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SARCOSYL-PAGE: a new method for the detection of MIRCERA- and EPO-doping in blood

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The detection of doping with MIRCERA (the brand name for Continuous Erythropoietin Receptor Activator, or CERA) is hampered by the limited excretion of the rather large molecule (approximately 60 kDa) in urine. Blood (serum, plasma) in combination with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) appears to be the ideal matrix for detecting all forms of doping with erythropoiesis-stimulating agents (ESAs) because the apparent molecular masses of ESAs are different from the mass of human serum erythropoietin (shEPO). While SDS-PAGE has proven the most sensitive method for the detection of doping with Dynepo, the sensitivity of SDS-PAGE for MIRCERA is drastically decreased. By exchanging the SDS for SARCOSYL (SAR) in the sample and running buffers the sensitivity problem was solved. SARCOSYL, a methyl glycine-based anionic surfactant, is only binding to the protein-part of MIRCERA but not to its polyethylene glycol (PEG)-chain, while SDS binds to both parts. In consequence, the monoclonal anti-EPO antibody (clone AE7A5) no longer interacts with the fully SDS-solubilized MIRCERA molecules. Only those molecules that contain SDS bound to the protein-chain are detected. Due to the inability of SARCOSYL to solubilize PEG-molecules, MIRCERA can be detected on SARCOSYL-PAGE with the same sensitivity as non-PEGylated epoetins. In a typical SAR-PAGE experiment, 200 μL of serum are used, which allows the *direct detection* of MIRCERA, recombinant epoetins (such as NeoRecormon, Dynepo, NESP), and shEPO *in a single experiment* and with high (i.e. femtogram) sensitivity.

Keywords: doping control; erythropoietin; MIRCERA; SARCOSYL-PAGE; SDS-PAGE

Introduction

Since its first approval in 1989, and aside from its misuse for doping purposes, recombinant erythropoietin has been used mainly for the stimulation of erythropoiesis in anaemia caused, for example, by chronic kidney disease or chemotherapy. The detection of doping with recombinant peptide and protein hormones – for example erythropoietin (EPO) and human growth hormone (hGH) – is generally one of the most challenging analytical problems in doping control.[1–4] The World Anti-doping Agency's (WADA) accredited method for the detection of doping with recombinant human erythropoietins (rhEPO) is based on isoelectric focusing (IEF).[5] In addition, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has proven a valuable tool for detecting Dynepo doping with increased sensitivity is also useful for ruling out active and effort-type urines.[2–3,6–7] However, both the SDS-PAGE and the IEF-PAGE methods use urine as sample matrix for the majority of EPO tests performed. Unfortunately, one of the latest generation of EPO-based pharmaceuticals, Continuous Erythropoietin Receptor Activator (CERA), RO0503821, or pegzyrepoetin, marketed under the brand name MIRCERA – a PEGylated epoetin beta) is hardly excreted in urine due to its prolonged serum half-life (approximately 130 h) and molecular mass (approximately 60 kDa) and hence should preferably be tested in blood.[8–15] In consequence, *up to four methods have to be performed* in order to detect unambiguously and confirm the misuse of recombinant eyrthropoietins – either the original innovator products or biosimilar epoetins or 'copy' epoetins[16–18] (currently numbered worldwide at approximately 80). These are the EPO-IEF method for urine and the SDS-PAGE method for additional evidence (for example, Dynepo[2–3], effort urines[3,19], degraded human urinary EPO (uhEPO[3]) and some biosimilars[7]), as well as an ELISA[20–21] and/or the IEF method for detecting the abuse of MIRCERA in blood.[1] Two different matrices have to be used – blood and urine. Due to the lower molecular mass of serum and plasma EPO (shEPO) compared to most recombinant erythropoietins,[22] SDS-PAGE holds the potential of detecting doping with the majority of rhEPO-forms in blood and *in a single experiment*. The isoform distribution of shEPO is less acidic than the distribution of uhEPO.[23] Thus, the detection of doping with erythropoiesis stimulating agents (ESA) without biotechnological (for example, darbepoetin alfa) or chemical modification (for instance, MIRCERA) is complicated by using the IEF method for blood samples (serum, plasma). Unfortunately, SDS-PAGE turned out to be less sensitive for MIRCERA than for the other rhEPOs. In the present work, the reason for this altered behaviour was identified and a new electrophoretic method with specifically enhanced sensitivity for MIRCERA was developed without altering the performance characteristics of SDS-PAGE for other epoetins.

