

Improvement of the separation of tumour cells from peripheral blood cells using magnetic nanoparticles

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Abstract

Circulating tumour cells are a key challenge in tumour therapy. Numerous approaches are on the way to achieving the elimination of these potential sources of metastasis formation. Antibody-directed magnetic cell sorting is supposed to enrich tumour cells, with high selectivity, but low efficiency. The short term application of carboxymethyl dextran (CMD) coated magnetit/maghemit nanoparticles allows the discrimination of tumour cells from leukocytes. In the present work we show that the interaction of CMD nanoparticles is cell-type specific and time dependent. The breast cancer cell line MCF-7 and the CML cell line K-562 are characterized by a rapid and high interaction rate, whereas leukocytes exhibit a decelerated behaviour. The addition of carboxymethyl dextran or glucose stimulated the magnetic labelling of leukocytes. The variation of the degree of substitution of dextran with carboxymethyl groups did not affect the labelling profile of leukocytes and MCF-7 cells. In order to verify the *in vitro* results, whole blood samples from 13 cancer patients were analysed *ex vivo*. Incubation of the purified leukocyte fraction with CMD nanoparticles in the presence of low amounts of plasma reduced the overall cell content in the positive fraction. In contrast, the absolute number of residual tumour cells in the positive fraction was 90% of the initial amount.

1. Introduction

Magnetic nanoparticles with various shells are widely used for specific labelling and detection of cells. Treatment of solid tumours and haematological disorders would benefit from an accurate and quantitative discrimination of tumour cells from healthy cells, e.g. in the peripheral blood. During the last decade a couple of nanoparticle based techniques have been developed

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to target tumour cells and eventually magnetic assisted cell sorting (MACS) has become a powerful tool for detecting and enriching circulating disseminated tumour cells (Wong *et al* 1995, Berois *et al* 1997, Eaton *et al* 1997, Mapara *et al* 1997, Naume *et al* 1997, Martin *et al* 1998).

Mostly magnetic nanoparticles require covering with a shell which plays an important role for the interaction with its target. For biomedical applications the biocompatibility of the magnetic nanoparticles, especially the shell, is essential. One of the most popular coatings is dextran and its derivatives. They show a low unspecific reactivity with peripheral blood cells (Pitha 1978) and loaded with specific ligands they could interact with peripheral blood cells specifically (Molday and MacKenzie 1982). The magnetic properties could then be used to enrich or deplete cell populations from peripheral blood or other complex cell mixtures *ex vivo* (Trickett *et al* 1991, Hancock and Kemshead 1993, Haukanes and Kvam 1993). The application of dextran coated magnetic nanoparticles *in vivo* has allowed discrimination of tumour infiltrated lymph nodes from lymph nodes free of metastasis (Anzai *et al* 1994). Several other approaches based on magnet resonance imaging are used to detect lymph node metastases with high precision (Harisinghani and Weissleder 2004, Will *et al* 2006). Interaction of magnetic nanoparticles with cells is not only restricted to the cell surface. The particles can be incorporated into cells by endocytosis and be accumulated in endosomes (Jordan *et al* 1996, 1997, Schoepf *et al* 1998, Wagner *et al* 2004), as demonstrated by electron microscopy.

We have shown previously that tumour cell line cells can be separated efficiently from a leukocyte–tumour cell suspension using magnetic nanoparticles with a carboxymethyl dextran (CMD) shell (Schwalbe *et al* 2005, Clement *et al* 2006). The enrichment of the tumour cells was dependent on factors such as the incubation time, the osmolality or the plasma concentration during incubation. The majority of the tumour cells could be labelled within 4–8 min whereas only a minority of leukocytes were significantly loaded with magnetic nanoparticles. These observations show that the dextran shell is an important prerequisite for separating tumour cells from leukocytes.

The aim of this study was to further investigate and improve the specific interaction of carboxymethyl dextran coated magnetic nanoparticles with leukocytes from peripheral blood and tumour cells. For this purpose we investigated the role of the degree of substitution of dextran with carboxymethyl groups and the incubation conditions with special regard to temperature and carbohydrate supplementation during nanoparticle cell interaction. Finally, *in vitro* observations were transferred to the *ex vivo* situation and peripheral blood samples from cancer patients analysed for tumour cell loading.

