Determination of Plasma Protein Adsorption on Magnetic Iron Oxides: Sample Preparation

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Purpose. The purpose of this study was to investigate the influence of the sample preparation on the plasma protein adsorption pattern of polysaccharide-stabilized iron oxide particles by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE).

Methods. The iron oxide particles were incubated in vitro in human plasma for five minutes. Thereafter, four different methods for particle recovery, including adsorbed proteins from surplus plasma, were investigated: centrifugation, magnetic separation, gel filtration and membrane-based static microfiltration. Adsorbed proteins were desorbed from the particle surfaces by surfactants and analyzed by 2-D PAGE, as described elsewhere (1,2).

Results. All the techniques investigated were able to separate small-size iron oxides (approx. 110 nm) and adsorbed proteins from excess plasma. The gels obtained by the different separation procedures displayed almost identical adsorption patterns. Major proteins identified were: fibrinogen, IgG, albumin and an unclassified protein of about 70 kDa with a pI value of 6.5–7.5.

Conclusions. Centrifugation was regarded as the most suitable separation method due to its speed and ease of use. In contrast to gel filtration, any washing media can be used. The magnetic separation process is restricted to particles with high inducible magnetic saturation, in particular, to iron oxides with overall sizes > 50 nm.

KEY WORDS: iron oxides; sample preparation; 2-D PAGE; plasma protein adsorption.

INTRODUCTION

Superparamagnetic iron oxide particles (SPIO) stabilized by dextran macromolecules are important organ-specific contrast media in magnetic resonance imaging (MRI) (3–5). The diagnostic potential of SPIO in the detection of small liver and spleen metastases is well-established, and several formulations of these MPS-type (mononuclear phagocytic system) contrast agents are under clinical investigation (6,7). The first representative of dextran-coated SPIO has already been approved for clinical use in Europe (Endorem). The size and surface properties of the particles determine their biological and pharmacological properties e.g. blood half life and tissue distribution (2,8,9). To make new indications accessible, e.g. application in MR lymphography (10,11), rapid uptake by the liver and spleen has to be prevented. The dependence on the biological fate of intravenously injected SPIO has already been elaborated (5), but there is a lack of knowledge concerning their in vivo behavior and surface properties. After injection, the particles interact with plasma proteins, in particular opsonins. In recent years attention has focussed on this protein adsorption because it is considered to be the key factor determining organ distribution (so called "differential adsorption"). Successful correlation of the physicochemical particle properties, protein adsorption and in vivo data should provide new tools for the design of advanced particle systems for medical purposes, e.g. lymphotropic contrast agents.

High-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) may be the method of choice to investigate plasma protein adsorption on SPIO particles because this method has already been successfully applied to the study of protein adsorption on several particulate systems. However, attention has to be paid to the sample preparation to avoid artificial protein adsorption due, for example, to desorption during the separation of the particles and excess plasma. The recovery of particles with adsorbed proteins from surplus plasma was identified as the main problem, especially for ultrasmall SPIO (diameter: appr. 30 nm) with sizes very close to the larger physiological proteins (e.g. ferritin, IgM). The aim of our study was to develop suitable separation procedures for small (100 nm) to ultrasmall (30 nm) SPIO particles. Four methods were investigated to assess their applicability: 1) centrifugation (2), 2) gel filtration, 3) static microfiltration and 4) use of the magnetic particles' properties, magnetic separation.

To qualify the results in terms of reproducibility, a comparative study was performed for the centrifugation method.

MATERIALS AND METHODS

Reagents

Three iron oxide preparations of different size were used for the investigations (12,13). All samples were composed of a magnetite-like iron oxide crystal core stabilized by a surrounding layer of polysaccharides. Sample A had a core size of about 8 nm. The core crystal was stabilized by a chondroitin-4-sulfate (CSA) layer to yield an overall diameter of 105 nm. Iron content was 17 mg/ml and the stabilizer amount was 33 mg/ml (Morgan-Elson assay). Sample B was of medium size (60 nm) and had a core crystal of about 5 nm. Iron was 57 mg/ml. This sample as well as the smallest sample C (core size: 3–4 nm, overall size: appr. 30 nm, 58 mg Fe/ml) were stabilized by a dextran derivative, in particular carboxydextran (CDx). The amount of stabilizer was 52 mg and 67 mg for samples B and C respectively, as measured by the anthrone sulfuric acid method.