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Experimental

Materials

Pharmaceutical formulations of recombinant and biotechnologically as well as chemically modified erythropoietins were from the manufacturers, namely Janssen-Cilag (Erypo; Vienna, Austria), Roche (NeoRecormon, MIRCERA; Mannheim, Germany), Amgen (NESP; Thousand Oaks, CA), and Shire (Dynepo; Hampshire, UK). The standard for human urinary erythropoietin (second international reference preparation) was obtained from the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, UK).

Sodium dodecyl sulfate (SDS), sodium decyl sulfate (SDecS), sodium dodecanoate (SDA, sodium laurate), sodium bis(2-ethylhexyl) sulfosuccinate (sodium docusate, AOT), sodium N-lauroylsarcosinate (SARCOSYL, SAR), sodium glycodeoxycholate (GDOC), polyethylene glycols (PEGs) of various average molecular masses (PEG 1500, PEG 2000, PEG 8000, PEG 20000, PEG 35000; numbers indicate molecular mass in Da), iodine, potassium iodide, barium chloride, dithiothreitol (DTT), phosphate buffered saline (PBS) tablets, 4-morpholinepropanesulfonic acid (MOPS), Tris hydrochloride, phenol red, ethylenediaminetetraacetic acid (EDTA), and glycerol were from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid, methanol (HPLC grade), and phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany).

Pre-cast polyacrylamide gels (NuPAGE BisTris gels, 10% T, 1.5 mm), 4-morpholinepropanesulfonic acid (MOPS) electrophoresis running buffer, lithium dodecyl sulfate (LDS) sample buffer (4x), sample reducing agent (10x), antioxidant, and molecular weight standard proteins (SeeBlue Plus2, Mark12) were provided by Invitrogen (Carlsbad, CA). Tris base and glycine were from GE Healthcare (Uppsala, Sweden). Coomassie Brilliant Blue R-250 was purchased from Serva (Heidelberg, Germany).

Devices for micro- (Steriflip filters, 0.2 µm) and ultrafiltration (Amicon Ultra-4, Microcon YM-30), as well as polyvinylidene difluoride (PVDF) membranes for Western blotting (Durapore, Immobilon-P), were from Millipore (Billerica, MA). The nominal molecular weight limit of all ultrafilters was 30 kDa. A combination consisting of a monoclonal mouse antibody (clone AE7A5; R&D Systems; Minneapolis, MN), a biotinylated polyclonal antibody (ImmunoPure goat anti-mouse IgG (H+L); Pierce; Rockford, IL), a streptavidin horseradish peroxidase complex (Biospa; Milano, Italy) and a substrate for enhanced chemiluminescence reaction (West Pico; Pierce; Rockford, IL) was used for the detection of erythropoietins on Western blots.

The Human 14 Multiple Affinity Removal System (MARS) column (10 × 100 mm) was from Agilent Technologies (Santa Clara, CA) and the enzyme-linked immunosorbent assay (ELISA) for erythropoietin-enrichment was bought from StemCell Technologies (Vancouver, Canada). GASepo version 1.3b2 (ARC; Seibersdorf, Austria) was used for all image analyses (image overlays, band quantitation).

Samples

Serum samples were obtained from four healthy volunteers who received single-dose subcutaneous applications of erythropoietin pharmaceuticals, i.e. 5000 international units (IU) of NeoRecormon (66 IU/kg), 3000 IU of Dynepo (35 IU/kg), and 50 µg of MIRCERA (0.67 and 0.58 µg/kg, respectively). Samples were collected for at least 14 days (NeoRecormon, Dynepo) or up to 42 days post-injection in the case of MIRCERA. During the first five days samples were taken on every day. Clot activator tubes (Vacuette system; Greiner Bio-One; Kremsmünster, Austria) were centrifuged at 2000 *rcf* (10 min) within the first 30 min after blood collection and then the obtained sera were aliquoted (250 µL) and stored at – 80 °C. All volunteers gave their written consent to using the anonymous samples for research purposes. The project was approved by the local ethics committee.