2. Materials and methods

2.1. Cell culture

The cell lines used for these investigations were obtained from DSMZ (Braunschweig, Germany) or ATCC (Rockville, USA) and are listed in table 1. The cell lines were cultivated under standard conditions as indicated. Adherent cell line cells were harvested by treatment with trypsin in order to detach the cells from the plastic surface of the cell culture flask.

2.2. Preparation of leukocytes from peripheral blood

Leukocytes from peripheral blood were prepared by erythrocyte lysis (Qiagen, Hilden, Germany) from whole blood samples of healthy volunteers or patients with informed consent. Before erythrocyte lysis the plasma fraction was removed by centrifugation ($300 \times g$, 5 min,

Table 1. Origin and cultivation of cell lines. (FCS: foetal calf serum.)

Cell line	Origin	Culture medium
BT-20	Human breast adenocarcinoma	DMEM + 10% FCS
BT-474	Human breast ductal carcinoma	RPMI1640 + 10% FCS
Caski	Human cervix epidermoid carcinoma	RPMI1640 + 10% FCS
CHRF	Human megakaryoblastic	RPMI1640 + 10% FCS
HBMEC	Human brain microvascular endothelium	RPMI1640 + 10% FCS
HepG2	Human hepatocellular carcinoma	DMEM + 10% FCS
K-562	Human chronic myeloid leukemia in blast crisis	RPMI1640 + 10% FCS
MCF-7	Human breast adenocarcinoma	DMEM + 10% FCS
NB-4	Human acute promyelocytic leukemia	RPMI1640 + 10% FCS
SK-BR-3	Human breast adenocarcinoma	RPMI1640 + 10% FCS
T-47D	Human breast ductal carcinoma, pleural effusion	RPMI1640 + 15% FCS

14 °C); the whole supernatant was defined as plasma. The leukocyte pellet was washed with erythrocyte lysis buffer twice and finally resuspended in PE buffer (phosphate-buffered saline (PBS) and 2 mmol EDTA).

2.3. Magnetic nanoparticles

Magnetic nanoparticles consisting of a superpara(ferro)magnetic core made from magnetite/maghemite were obtained from N Buske (Magneticfluids, Berlin, Germany). The TEM size diameter of the core varied between 3 and 15 nm. The average diameter was 5 nm. The nanoparticles were coated with carboxymethyl dextran as previously described (Wagner *et al* 2004). The average degree of substitution of the dextran with carboxymethyl groups was 0.8. Repeating of the procedure resulted in higher DS values (Wagner *et al* 2004). The hydrodynamic diameter of the generated nanoparticle clusters was 200–300 nm. The saturation magnetization ranged from 4.5 to 6.2 mT. Iron oxide nanoparticles coated with citric acid, carboxymethyl beta-cyclodextrin, bovine serum albumin and L-glutamic acid as well as cobalt ferrit nanoparticles were kindly provided by N Buske. The average nanoparticle size was 10 nm. The barium ferrit nanoparticles were a generous gift from R Müller (IPHT, Jena, Germany). These particles with a core size of 70–100 nm were coated with CMD as previously described (Schwalbe *et al* 2006).

2.4. Incubation of cells with magnetic nanoparticles

For kinetic analysis cell line cells (1×10^6 cells per 500 μ l PE) and/or leukocytes (2.5×10^6 cells per 500 μ l PE) were incubated in short term incubation with 2.5 μ l/500 μ l PE magnetic nanoparticles at 37 °C. Preceding experiments showed that addition of 2.5 μ l of a nanoparticle solution led to an optimal interaction rate independent of the batch. A further increase of the nanoparticle concentration in the reaction did not increase the interaction behaviour of the cells indicating that the nanoparticles are in far excess. For *ex vivo* investigations the white blood cells, containing the putative tumour cell fraction, derived from 1 ml tumour patient blood were incubated for the times indicated in a plasma–PE mixture supplemented with different amounts of plasma (0, 1, 5%). After the treatment magnetically labelled cells were separated using a SuperMACS and MS columns (Miltenyi-Biotec, Bergisch-Gladbach, Germany). The separated cells were designed as positive fraction (retained in column) and negative fraction (effluent). The number of cells in each fraction was enumerated (Particle Count & Size Analyzer Z2, Beckman-Coulter, Krefeld, Germany). For determination of the tumour cell content in the