Sample A was used to investigate variations within one run, where up to eight gels can be run simultaneously, and sample B was used to determine the reproducibility between three different and independent runs. The overall sizes were estimated by photon correlation spectroscopy (Zetasizer 4, Malvern Instruments, UK), whereas the core sizes were calculated from electron micrographs (Siemens EM 102, Germany, magni-

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ABBREVIATIONS: 2-D PAGE; two-dimensional polyacrylamide gel electrophoresis, ddw; double distilled water, MRI; magnetic resonance imaging, MPS; mononuclear phagocytic system, SDS; sodium dodecylsulfate, SPIO; superparamagnetic iron oxides.

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fication 1:600,000). Iron was measured by AES-atomic emission spectroscopy (Fisons, ARL, UK).

All the chemicals for electrophoresis were of analytical grade. Acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), ammonium persulfate and piperazine diacrylamide (PDA) were obtained from Bio-Rad (Germany). Carrier ampholytes with a pH of 3.5–10 and 4–8 were obtained from BDH (Poole, UK). Cholamidopropyldimethylhydroxypropanesulfonate (CHAPS), Nonidet-P 40 and TRIS were purchased from Sigma (Germany). All other chemicals were the same as those in (1) and either obtained from Fluka Chemie AG (Switzerland) or Merck (Germany).

Sample Preparation

The particles were incubated at a constant volume ratio (iron oxide to plasma of 1 + 9) for five minutes in citrated human plasma at 37°C in 1.5 ml Eppendorf vials (Eppendorf, Germany). The plasma used was drawn from healthy volunteers donated at the center for blood donation, Deutsches Rotes Kreuz, Berlin, Germany. The particles and adsorbed proteins were then separated from excess plasma using four different methods:

Centrifugation

Centrifugation was performed at 15,000 g for 100 min (sample A, B) or 160 min (sample C) in a Biofuge 22R (Heraeus Sepatech, Germany). The supernatant was particle-free, as checked by measuring the iron content with AES, and discarded. The pellet was redispersed in 333 µl of double distilled water (ddw). Centrifugation and redispersion were repeated four times. Finally, the pellet was dispersed in 30 µl of ddw.

Gel Filtration

Gel filtration was done using 10 ml columns with a Sepharose 2B matrix (Pharmacia, Germany). The columns were packed at a flow rate of 25 ml/h using a peristaltic pump (Millipore, Germany) and isotonic Krebs buffer. The resolubilized samples were carefully layered on top of the gel matrix, and elution started immediately. Prior to use the matrix was equilibrated with Krebs buffer at a flow rate of 18 ml/h. Fractions of about 1 ml were collected (20 drops per fraction). To determine the efficacy of the separation of the particles from non-adsorbed plasma components, the fractions were analyzed by UV spectrometry at 350 nm (Uvikon 710, Kontron Instruments) (14). Particle-positive fractions were pooled and concentrated by centrifugation (15,000 g, 100 min). The pellet was redispersed in 30 µl of ddw.

Static Microfiltration

After plasma incubation sample A was filtered using a polycarbonate filter (Millipore, Germany) with a pore size of 50 nm. The filter was thoroughly rinsed with ddw, and, finally, the particles with the adsorbed proteins were washed off the filter by shaking in a beaker of ddw. Again, the dispersion was concentrated by centrifugation (15,000 g, 100 min) and the pellet redispersed in 30 µl of ddw for analysis.