Depletion of high abundant serum proteins

A high-capacity multi-immunoaffinity column (MARS system) was used for removing the majority of high abundant serum proteins (albumin, IgGs, antitrypsin, IgAs, transferrin, haptoglobin, alpha-2-macroglobulin, alpha-1-acid glycoprotein, IgMs, apolipoprotein AI, apolipoprotein AII, complement C3, transthyretin, remnants of plasma fibrinogen), thus leading to a reduction in serum protein content of approximately 94% (alternatively, the HiTrap Albumin & IgG Depletion system from GE Healthcare can be used). The column was connected to a HPLC system consisting of a pump series P580, an AS-100 autosampler, and a UVD340U diode array detector (Dionex; Germering, Germany). All depletion steps were performed as described in the manufacturer's protocol and elsewhere.[3] Briefly, 200 µL of serum were diluted with 600 µL of buffer A (Agilent; a salt-containing neutral buffer, pH 7.4; proprietary formulation) and filtered through a 0.2 µm spin filter for 1 min at 14000 *rcf* (Nanosep MF, Pall; Ann Arbor, MI). Then the diluted sample (800 µL) was injected in a single step and separated on the MARS column at a flow rate of 500 µL/min. The flow-through fraction was collected, buffer-exchanged (50 mM Tris-HCl, pH 7.4) and concentrated down to approximately 10 – 20 µL by two ultrafiltration steps (Amicon Ultra-4 at 4000 *rcf*/30 min, Microcon YM-30 at 14000 *rcf*/approximately 15 min). The retentates were stored at – 80 °C. Column regeneration was done under acidic conditions (Agilent buffer B; a low-pH urea buffer; proprietary formulation) and at a flow rate of 3 mL/min. After re-equilibration with buffer A the column was ready for another round of sample depletion.

EPO-immunoaffinity extraction by ELISA

A commercial ELISA kit (StemCell Technologies) was used for extracting both the endogenous and recombinant epoetins present in the depleted serum samples. The extraction protocol was based on a previously published protocol[3] with slight modifications. After singularizing the ELISA wells and inserting them into Microcon tubes, 50 µL of PBS were added to each well and mixed with the retentates (10 to 20 µL) of the depleted serum samples. Then the wells were covered with an adhesive strip and incubated overnight without shaking in the coldroom. After washing the wells five times with 400 µL of PBS, the wells were carefully tapped dry on adsorbent paper to remove excess liquid. Bound epoetins were eluted by heating the wells at 95 °C for 5 min in a Thermomixer (1200 rpm) and with 30 µL of LDS sample buffer (1x). After cooling the eluate to room temperature and re-diluting it to 30 µL with Milli-Q water, the sample was ready for application on SDS-PAGE. In case of a non-SDS-based PAGE (e.g. SDecS-PAGE, SDA-PAGE, AOT-PAGE, SARCOSYL-PAGE, GDOC-PAGE) the LDS in the sample buffer was replaced by the respective anionic detergent. The final composition of sample buffers (1x) was 106 mM Tris hydrochloride, 141 mM Tris base, 2% detergent, 10% glycerol, 0.51 mM EDTA, and 0.175 mM phenol red.[24] All sample buffers were supplemented with reducing agent (Invitrogen; 1x) before using as eluent.