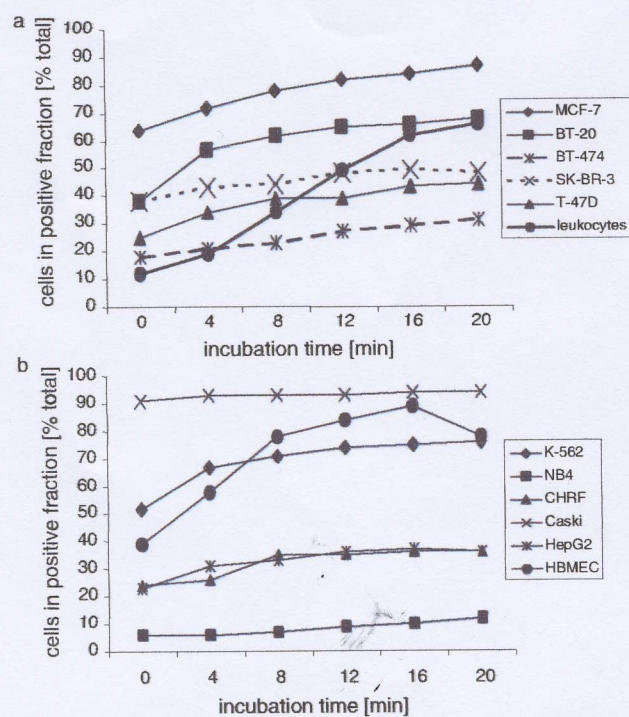


Figure 1. Time-dependent labelling of cell line cells and leukocytes with CMD coated magnetic nanoparticles. Cells were incubated with nanoparticles in PE for the times as indicated and separated by MACS. Cell content in the positive and negative fraction was estimated. (a) Breast cancer cell lines and leukocytes; (b) various cell lines.

ex vivo experiments an aliquot of the original sample, of the positive and the negative fraction was analysed by flow cytometry (FACS Calibur, Becton-Dickinson, Heidelberg, Germany) using epithelium-antigen specific staining for the detection of tumour cells (Anti-HEA FITC, Miltenyi biotec, Bergisch-Gladbach, Germany) and CD45 for the estimation of the leukocyte content (CD45 PE, Miltenyi Biotec, Bergisch-Gladbach, Germany).

3. Results

3.1. Cell-type specific interaction of cells with magnetic nanoparticles

Magnetic nanoparticles provide a suitable strategy for separating distinct subpopulations of cells from cell suspensions where the interaction of nanoparticles with the cells is usually directed by antibodies attached to the nanoparticles (Kandzia *et al* 1981, Abts *et al* 1989). We have, however, shown previously that there is a differential interaction of only carboxymethyl dextran coated magnetic nanoparticles with tumour cells and leukocytes (Schwalbe *et al* 2005). Tumour cells showed a more rapid interaction than leukocytes during an 8 min exposure. The difference could be further enhanced by a slightly increased pH of the incubation solution and by the addition of up to 5% plasma. In order to further improve the preferential labelling of tumour cells we investigated the time dependence of the interaction. Various cell lines (table 1) and leukocytes from peripheral blood were incubated with CMD coated nanoparticles. At defined time points the cells were magnetically separated (figure 1). The breast cancer cell