Magnetic Separation

To take advantage of the magnetic properties of the particles, high gradient magnetic separation of the particles was performed. RS-type Mini MACS columns (Miltenyi Biotech GmbH, Germany) were placed between the poles of a $1^1/_2$ inch electromagnet (Oxford Instruments Limited, UK). The sample was placed on top of the column with the magnetic field switched on (magnetic flux density: 1 Tesla, cf. Fig. 1). The magnetic particles were retained in the matrix by magnetic means, the plasma running through the column. After the excess plasma was washed off, the column was removed from the magnetic field and the particles recovered by elution with 1.5 ml of ddw. The magnetic separation and washing was done again three times. Finally, the volume of the dispersion (1.5 ml) was reduced by centrifugation (15,000 g, 100 min) and the obtained pellet redispersed in 30 µl of ddw.

2-D Page

The samples obtained by the four separation procedures (30 µl each) were incubated with solubilizing solution containing 10% sodium dodecylsulfate (SDS) and 2.3% dithioerythritol (DTE) at elevated temperature (95°C) for desorption of the adsorbed proteins. 80 µl of the prepared samples were analyzed by 2-D PAGE following elaborated protocols (1,15). Silver staining of the proteins was subsequently done and the gels digitized for densitometric measurements (Personal Densitometer, Molecular Dynamics, Germany). The data were processed on a SUN SPARC 10 using MELANIE II software (16). The protein spots obtained were identified by comparison of the gel with known coordinates from human plasma reference maps (17,18).

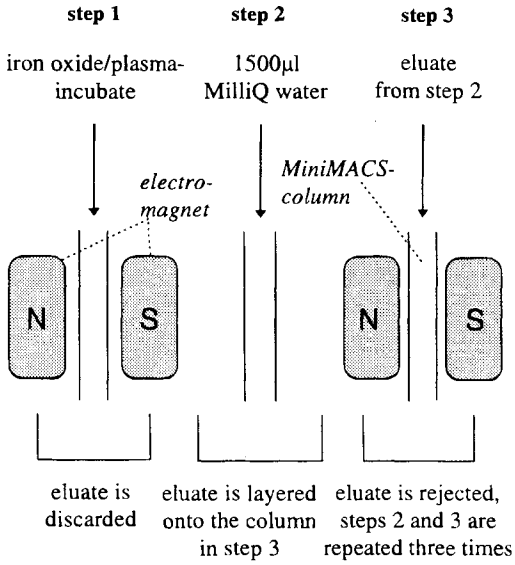

Fig. 1. Experimental set-up for magnetic separation: The MiniMACS-column is filled with iron pellets (diameter appr. 300 µm) that allow particles smaller than appr. 35 µm to pass. Being exposed to a magnetic field, the iron pellets themselves are magnetized (see text for details).

RESULTS

Separation Methods

Centrifugation

With the centrifugation procedure it is possible to follow the separation efficacy by visual means. The dark-coloured particles were clearly separated from the excess plasma, and recovery of the particles was complete, which was checked by measurements of the iron in the supernatant. Redispersion of the pellet was successful and the suspension obtained homogeneous. A representative plasma protein adsorption pattern for sample A is shown in Fig. 2 (upper left). Major proteins detected were fibrinogen (24%), IgG γ chain (21%), an unidentified protein called protein B (8%, named after (2)) and Ig light chains (5%). Quantitative values are referenced to the total amount of detected proteins.

Gel Filtration

Gel filtration revealed a considerable overlap of particle elution with the chromatographic profile of blank plasma. Thus, for sample A only three fractions were considered to be free of excess plasma. These fractions represented only about 70% of the total iron oxide. The adsorption pattern obtained using gel filtration (Fig. 2, upper right) is thus due only to the adsorption of proteins on the larger particles, which may not be representative of the whole dispersion. Fibrinogen (42%), albumin (9%) and C3β (6%) were major proteins identified, whereas adsorption of IgG was low (1%). These findings were obviously different from the adsorption patterns obtained by other methods and will be discussed below in the context of the Krebs buffer used.

