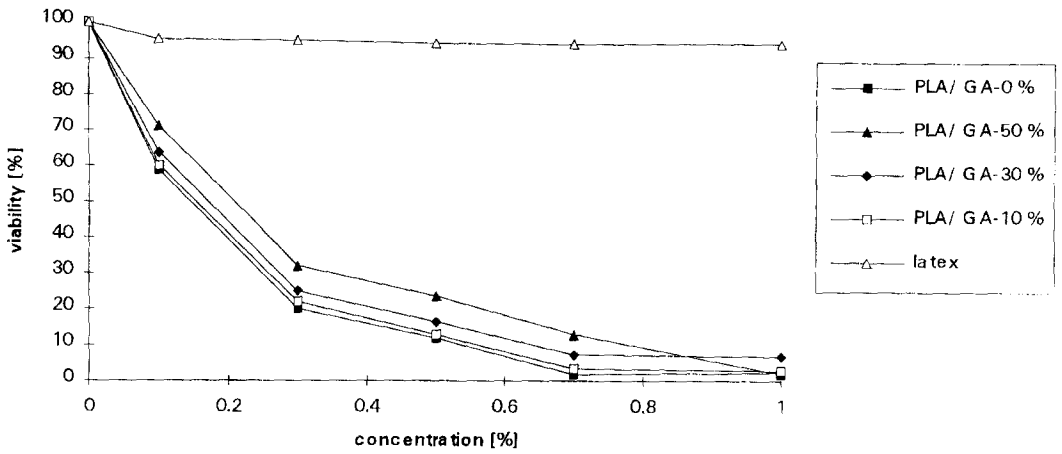

Fig. 3. Viability of human granulocytes after incubation with increasing concentrations of polylactide/glycolide particles without magnetite (PLA/GA-0%) and with increasing magnetite content (10%, 30%, 50%) compared to non-cytotoxic 498-nm polystyrene particles (Latex).

phate buffer (PBS) [NaCl: 800 mg, KCl: 200 mg. Na_2HPO_4: 1150 mg, KH_2PO_4: 200 mg, $MgCl_2 \times 6$ H_2O: 100 mg, $CaCl_2 \times 2$ H_2O: 135 mg and aqua bidest. ad 1000 ml] and the cell number adjusted to 5 x 10^6 cells/ ml; 250 μl of this cell suspension was added to each well of the microtitre plate. For the assay, 50 μl of particle suspension (varying concentrations) was added and incubated for 2 h. The particle concentrations given in the figures and tables are the final concentrations in the well. Then MTT solution (3 mg/ ml PBS) was added and incubated for 2 h. Living cells take up the MTT which is reduced in the mitochondria to the blue tetrazolium salt. The supernatant was removed, the formed blue crystals dissolved in 100 μl isopropanol and the absorption measured at 500 nm (titertek II microtitre photometer, Flow Laboratories). The experiments were performed in triplicate, the relative standard deviations were typically in the range 7–10%.

The uptake of particles by human granulocytes was compared using chemiluminescence (CL) (Amersham research luminometer, Amersham-Buchler, Braunschweig) as described previously (Rudt and Müller, 1992; Rudt and Müller, 1993a; Rudt and Müller, 1993b; Rudt and Müller, 1993c; Rudt and Müller, 1993d) Briefly, particles were added to the cells and the CL intensity (arbitrary units) recorded for 120 min. The uptake was quantified by calculating the area under the curve (AUC) of the intensity/time profiles (integration of CL in arbitrary units, time in minutes, yielding arbitrary AUC units). The AUC was found to be correlated with the total particle mass internalized at the analytical parameters applied in this assay (Rudt and Müller, 1993d).

3. Results and discussion

Non-biodegradable polystyrene particles showed no cytotoxic effects at concentrations up to 1%, the viability was in the range of 94.1–95.3% of the control cells not incubated with particles (Fig. 1). Incubation with low molecular weight polylactide particles led to a distinct reduction in viability when increasing the particle concentrations from 0.1% to 0.3% (Fig. 1). At concentrations above 0.5%, the viability remained unchanged at approx. 30% of the control. The viability/concentration curves were identical for magnetite-free and magnetite-loaded particles. The magnetite is of low toxicity and found to be well tolerated in the human body. Incorporation of magnetite was therefore not expected to increase the cytotoxicity of the polyester particles. The viability/concentration curve of the 50% magnetite containing polylactide particles (PLA-lw-