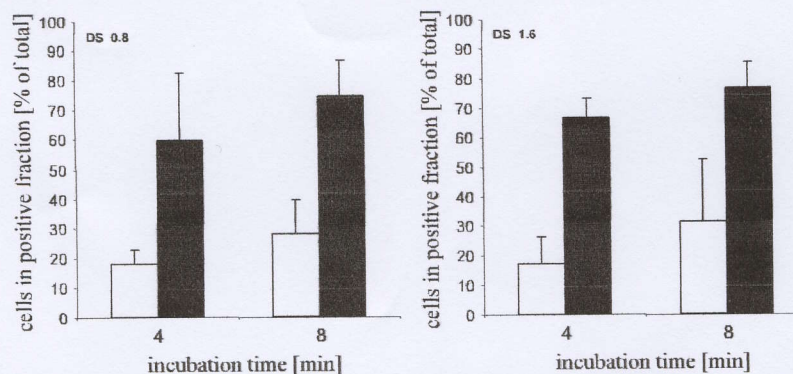


Figure 2. The degree of substitution (DS) of dextran with carboxymethyl groups does not affect the labelling efficiency. MCF-7 cells (black bars) and leukocytes from peripheral blood (white bars) were incubated with CMD coated nanoparticles with DS 0.8 and DS 1.6 for 8 min in PE. Cells were separated by MACS and the cell content in the positive and negative fraction was estimated. MCF-7: $n = 9$; leukocytes: $n = 14$.

lines MCF-7, BT-20, BT-474, SK-BR-3 and T-47D showed a continuous increase of labelled cells over a time period of 20 min. MCF-7 was the most active cell line with a maximum of nearly 90% labelled cells whereas BT-474 exhibited only a low degree of labelling (30%). In contrast to the breast cancer cell lines the blood-derived leukocytes showed a sigmoidal curve with a sharp increase between 4 and 16 min (figure 1(a)). The interaction of CMD coated magnetic nanoparticles with Caski, HepG2, CHRF and NB-4 was more or less unaltered over the whole incubation period. The interaction profile of K-562 was similar to that of MCF-7 whereas HBMEC cells displayed a steady increase during the first 8 min with a flattening during the following 12 min (figure 1(b)). The degree of labelling was cell-type specific. MCF-7, Caski and HBMEC showed a high degree of labelling. In contrast, NB-4 was nearly not affected by the CMD coated nanoparticles.

3.2. The nanoparticle shell is critical for the nanoparticle–cell interaction

Magnetic nanoparticles without a carbohydrate shell interact instantaneously with most cell types. This holds true for magnetite/maghemite nanoparticles as well as for cobalt ferrite and barium ferrite nanoparticles, which are toxic for the cells.

A broad variety of carbohydrates is, therefore, used to cover magnetic nanoparticles, e.g. for biocompatibility reasons. Such coatings may influence the interaction of the particles with different cell surfaces. We studied several of these coatings in order to maximize differential interaction of magnetic nanoparticles with different cells. In our hands carboxymethyl dextran proved to optimally differentiate tumour cells from leukocytes (Schwalbe *et al* 2006). Carboxymethyl groups are negatively charged and therefore the degree of substitution of dextran with carboxymethyl groups alters the overall charge and in consequence may influence the interaction of the coated nanoparticles with cells. In order to optimize the charge of the CMD nanoparticles we investigated the role played by the degree of substitution (DS) of dextran with carboxymethyl groups. In figure 2 the interaction profiles of CMD nanoparticles with a DS of 0.8 and 1.6 are shown. With both types of CMD nanoparticles leukocytes could clearly be discriminated from MCF-7 cells after a 4 min as well as 8 min incubation. There was only a minor difference between these two preparations. For further analysis we used carboxymethyl dextran with DS 0.8.

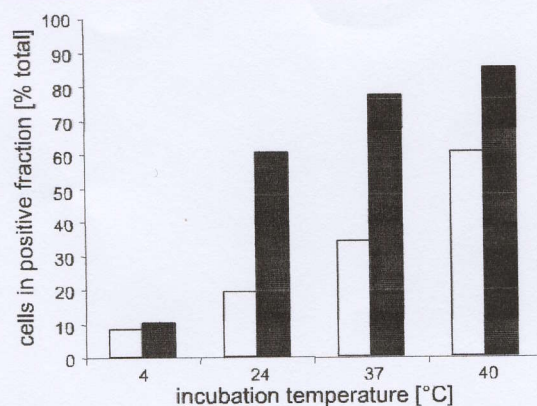


Figure 3. The labelling of cells with magnetic nanoparticles is temperature dependent. Cells and incubation medium were adjusted to the indicated temperatures for 30 min. After 8 min of incubation cells were separated by MACS. The cell content of the positive and negative fraction was estimated. MCF-7 (black bars): $n = 2$; leukocytes (white bars): $n = 2$.