SDS-PAGE and non-SDS-based anionic detergent PAGE systems

Sodium dodecyl sulfate as well as all non-SDS-based anionic detergent polyacrylamide gel electrophoreses (PAGE) were performed on pre-cast BisTris-gels (Invitrogen; 10% T, 1.5 mm, 10 wells) using a MOPS-based running buffer system (50 mM MOPS, 50 mM Tris base, 0.1% (0.2%) detergent, 1 mM EDTA).[24] The buffer was supplemented with 500 µL of antioxidant/L. Standards of uhEPO and EPO-pharmaceuticals (Erypo, NeoRecormon, NESP, Dynepo, MIRCERA) were denatured under reducing conditions by heating for 5 min at 95 °C and in detergent-containing sample buffers (see above). Electrophoresis was performed at constant voltage (200 V) and until the phenol red front arrived at the end of the gel (typically after approximately 55 min). In order to evaluate the sensitivity of the different PAGE systems for Western blotting a twofold serial dilution of a mixture containing three EPO-pharmaceuticals was prepared (highest concentration: 0.09 ng Dynepo, 0.07 ng NESP, 0.4 ng MIRCERA; lowest concentration: 0.7 pg Dynepo, 0.5 pg NESP, 3.0 pg MIRCERA). Between 1.0 and 2.2 µg of recombinant epoetins were applied on those gels that were stained with Coomassie R-250 or a PEG-specific stain. Pre-stained molecular weight markers (SeeBlue Plus2; 10 µL) were used in order to control the separation behaviour of the electrophoretic system during the run and also the transfer-efficiency of the Western blot. Unstained molecular weight marker proteins (Mark12; 10 µL) were only used for Coomassie- and PEG-stained gels. For testing the migration behaviour of polyethylene glycols, between 5 and 20 µg of PEGs with different average molecular masses (PEG 1500, PEG 2000, PEG 8000, PEG 20000, PEG 35000) were applied on the gels and after heat treatment in the respective sample buffer. The standard for uhEPO was used on Coomassie stained gels as a standard for a mixture of urinary proteins only, since it contained approximately 2 µg of urinary proteins per μL in addition to only 0.01 IU/μL uhEPO (undetectable by Coomassie staining).

Coomassie R-250 stain

Polyacrylamide gels were fixed in 10% acetic acid/50% methanol (v/v) for 60 min and then stained overnight with 0.1% (w/v) Coomassie R-250 in 10% acetic acid/40% methanol (v/v). Subsequently, gels were destained for several hours in a solution containing 10% acetic acid and 20% methanol (v/v). The Coomassie R-250 solution was freshly prepared and filtered through a 5951/2 fold filter (Whatman; Dassel, Germany) immediately before usage.

PEG stain

After polyacrylamide gel electropohoresis PEGs and PEG groups of MIRCERA were stained according to Zheng *et al*.[25] Briefly, gels were first incubated in a 5% (w/v) solution of barium chloride in Milli-Q water (15 min), followed by a 15 min incubation in an aqueous solution of 1% iodine/1.5% potassium iodide (w/v). Proteins without PEG groups are not stained by this method.

Western blot with chemiluminescence detection

After electrophoresis, each gel was equilibrated for 3 × 5 min in Bjerrum buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol)[26] and then proteins were blotted on an Immobilon-P membrane (semi-dry method; 0.8 mA/cm^2, 60 min). One sheet of thick blotting paper (BioRad; Hercules, CA) was put on each side of the blotting sandwich. All subsequent steps were performed as described by Lasne[27–28] with minor modifications. After the transfer membranes were first incubated 5 mM DTT/PBS (37 °C, 60 min), then blocked in 5% non-fat milk (BioRad; Hercules, CA) in PBS (60 min), and incubated overnight at 4 °C to 8 °C in a solution of the primary antibody (mouse monoclonal anti-EPO antibody, clone AE7A5) in 1% non-fat milk (1 µg/mL). The membranes were washed (0.5% non-fat milk in PBS; 3 × 10 min) and double-blotted (0.8 mA/cm^2, 10 min) using 0.7% acetic acid. The receptor membrane was blocked (5% non-fat milk in PBS, 60 min) and incubated in a 1 : 2000 dilution of the biotinylated secondary goat anti-mouse IgG (H+L) antibody (1% non-fat milk, 60 min). After washing for 3 × 10 min in 0.5% non-fat milk the membrane was transferred to a 1 : 2000 dilution of the streptavidin horseradish peroxidase (HRP) complex in 1% non-fat milk (60 min) and then washed with PBS (3 × 10 min). Immediately after the last washing step the membrane was incubated in chemiluminescence substrate (West Pico). A CCD camera (LAS-4000; Fujifilm, Tokyo, Japan) was used for acquiring images of variable exposure time. Finally, images were analysed using GASepo (version 1.3b2) software.[29] All blotting steps were performed on a semi-dry blotter with metal electrodes (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell; BioRad, Hercules, CA).