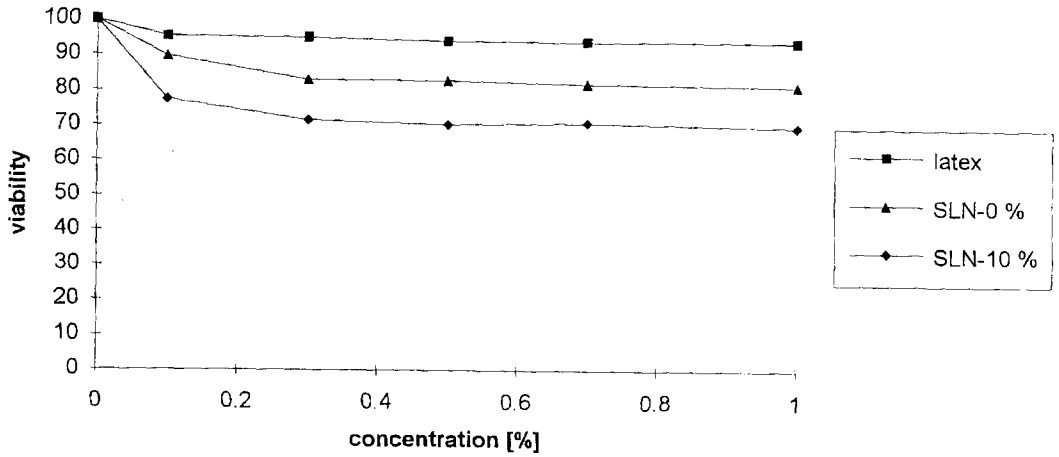

Fig. 4. Viability of human granulocytes after incubation with increasing concentrations of solid lipid nanoparticles without magnetite (SLN-0%) and with 10% magnetite content compared to non-cytotoxic 498-nm polystyrene particles (Latex).

50%) showed the highest viabilities of the investigated particles (Fig. 1). However, this difference was not significant and might therefore only be interpreted as a tendency (cf. below).

The unchanged viability (cytotoxicity) of PLA-lw particles above 0.5% was attributed to the cells reaching their maximum uptake capacity in 0.5% particle suspensions. From this, conclusions could be drawn regarding the cytotoxic mechanism. The cytotoxicity observed is caused by particles internalized by the cells. The viability decreases with increasing particle concentration. At 0.5%, the cells are filled with particles (maximum uptake), the cytotoxicity remains unchanged. The unchanged cytotoxicity supports the assumption that no extracellular cytotoxic effects occur, e.g. caused by adherence to the cell membranes. Such extracellular effects were observed, for example, with cyanoacrylate nanoparticles. They adhere to the cell membrane, are degraded by hydrolysis and release toxic degradation products damaging the cell membrane (Lherm et al., 1992). Degradation of PLA and PLA/GA nanoparticles by hydrolysis in water is relatively slow (Wallis and Müller, 1993) and, therefore, not expected to contribute essentially to a cytotoxic effect within an incubation time of 2 h. The in vivo degradation of polyester implants is considered to take place solely by hydrolysis and not by enzymatic degradation (Tice and Cowsar, 1984). However, the environment and degradation capabilities of granulocytes differ from the body fluids leading potentially to a faster intracellular nanoparticle degradation. Even assuming similar degradation velocity inside and outside the cell, the phagocytic uptake of particle leads to a polymer accumulation inside the cell. Subsequently, the concentration of degradation products inside the cell is higher than in the extracellular environment. For PLA-lw particles, an extracellular cytotoxicity appears therefore unlikely, the cytotoxic effect is caused by internalized particles, e.g. degradation products.

To study the effect of degradation on the cytotoxicity, polylactide particles were produced using a high molecular weight polymer. The degradation velocity decreases with increasing molecular weight of the polymer. The lower intracellular concentration of degradation products should consequently reduce the cytotoxicity. This was confirmed by the significantly higher viabilities observed with the PLA-hw particles (Fig. 2). (t-test double sided).

As expected from the role of degradation products, distinctly higher cytotoxicities should be observed when using particles from relatively fast degrading polymers. The 50:50 copolymer of lactic and glycolic acid is known as the polyester