Table 2. Interaction of nanoparticles with various shells with cells.

Core	Magnetic nanoparticles		Cells in positive fraction (%) ^a	
	Shell		MCF-7	Leukocytes
Iron oxide	Carboxymethyl beta-cyclodextrin		96	94
Iron oxide	Citric acid		92	98
Iron oxide	Bovine serum albumin		81	79
Iron oxide	L-glutamic acid		84	82
Iron oxide	Without		91	88
Cobalt ferrit	Without		97	98
Barium ferrit	Without		91	93

^a Incubation time: 8 min.

An interesting carbohydrate is carboxymethyl beta-cyclodextrin which forms tube-like structures. Magnetic nanoparticles coated with carboxymethyl beta-cyclodextrin did not allow discrimination between the tumour cell lines MCF-7, K-562 and peripheral blood leukocytes (table 2). Immediately after addition of the nanoparticles more than 85% of the cells, independent of their origin could be separated by magnetic force. Interestingly, the maximum amount of labelled MCF-7 and K-562 cells was reached already after 4 min whereas leukocytes required 8 min of interaction to become separable to the same extent. The coating of magnetic nanoparticles with various shells, e.g. citric acid, L-glutamic acid or bovine serum albumin did not allow separation of cells in a cell-type specific manner after an 8 min incubation (table 2).

3.3. Temperature and soluble carbohydrates affect the nanoparticle-cell interaction

Besides core and shell of the nanoparticles the buffer conditions might also modulate the interaction with cells. Usually cell culture cells are cultivated at 37 °C comparable to the mean temperature conditions of the human body. The temperature distribution in the human body however is not homogeneous. The temperature of the torso is quite uniform but in the limbs for instance the temperature is reduced. Therefore we performed incubations of CMD nanoparticles with leukocytes and MCF-7 cells at 4, 24, 37 and 40 °C (figure 3). An increase in temperature from 37 to 40 °C during an incubation for eight minutes doubled the amount

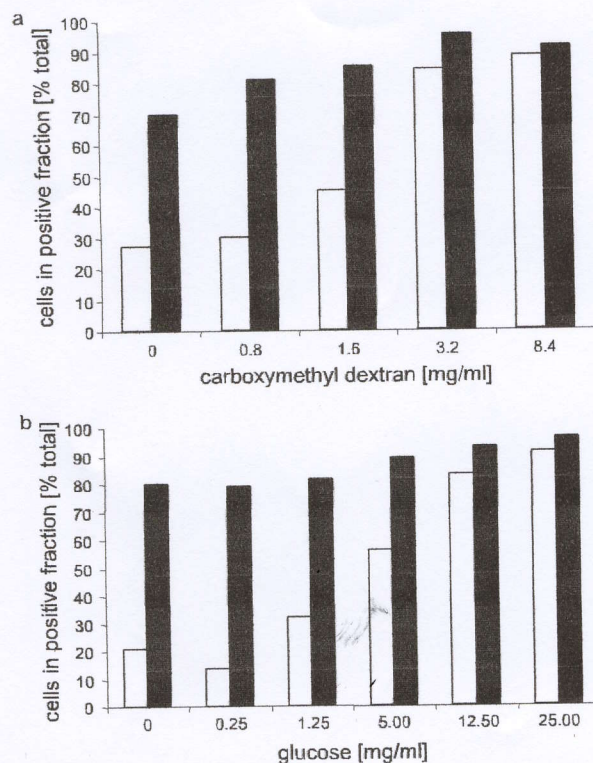


Figure 4. The addition of soluble carboxymethyl dextran and glucose enhances the interaction of CMD nanoparticles and leukocytes. MCF-7 cells and leukocytes were incubated with the nanoparticles in PE and the indicated concentrations of carboxymethyl dextran and glucose for 8 min. Cells were separated by MACS. The amount of cells in the positive and the negative fraction was estimated. (a) Addition of carboxymethyl dextran; (b) supplementation of glucose. MCF-7 (black bars): $n = 3$; leukocytes (white bars): $n = 3$.