Results and Discussion

SDS-PAGE of MIRCERA and other recombinant epoetins

For comparing the sensitivity of SDS-PAGE for detecting MIRCERA by Western blotting a twofold serial dilution of Dynepo (0.09 ng to 0.7 pg), NESP (0.07 ng to 0.5 pg), and MIRCERA (0.4 ng to 3.0 pg) was used. While the intensity of the Dynepo and NESP bands gradually declined according to the reduction in their concentration, no such behaviour was observed for the MIRCERA bands. The signal was not only much weaker but also rapidly lost intensity. However, it stayed at a low level until a concentration of approximately 12 pg was reached and then completely disappeared at approximately 3 pg (absolute amount on gel). In order to clarify the reason for the reduced sensitivity, MIRCERA, Erypo, NeoRecormon, NESP, and Dynepo were separated on SDS-PAGE and subsequently stained with Coomassie R-250. Bands were visually evaluated according to their shape and apparent molecular mass distribution. While Erypo, NeoRecormon, NESP, and Dynepo showed bands characteristic for these glycoproteins and their respective glycoform distributions, the band of MIRCERA behaved markedly differently from these epoetins. Obviously due to the PEGylation of MIRCERA – which is an epoetin beta carrying a approximately 30 kDa methoxy-polyethylene glycol-group predominantly on Lys 52 or Lys 45[30] – the front (lower) end of the band (with the lowest apparent molecular mass) stained with highest intensity and the back (upper) end with lowest intensity, indicating a concentration gradient within the band that was not due to the glycoform distribution of epoetin beta (NeoRecormon). Strangely enough, MIRCERA produced a very sharp band after Western blotting, comparable to the band sharpness of Dynepo. Upon closer examination of the Coomassie R-250 stained MIRCERA band and the MIRCERA band detected by the EPO-antibody (clone AE7A5) – by virtual overlaying of both images – it turned out that the antibody was only able to stain the back end of the band – the part of the band with the lowest concentration of MIRCERA (Figures 1A to 1C). Hence, what was actually detected by Western blotting was much less from what