Static Filtration

Several problems appeared when the 105 nm particles of sample A were filtered. On the one hand, the method was ineffective due to obstruction of the filter pores and, more importantly, the filtrate darkened due to leakage of some of the particles through the filter. Thus, as already mentioned in respect to the gel filtration procedure, only part of the sample, in particular the larger particles, were recovered. The resulting adsorption pattern is shown in Fig. 2 (lower left). Fibrinogen

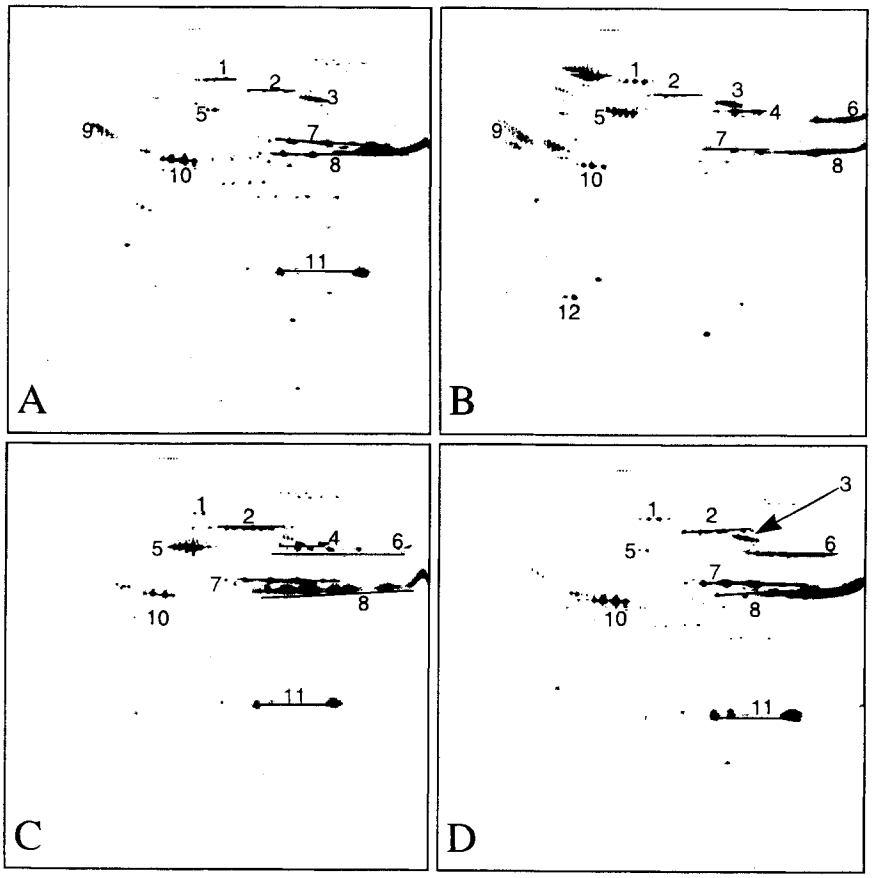

Fig. 2. Plasma protein adsorption patterns of iron oxide colloids (appr. 105 nm) stabilized by chondroitin-4-sulfate. The separation technique employed was centrifugation (A), gel filtration (B), static microfiltration (C) and magnetic separation (D). Isoelectric point pI 4.0–8.0 (from left to right, not linear), molecular weight MW 6–250 kDa (from bottom to top, not linear). The spots indicated are the most relevant proteins in this study. 1: protein A (named after (2)); 2: IgM μ chain; 3: unidentified protein B (named after (2)); 4: C3β; 5: albumin; 6: fibrinogen α chain; 7: fibrinogen β chain; 8: IgG γ chain; 9: α1-antichymotrypsin; 10: fibrinogen γ chain; 11: Ig light chains; 12: ApoA-I. The coordinates of C3β are not completely assured yet.

and IgG were detected as major spots on the gel (29% and 27% respectively). Compared to the other techniques, albumin displayed increased adsorption (19%).

Magnetic Separation

The qualitative adsorption pattern obtained after magnetic separation (Fig. 2, lower right) is comparable to that identified after centrifugation. On a quantitative basis, the adsorption of fibrinogen (36%), IgG (33%) and Ig light chains (12%) observed was, compared to all the others, the highest with the magnetic separation method. Additionally, the efficacy in terms of particle recovery was very high with magnetic separation. About 98% of the iron oxides were found in the pooled eluates. Thus, the adsorption pattern of the investigated sample is thought to be representative of the whole dispersion. Fig. 3 summarizes the results obtained with the four different separation methods.