of separable leukocytes, whereas at a temperature of 24 °C the leukocyte pool in the positive fraction was reduced to 50% as compared to 37 °C. MCF-7 cells were also affected by the temperature but to a lesser extent. The amount of labelled MCF-7 cells was reduced from 85% at 40 °C to 60% at 24 °C. The interaction of CMD nanoparticles with leukocytes and with MCF-7 is nearly completely abolished at 4 °C.

The carbohydrate shell plays an important role for the cell-type specific and time-dependent interaction of the nanoparticles with cells. The addition of soluble carbohydrates during incubation might influence that interaction. We tested several carbohydrates known to be biocompatible for their ability to interfere with the cell-type specific magnetic labelling of tumour cells and leukocytes during incubation (figure 4). Addition of carboxymethyl dextran in a range from 0.8 to 8.4 mg ml⁻¹ enhanced the labelling of leukocytes with CMD nanoparticles from 30% to 80% during a incubation of eight minutes. The portion of MCF-7 cells in the positive fraction was only marginally increased from 80% to 90%. Other polysaccharides like methylcellulose or polysucrose did not alter the labelling profile of leukocytes or MCF-7 cells. Among the monosaccharides only glucose interfered with the cell-type specific magnetic labelling of cells. In the range from 0.25 to 25 mg ml⁻¹ glucose the portion of leukocytes in the

positive fraction raised from 20% up to 90% within 8 min of incubation. Glucose induced only a minor increase of magnetically labelled MCF-7 cells. Other monosaccharides like fucose or mannose had no effect on the cell-type specific interaction of tumour cells and leukocytes.

3.4. Separation of epithelial tumour-derived cells from peripheral blood

These experiments show that the cell-type specific interaction of magnetic nanoparticles with tumour cells can be modulated by the nature of the carbohydrate shell as well as the incubation conditions. In addition, we could recently demonstrate that human plasma affects the interaction of CMD magnetic nanoparticles with cells (Schwalbe *et al* 2005). In brief, leukocyte preparations spiked with various amounts of MCF-7 cells incubated with CMD magnetic nanoparticles and subsequent separation led to a dramatic reduction of the cell number in the positive fraction. Surprisingly, the total number of MCF-7 cells retained in the positive fraction was almost constant at a plasma content of 1% and slightly reduced at a plasma content of 5%.

To prove the hypothesis that our approach is suitable for the separation of tumour cells from the peripheral blood of cancer patients we analysed blood samples from 13 patients suffering from various solid tumours (e.g. breast cancer, non-small cell lung cancer, gastric cancer). After erythrocyte lysis of the whole blood, the remaining leukocyte fraction was incubated with CMD nanoparticles without plasma or in the presence of 1% and 5% plasma. After magnetic separation the positive and negative fraction was analysed by FACS and compared to a control sample acquired before the incubation with magnetic nanoparticles. Epithelial cells were labelled with a HEA specific antibody, whereas the leukocytes were detected using anti-CD45. Taking into account that usually no epithelial-like cells are detectable in peripheral blood we consider HEA-positive cells as disseminated tumour cells. The average proportion of leukocytes and tumour cells in the untreated samples was $99.83 \pm 0.46\%$ versus $0.17 \pm 0.46\%$. Incubation of the leukocyte fraction with CMD nanoparticles for 8 min in the presence of plasma led to a dramatic reduction of cell number in the positive fraction (figure 5(a)). Without addition of plasma the positive fraction contained 81% of the initially applied cells. In the presence of 1% plasma the number of magnetically labelled cells in the positive fraction was reduced to 51% and in the presence of 5% plasma to 17%. Inversely cells accumulated in the negative fraction. In contrast the HEA-positive cells increased up to threefold with the increment of plasma in the positive fraction (figure 5(b)) and declined in the negative fraction (figure 5(c)). The absolute number of tumour cells in the positive fraction was 90% of the initially measured content in the not separated sample. This was independent of the addition of plasma.