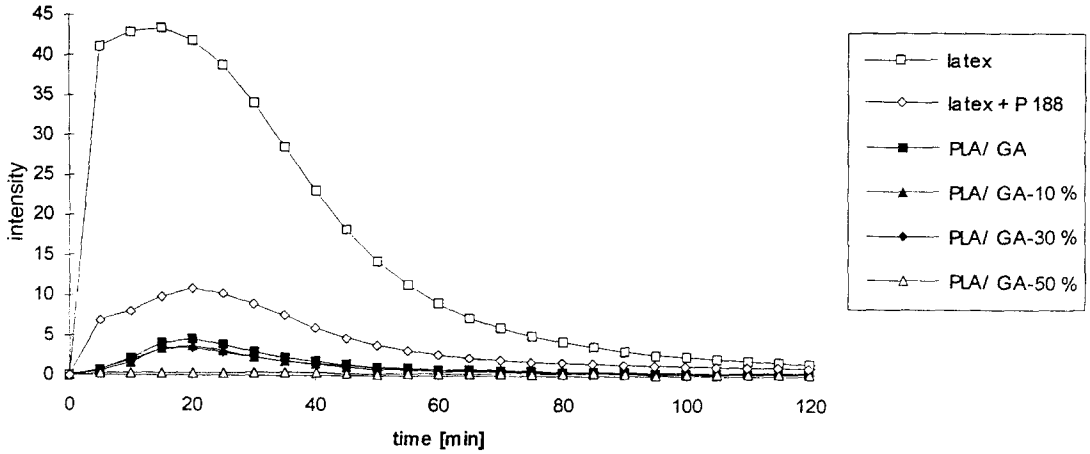

Fig. 5. Quantification of phagocytic uptake: Chemiluminescence intensity/time profiles of human granulocytes incubated with polystyrene particles, polstyrene particles with adsorbed poloxamer 188, and polylactide/glycolide particles, magnetite free and with 10%, 30% and 50% magnetite).

polymer with fastest hydrolytic degradation (Vert et al., 1981). In contrast to PLA implants with a degradation time of 1–2 years, the 50:50 copolymer degrades within 3–6 months. In addition, the size and the surface of the polymeric unit to be degraded plays an important role. Smaller implants (e.g. zoladex injection zylinders, 1.5 diameter, 1.5 cm length) or even microparticles (decapeptyl) degrade within 4–5 weeks (Wallis and Müller, 1993). The PLA/GA particles investigated possessed smaller sizes than the PLA particles (Table 1, e.g. PCS diameter 456–536 nm for PLA/GA but up to 768 nm for PLA-lw). This should additionally accelerate the fast degradation due to the polymer composition. Compared to PLA particles, the viability decreased faster with increasing particle concentration reaching practically zero at 0.7% (Fig. 3). The effective dose to reduce viability to 50% (ED 50%) was 0.35% for PLA-hw, 0.30% for PLA-lw and 0.15% for PLA/GA showing a correlation to the increase in degradation velocity. As observed above with PLA-lw-50% particles, the 50% magnetite loaded particles possess again a lower cytotoxicity than the other PLA/GA particles (Fig. 3, e.g. viabilities at 0.3% particle concentration are significantly different with a confidence interval of 80%).

The viability/ concentration profiles prove a cytotoxicity for polyester particles. To judge the particles regarding their cytotoxicity, they need to be compared with other particles. The ED 50% of human granulocytes incubated with polyhexylcyanoacrylate nanoparticles was 0.001%, indicating a more than 1000-fold lower cytotoxicity for polyester particles. In contrast, the ED 50% could not be reached with solid lipid nanoparticles up to concentrations of 10% in the suspension of human granulocytes (Maaβen et al., 1993). The cytotoxicty of magnetite-free and magnetite-loaded SLN was distinctly lower compared to all polyester particles (Fig. 4).

The minor reduction in viability indicates that the degradation products of SLN are well tolerated inside the cell. Intravenously, as oil in water emulsions, injected liquid triglycerides (e.g. Lipofundin MCT) are degraded very fast within 3–4 h (Davis et al., 1990). The SLN as a natural triglyceride are therefore expected also to be degraded relatively fast, faster than polyesters but, due to their solid character, slower than emulsions. The concentration of degradation products inside the cell is therefore higher than with polyester particles but the cytotoxicity is more then 10-fold lower. The SLN represent, therefore, an alternative colloidal carrier for the intravenous injection