To be able to compare the results obtained by centrifugation (Fig. 2, upper left) and gel filtration (Fig. 2, upper right), experiments were carried out with the same washing medium, in particular Krebs buffer. Krebs buffer was chosen because it is the most physiological system of the buffers investigated, and it fits requirements for gel filtration, e.g. suitable ionic strength. The adsorption of fibrinogen (33%), IgG γ (4%), albumin (8.5%) and C3β (6.5%) was comparable to centrifugation and gel filtration when Krebs buffer is used in both systems (Fig. 4).

REPRODUCIBILITY STUDIES (CENTRIFUGATION PROCEDURE)

Reproducibility Within One Run

To assess the reproducibility of the electrophoresis procedure, the same sample was applied to four gels in one single run. The whole procedure was almost identical for all samples. The matching feature of the MELANIE II software was used to investigate the reproducibility of the plasma protein adsorption patterns obtained. Two gels are superimposed on each other by the program and one gel used as the reference. The spots on the gels that do not have a counterpart on the second gel are

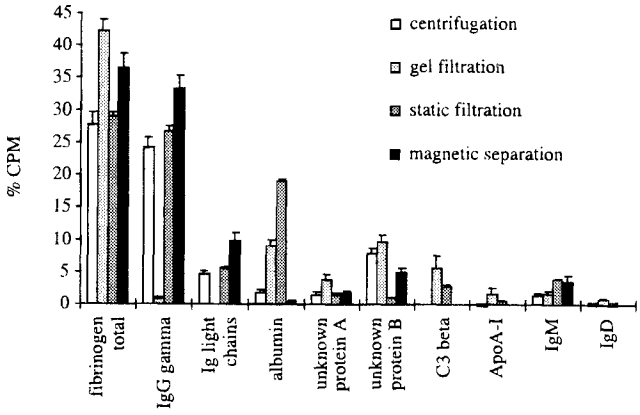

Fig. 3. Quantitative comparison of the protein adsorption patterns obtained using the four separation methods (each: n = 2). CPM is an arbitrary unit and a quantitative measure for the amount of detected proteins determined by MELANIE II software.

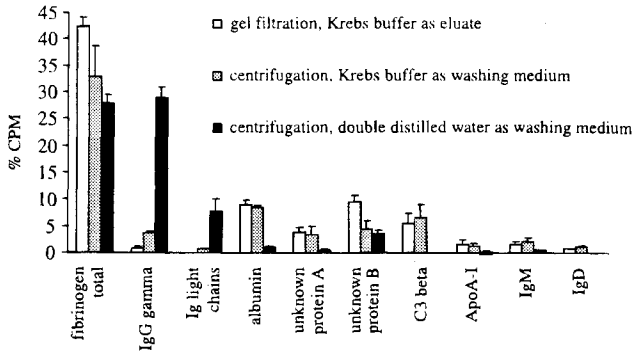

Fig. 4. Quantitative comparison of the protein adsorption patterns. The samples were prepared by gel filtration using Krebs buffer (n = 2), centrifugation using Krebs buffer (n = 3) and centrifugation using double distilled water (n = 2).

determined quantitatively. The number of unpaired spots and the quantitative amount represented by these spots (% of total proteins) are used as a measure of conformity. Sample A (large particles) and sample C (small particles) were used throughout the procedure as reference particles.

The unpaired spot with the highest amount of protein accounted for only 0.85% in the case of sample A (cf. Tab. I). At least 75% of the sample A spots were identified on all gels. Comparable results were found for small particles (sample C). The largest unpaired spot displayed 1.2% of the overall proteins detected, and more than 90% of the protein spots were identified on all gels (Table II), but the coefficient of variation (CV) was increased especially for the dominant proteins fibrinogen (CV = 14%) and the IgG γ chain (CV = 16%).