4. Discussion

The separation of selected cell populations is a vital problem in biomedicine. Magnetic nanoparticles have become an important tool for meeting this challenge. An essential component of a biocompatible magnetic nanoparticle is the shell and its functional groups. In case of antibody-directed cell separation antibodies against cell surface markers are attached to the nanoparticles. They allow a precise and high enrichment of the cells bearing the antigenic determinant (Abts *et al* 1989, Miltenyi *et al* 1990). However, the separated fraction has high homogeneity but usually a low recovery with a substantial amount of positive target cells remaining in the flow through. This might contribute to the hitherto disappointing results of clinical trials evaluating the effect of *in vitro* purging in the setting of autologous transplantation for aggressive lymphomas (Jacobsen and Freedman 2004). In attempts to purify human blood

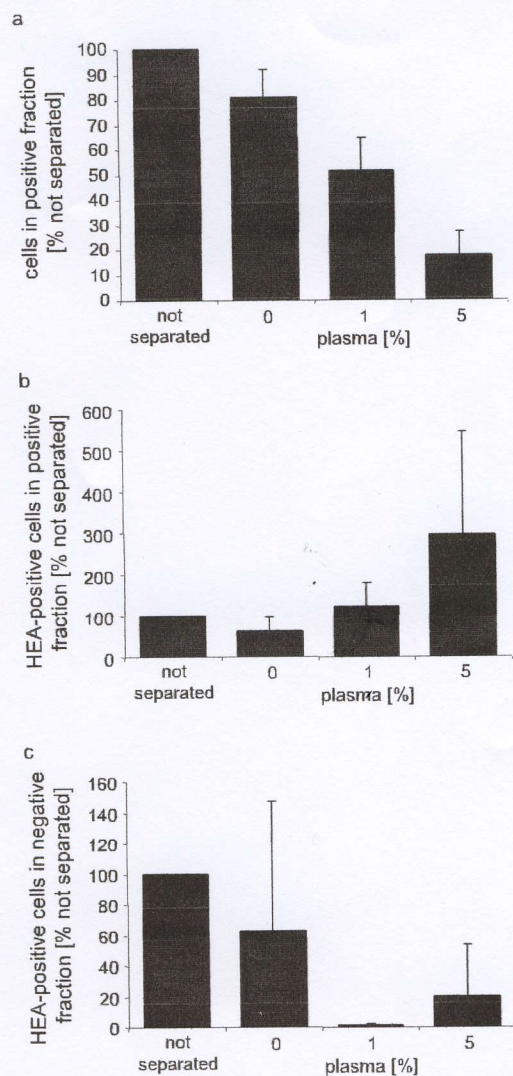


Figure 5. Enrichment of HEA-positive cells from the peripheral blood of tumour patients. The leukocyte fraction from the peripheral blood of tumour patients was incubated with CMD nanoparticles for 8 min in the presence of plasma as indicated. The cells were separated by MACS. For FACS analysis epithelial cells were labelled with HEA and leukocytes with CD45. (a) The total amount of cells is reduced in the presence of increasing amounts of plasma. (b) The HEA-stained cells are enriched in the positive fraction with rising concentrations of plasma. (c) The amount of HEA-positive cells is reduced in the negative fraction. $n = 13$.

from tumour cells any residual tumour cell might be hazardous. Therefore, other approaches besides antibody coating might be helpful to differentiate between tumour cells and leukocytes in peripheral blood.