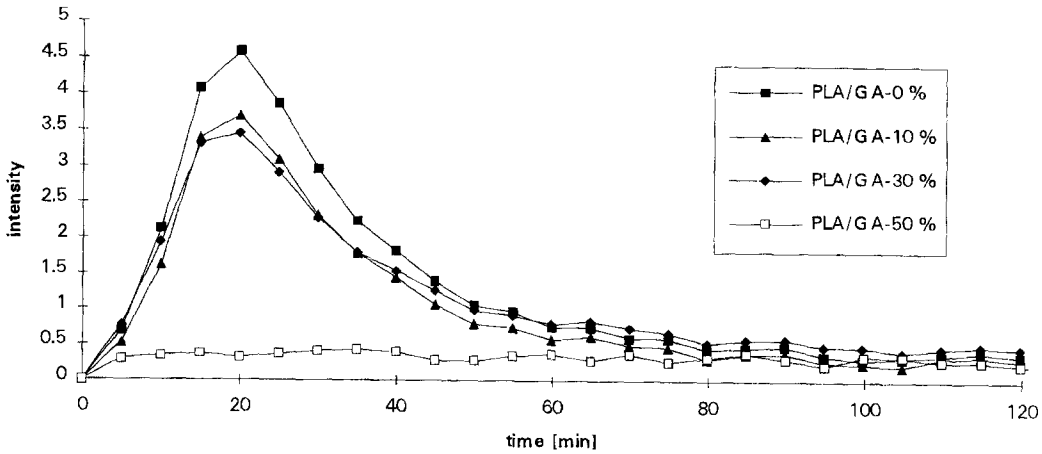

Fig. 6. Quantification of phagocytic uptake: Chemiluminescence intensity/time profiles of human granulocytes incubated with polylactide/glycolide particles, magnetite free and with 10%, 30% and 50% magnetite (identical curves as in figure 5 but with an enlarged scale on the y-axis).

of magnetite. At present, the production of magnetite-loaded SLN is being optimized to achieve a high payload similar to polyester particles. A higher payload is not necessarily desirable for magnetic resonance imaging but potentially of interest for the use of SLN as magnetic carriers for drug targeting similar to albumin particles by Widder et al. (1980).

A comparison of cytotoxicity as performed above is only meaningful if the particles are internalized by the cells to a similar extent. To check this, the phagocytic uptake was studied using chemiluminescence. Polystyrene particles are phagocytosed heavily due to their hydrophobic surface (Fig. 5, upper curve). Compared to the latex particles, the uptake of both polyester particles (e.g. PLA/GA, Fig. 5, lower curves) and SLN was distinctly lower and of similar extent. The low uptake was attributed to a hydrophilic surface of the polyester particles created by the adsorption of the poloxamer 188 used as stabilizer in the dispersion. This could be confirmed by stabilizing the polystyrene particles with 1% poloxamer leading to a distinct reduction in phagocytosis (Fig. 5, middle curve). Poloxamer 188 adsorbs on particles as steric stabilizer, e.g. on polystrene particles in a layer of 7.6 nm thickness (Müller, 1991). This adsorption layer increases the surface hydrophilicity (Müller, 1991) minimising adherence to cell membranes by hydrophobic interaction, the first required step for phagocytosis.

The uptake was similar when comparing PLA, PLA/GA and lipid particles, apart from particles loaded with 50% magnetite (e.g. shown for PLA/GA, Fig. 6). The AUC was typically approx. 170 units, but only 66 units for e.g. PLA/GA-50% (polystyrene particles: 2041 units; poloxamer stabilized polystyrene particles: 568 units). Such a distinct reduction in uptake was previously observed with particles carrying a relatively thick adsorption layer of poloxamer 188 (Rudt and Müller, 1993a). In general, the uptake decreases with increasing adsorption layer thickness. This is due to an increase in the steric stabilisation effect of the adsorption layer and simultaneously increase in surface hydrophilicity of the adsorption layer (Müller, 1991). At 50% magnetite load, a large part of the magnetite will be exposed on the surface of the polyester particles. This might affect the surface hydrophobicity of the polyester particles and, therefore, subsequently the adsorption of the stabilizer poloxamer 188. The reduction in uptake of 50% magnetite loaded particles suggests a thicker poloxamer layer, or at least a difference in the conformation of adsorbed poloxamer exposing more hydrophilic parts of the

molecule. The reduced uptake is well in agreement with the above discussed lower cytotoxicity of PLA-lw-50% and PLA/GA-50% (higher viabilities in Figs. 1 and 3 than other polyester particles).

The cytotoxicity of the PLA and PLA/ GA magnetite-loaded particles is relatively low qualifying them as potential formulation for intravenous injection with regard to the toxicological acceptance. However, the fraction of particles larger than 5 μm needs to be further reduced to avoid capillary blockade. Further optimization of the magnetite loaded particles should achieve a similar quality with regard to particle size as, for example, magnetite-free PLA/GA particles (mean diameter: 570 nm, 100% below 1.80 μm in the volume distribution as determined by laser diffractometry). Solid lipid nanoparticles are more than 10-fold less cytotoxic compared to the polyester particles and the amount of large particles is smaller. This promotes their use as an alternative carrier for magnetite.

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