A remarkable amount of unpaired spots was found (105 nm particles as high as 15% or 55 spots and 30 nm particles

Table I. Reproducibility of 2-D Analysis Within One 2-D PAGE Run (n = 4) Using Centrifugation as the Separation Method (Washing Medium: Double Distilled Water)

Percentage	gel A	gel B	gel C	gel D	x	s	CV
albumin	1.2	0.8	0.6	1.4	1.0	0.3	28.8
fibrinogen total	27.1	25.6	28.3	30.1	27.8	1.6	5.9
IgG γ chain	31.9	27.1	29.0	27.5	28.9	1.9	6.7
IgM μ chain	0.6	0.5	0.5	0.4	0.5	0.1	8.8
Ig light chains	4.8	11.2	8.3	6.9	7.8	2.3	32.2
α1-antichymotrypsin	4.0	5.2	4.2	5.0	4.6	0.5	11.8
α-microglobulin	0.7	0.5	0.5	0.7	0.6	0.1	16.6
ApoA-I	0.2	0.2	0.1	0.1	0.2	0.1	28.3
ApoA-II	0.4	0.5	0.3	0.5	0.4	0.1	20.4
unidentified protein A	0.5	0.6	0.5	0.6	0.6	0.1	8.8
unidentified protein B	4.6	2.8	3.8	3.0	3.6	0.7	19.7
total percentage	76.0	75.0	76.1	75.2	75.7	0.4	0.6
no. of spots total	334	376	357	360			
no. of paired spots	—	322	314	311			
no. of unpaired spots	—	55	43	49			
largest unpaired spot (%)	—	0.85%	0.60%	0.63%			

Note: Iron oxides of appr. 105 nm were investigated (x = average; s = standard deviation; CV = coefficient of variation); three gels were matched with one gel (gel A) with the lowest number of detected spots. Designation of the paired and unpaired spots was dispensed with for gel A; the values were the lowest in each matching process.

Table II. Reproducibility of 2-D Analysis Within One 2-D PAGE Run (n = 4) Using Centrifugation as the Separation Method (Washing Medium: Double Distilled Water)

Percentage	gel A	gel B	gel C	gel D	x	s	CV
plasminogen	0.2	0.1	0.0	0.0	0.1	0.1	110.6
albumin	0.4	0.4	0.3	0.6	0.4	0.1	25.6
fibrinogen total	24.8	29.0	37.0	31.1	30.5	4.4	14.4
IgG γ chain	40.1	34.6	28.1	27.0	32.5	5.3	16.3
IgM μ chain	8.4	7.6	6.3	8.6	7.7	0.9	11.7
IgD δ chain	2.9	4.0	3.4	4.3	3.7	0.5	14.8
IgA α chain	0.8	0.5	0.4	1.0	0.7	0.2	35.3
Ig light chains	11.7	12.6	10.9	13.0	12.1	0.8	6.8
α-microglobulin	0.1	0.1	0.1	0.1	0.1	0.0	0.0
ApoA-I	0.5	0.8	0.8	0.9	0.8	0.2	20.0
ApoA-II	0.1	0.1	0.3	0.2	0.2	0.1	47.4
unidentified protein A	1.2	1.0	0.9	1.1	1.1	0.1	10.7
C3β	1.9	2.7	2.4	2.8	2.5	0.4	14.3
total percentage	93.1	93.5	90.9	90.7	92.1	1.3	1.4
no. of spots total	385	337	351	364			
no. of paired spots	325	—	307	315			
no. of unpaired spots	60	—	44	49			
largest unpaired spot (%)	1.21	—	1.08	1.24			

Note: Iron oxides of appr. 30 nm were investigated (x = average; s = standard deviation; CV = coefficient of variation); three gels were matched with one gel (gel B) with the lowest number of detected spots. Designation of the paired and unpaired spots was dispensed with for gel B; the values were the lowest in each matching process.