We were able to show previously that carboxymethyl dextran coated magnetit/maghemit nanoparticles interact preferentially with tumour cells and less with peripheral blood leukocytes

in vitro (Schwalbe *et al* 2005, Clement *et al* 2006). The addition of low amounts of human plasma amplified that effect. The labelling of the cells increases over a time period of 20 min. The leukocytes show a delayed interaction compared to the breast cancer cell line MCF-7. The analysis of the interaction kinetics of CMD nanoparticles with various cell lines and leukocytes from peripheral blood showed that the interaction process is cell-type specific and time dependent. The magnetic separation in columns does not distinguish between incorporated and attached magnetic nanoparticles. Chithrani *et al* (2006) showed that the uptake of gold nanoparticles with a diameter of 14 nm into HeLa cells started immediately after application of the nanoparticles and reached a half-maximal uptake after more than 2 h. This indicates that only a minor portion of the nanoparticles are expected to be inside the cells. Thus the initial contact of the CMD nanoparticles with the outer cell membrane might contribute to the differences in magnetic labelling. In a first attempt, we varied the degree of substitution of dextran with carboxymethyl groups. In general, an increasing amount of carboxymethyl groups makes the nanoparticles more anionic. However, the degree of substitution did not remarkably influence the amount of magnetically separable cells and the leukocyte–tumour cell ratio in the positive fraction. The cell surface composition of tumour cells differs from that of normal cells. Altered expression of membrane proteins as well as the exposed sugar residues might, therefore, affect the overall charge of the cell surface and contribute to the differences between leukocytes and tumour cells, especially MCF-7 and K-562 more than the substitution degree of the shell. Another important aspect is the membrane recycling which is driven by endocytosis. The rate of plasma membrane turnover is cell-type specific, e.g. fibroblasts ingest 3% of its plasma membrane each minute. In contrast, macrophages as professional phagocytic cells ingest 25% of their plasma membrane per minute (Alberts *et al* 2002). During malignant transformation the composition and the properties of the cell membrane change. The structure of glycoproteins and glycolipids is altered, e.g. the amount of the high molecular weight cell surface glycoprotein LETS is reduced during transformation of fibroblasts (Yamada *et al* 1976). In addition, the substrate intake of tumour cells is enhanced compared to that for normal cells. Thus further investigations on the properties of endocytotic mechanisms of tumour cells and leukocytes are required to understand the differential interaction behaviour in more detail.

Incubation conditions play an important role for the interaction of nanoparticles with cells. The labelling of leukocytes as well as MCF-7 cells is temperature dependent. Leroux *et al* (1994) showed that the uptake of poly(D,L-lactic acid) nanoparticles by monocytes and lymphocytes is increased from 4 to 37 °C and thus should be, at least in part, an energy-requiring process. Another approach for modifying the cellular uptake of nanoparticles is adding proteins or carbohydrates during incubation. Supplementation of soluble carboxymethyl dextran or glucose during nanoparticle–cell interaction markedly increased the leukocyte content in the positive fraction. The elevation of the number of magnetically separable cells by glucose might be due to an increase of the intracellular Ca^{2+} level and a resulting higher capacity of endocytotic vesicles (MacDonald *et al* 2005).

The manipulation of the magnetic labelling procedure of tumour cells and leukocytes *in vitro* offers a range of opportunities to optimize the separation efficiency of both types of cells. In a biomedical setting a maximum of tumour cells in the positive fraction in concert with a minimum level of leukocytes is preferred. The analysis of the first patient samples *ex vivo* supports the idea that short term incubation of leukocytes from the peripheral blood of tumour patients with carboxymethyl dextran coated magnetic nanoparticles in the presence of low amounts of plasma allows magnetic tumour cell depletion.

In order to further validate our procedure we intend to study a larger cohort of patients. The variety of tumours and the resulting diversity of circulating tumour cells may require an adaptation of our approach. Finally, a better understanding of the processes at the cellular level

which are responsible for the nanoparticle–cell interaction and also the uptake are of future interest.

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	nr of patients	patients in complete remission	relapsed patients	p-value
	19	6		
mean age	48	34	0.038	
ER pos %	56	0	0.029	
ER neg %	22	22	0.005	
node neg %	68	0		
node pos %	20	12		
cell number before therapy in 5L blood	1010835	90257	n.s.	
cell number end of therapy in 5L blood	952181	131288	n.s.	
increase >10 fold %		24	<0.0001	
decrease or no change %	76	0		

Table 2a

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