Table III. Reproducibility of 2-D Analysis of Three 2-D PAGE Runs Using Centrifugation as the Separation Method (Washing Medium: Krebs Buffer pH 7.4)

Percentage	Run 1	Run 2	Run 3	x	s	CV
albumin	8.8%	8.1%	8.5%	8.5%	0.3	3.4
IgG γ chain	3.3%	3.5%	4.2%	3.7%	0.4	10.5
IgM μ chain	7.6%	7.1%	4.0%	6.2%	1.6	25.5
IgD δ chain	3.4%	6.7%	4.8%	5.0%	1.4	27.2
Ig light chains	0.4%	0.8%	0.8%	0.7%	0.2	28.3
α1-antichymotrypsin	0.5%	1.2%	1.0%	0.9%	0.3	32.7
ApoA-I	1.4%	0.7%	1.8%	1.3%	0.5	35.0
ApoC-III	0.6%	0.5%	0.3%	0.5%	0.1	26.7
C3α	0.7%	0.9%	0.7%	0.8%	0.1	12.3
C3β	6.5%	9.5%	3.4%	6.5%	2.5	38.5
antithrombin III	0.3%	0.6%	0.3%	0.4%	0.1	35.4
IgA α chain	0.1%	0.4%	0.4%	0.3%	0.1	47.1
serum amyloid P	0.5%	0.2%	0.8%	0.5%	0.2	49.0
unidentified protein A	5.2%	3.4%	1.5%	3.4%	1.5	44.9
unidentified protein B	6.2%	4.5%	2.3%	4.3%	1.6	36.8
unidentified protein C	2.3%	5.5%	6.6%	4.8%	1.8	38.0
fibrinogen total	38.5%	24.7%	35.3%	32.8%	5.9	18.0
total percentage	86.3%	78.3%	76.7%	80.8%	4.7	5.8
total cpm	1143	1026	1728	1299.0	307.1	23.6

Note: Iron oxides of appr. 60 nm were investigated (x = average; s = standard deviation; CV = coefficient of variation). CPM is an arbitrary unit and a quantitative measure for the amount of detected proteins determined by MELANIE II software.

as high as 16% or 60 spots) for both batches, but these findings were as expected. The matching procedure requires a certain level of spot intensity, which is set by the user (threshold). Thus, very small spots were sometimes just above the threshold, or, vice versa, just below the level. In conclusion, the very small spots in particular account for the high number of unpaired spots, but the importance of this number seems to be low because the mass contribution to the overall adsorbed protein is very low. This is emphasized by the low percentages of the largest unpaired spot (105 nm particles: 0.85%; 30 nm particles: 1.24%).

Reproducibility Within Independent Runs

The evaluation of protein adsorption performed in three different runs is summarized in Tab. III. Again, the centrifugation procedure was used to prepare the samples for the 2-D PAGE analysis. As expected, the reproducibility is lower than for the gels within one run. The quantitative data are presented in Tab. III.

DISCUSSION

Four methods were investigated for sample preparation prior to 2-D PAGE analysis of the plasma protein adsorption on superparamagnetic iron oxides. Compared to gel filtration and static filtration, centrifugation and magnetic separation were superior in regard to the separation of SPIO and adsorbed proteins from bulk plasma. Centrifugation is time-consuming (10–11 h per sample) but seemed to be most suitable due to its general applicability to even the smallest particles of only 30 nm. Furthermore, sample recovery was quantitative, a representative particle fraction is analyzed. Additionally, the method is not restricted in terms of the washing media used. The medium selected for the washing steps is of great importance because it has already been shown that the adsorption pattern is influenced by the choice of washing medium (2). For comparative studies using different separation techniques it would be advantageous for the separation technique to permit the use of different washing media. This requirement was not fulfilled by magnetic separation, because irreversible particle aggregation in the RS-type column was observed for the Krebs buffer. Further prerequisites for successful magnetic separation are a high inducible magnetic moment as well as superparamagnetic behavior of the particles. In addition, the separation equipment is not widely available. These requirements are met by SPIO particles with relatively large core sizes but not by the ultrasmall SPIO. These findings are based on the known dependence of the magnetic properties on the core size. Thus, magnetic separation is restricted to selected SPIO particles. However, the method is fast and was found to be a very sophisticated separation method if the aforementioned particle properties are complied with. In that case the gels resulting from centrifugation or magnetic separation are in good agreement and exhibit good conformity.

The overall particle size limits the use of gel filtration due to an overlap of particle elution with blank plasma. Iron oxides smaller than appr. 100 nm in diameter were not sufficiently separated. In the range of appr. 105 nm a distinct fraction of the population—the smaller particles—was lost. Thus, the sample analyzed by 2-D PAGE may be not representative of the whole suspension, confirming the restricted applicability of this method. The selection of larger particles due to the gel filtration procedure was the most likely reason for differences

in the quantitative plasma protein adsorption pattern when compared to the other separation methods. The 2-D PAGE pattern resulting from gel filtration also displayed diminished adsorption of particular proteins (e.g. IgG, cf. Fig. 3). In order to estimate whether the technique or the Krebs buffer used is responsible for these findings, centrifugation was performed using Krebs' buffer to generate a reference map (cf. Fig. 4). When centrifugation was performed using Krebs' buffer the adsorption of IgG was strongly reduced, whereas adsorption of albumin, the unclassified protein A and C3β was increased. These adsorption values agree well with those obtained using gel filtration and the same buffer. Thus, the differences in the protein adsorption were influenced more by the washing medium used than by the separation technique. It is assumed that a higher solubility of proteins such as IgG in the buffer lead to decreased adsorption on the surface. Opposite effects were thought to be responsible for proteins such as albumin, C3β or the unclassified protein A, which are adsorbed to greater extent. Furthermore, the amount of adsorbed proteins may be influenced by changes in protein conformation.

The adsorption pattern on 105 nm particles resulting from static microfiltration was qualitatively comparable to those obtained with the centrifugation and magnetic separation techniques. However, this method is not suitable for sample preparation of ultrasmall iron oxides because of a remarkable loss of particles.

Reproducibility Studies

Reproducibility studies using the centrifugation procedure showed that particles of appr. 105 nm could be separated quantitatively from bulk plasma, and 2-D PAGE plasma protein adsorption patterns were highly reproducible. For SPIO of appr. 30 nm in diameter the reproducibility was lower, which is reflected by an increase in the standard deviation, which is most marked for the dominant protein spots. This observation was most likely explained by the fact that the separation of the supernatant and the bulk plasma was not as distinct as compared to the larger particles, leading to a less reproducible recovery of the pellet after centrifugation. For these very small particles of only 30 nm in size, magnetic separation was not very efficient due to the low magnetization of the tiny iron oxide crystals. Thus, as already described in regard to the gel filtration procedure, some fractionation of the particles was observed for the ultrasmall SPIO, which leads to an incomplete pattern of adsorbed proteins.

Centrifugation was considered to be suitable for the sample preparation of even ultrasmall particles for subsequent 2-D PAGE analysis. The reproducibility results obtained in the course of three independent runs (Table III) allows estimation of the method-inherent variation in observed adsorption patterns (cf. Fig. 2).

In conclusion, it was possible to prepare samples for the in vitro adsorption pattern on large (100 nm) to ultrasmall (30 nm) superparamagnetic iron oxide particles by different separation methods. Only small differences in the qualitative and quantitative protein adsorption patterns are found for the different separation techniques. The variations were most likely due to a loss of smaller particles in some of the separation techniques, in particular gel filtration and static microfiltration. However, remarkable differences resulting from the washing medium used during particle separation from bulk plasma were identified. Thus, the plasma protein adsorption pattern was influenced much more by the washing medium than by the separation technique itself.

A centrifugation procedure for the sample preparation of SPIO particles for 2-D PAGE analysis was established. Separation in a magnetic field is also suitable when certain particle requirements are met and the stability of the SPIO particles in the washing medium is confirmed. The separation efficacy of ultrasmall SPIO by gel filtration and static filtration is too low and not suitable for general use.

We have further shown that the washing media have strong impact on the in vitro protein adsorption pattern on the iron oxide particles. Thus, alternative buffer systems have to be carefully evaluated for the reliability of the results. 2D-PAGE analysis of the plasma protein adsorption in vitro may be an important tool to predict the in vivo behavior of i.v. injected iron oxides but the influence of the sample preparation, in particular, the choice of the washing media, on the protein adsorption pattern has to be considered.

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