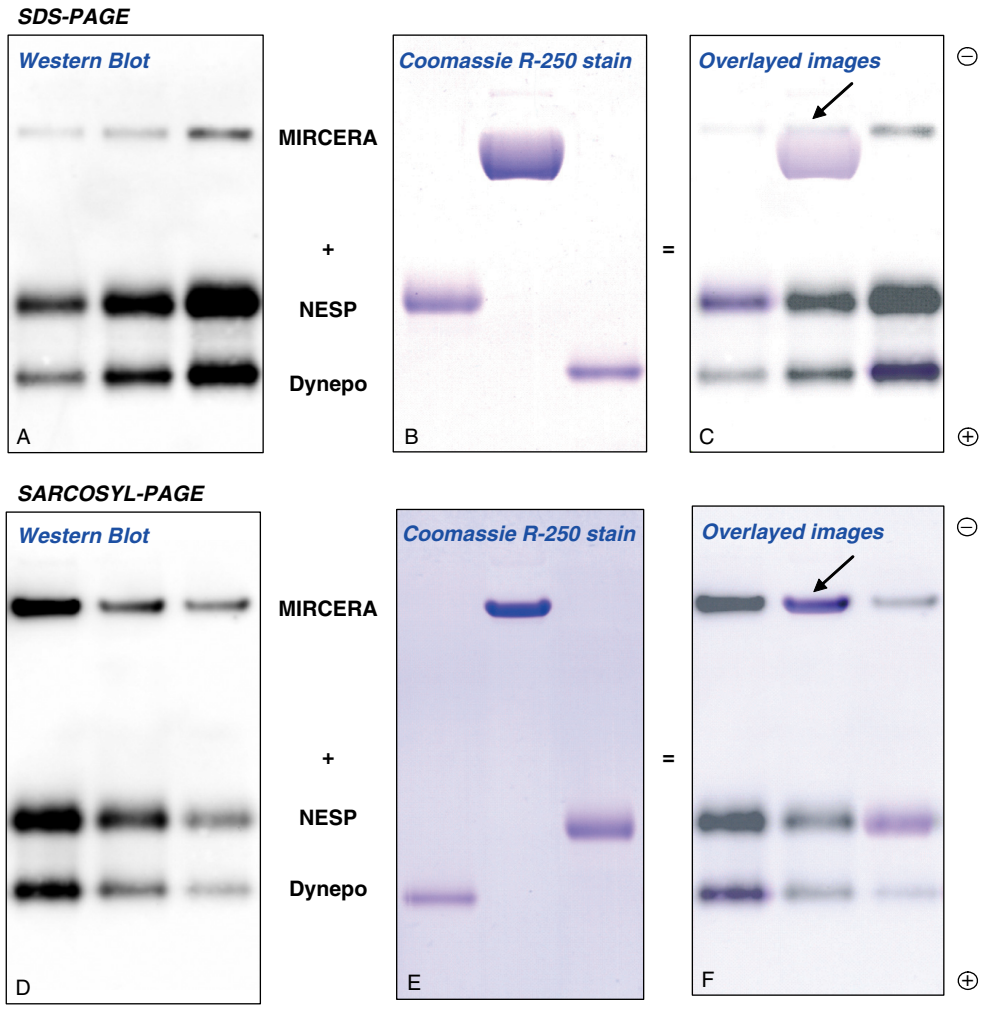


Figure 1. MIRCERA, NESP and Dynepo as detected by Western blotting and Coomassie R-250 stain on SDS-PAGE (Figure 1A to 1C) and SARCOSYL-PAGE (Figure 1D to 1F). The composite images on the right (Figure 1C, 1F) were generated by overlaying the two images on the left (Figure 1A and 1B, Figure 1D and 1E). Only on SARCOSYL-PAGE the Coomassie R-250 stained MIRCERA-band perfectly matches the band shape as obtained on the Western blot.

was actually present on the gels (as concluded from the Coomassie R-250 stain, Figure 1B).

Behaviour of PEG on SDS-PAGE

From the previous experiments it was concluded that the altered Western blotting behaviour of MIRCERA was due to its PEGylation. Hence, PEGs of different average molecular masses (PEG 1500, PEG 2000, PEG 8000, PEG 20000, PEG 35000) were separated on SDS-PAGE and visualized with a PEG-specific staining method. The method is based on the formation of a iodine complex with the PEG molecules resulting in an orange-to-brownish colouring.[25,31–32] Proteins that do not contain PEG groups are not stained by this method. It was demonstrated that SDS binds to PEG and thus leads to the migration of the uncharged PEG molecules in the electric field. However, due to a limited solubilizing power of SDS, PEGs – regardless of molecular size – migrate as broad and smeared bands on SDS-PAGE. By comparing the PEG-stained band of MIRCERA with the Coomassie R-250 stained band it became further evident that both bands were identical in the distribution of their intensity. Consequently, the monoclonal EPO-antibody (clone AE7A5) was indeed binding only to that part of the band that contained the lowest MIRCERA – and therefore also absolute PEG – concentration (Figure 2).

SARCOSYL-PAGE: Redesign of SDS-PAGE for MIRCERA

As evidenced so far, the binding of SDS to both the protein and PEG parts of MIRCERA led to the broad band with the atypical (for an epoetin beta) but significant (for PEG) density distribution. And as further substantiated by the smeared bands, SDS – as an anionic detergent – was not capable of fully solubilizing PEGs regardless of their molecular size (Figure 2). Hence, it was concluded that the decreased sensitivity of MIRCERA on Western blots might be due to an SDS-based solubility problem of MIRCERA's PEG-part or an SDS-based problem concerning a non-homogenous binding of SDS to the polymer. Consequently, two strategies seemed to be promising for solving the problem: either (1) using a detergent with higher solubilizing power for PEGs than SDS (or using SDS at an increased concentration), or – less obvious – (2) using (i.e. locating) a detergent that does only bind to the protein-part of MIRCERA but not to the PEG-moiety. Increasing the concentration of SDS from 0.1% (w/v) to 0.2% (w/v) in the running buffer did not improve the band shape of MIRCERA (Figure 3). Consequently, it was decided to replace the SDS in SDS-PAGE by anionic detergents

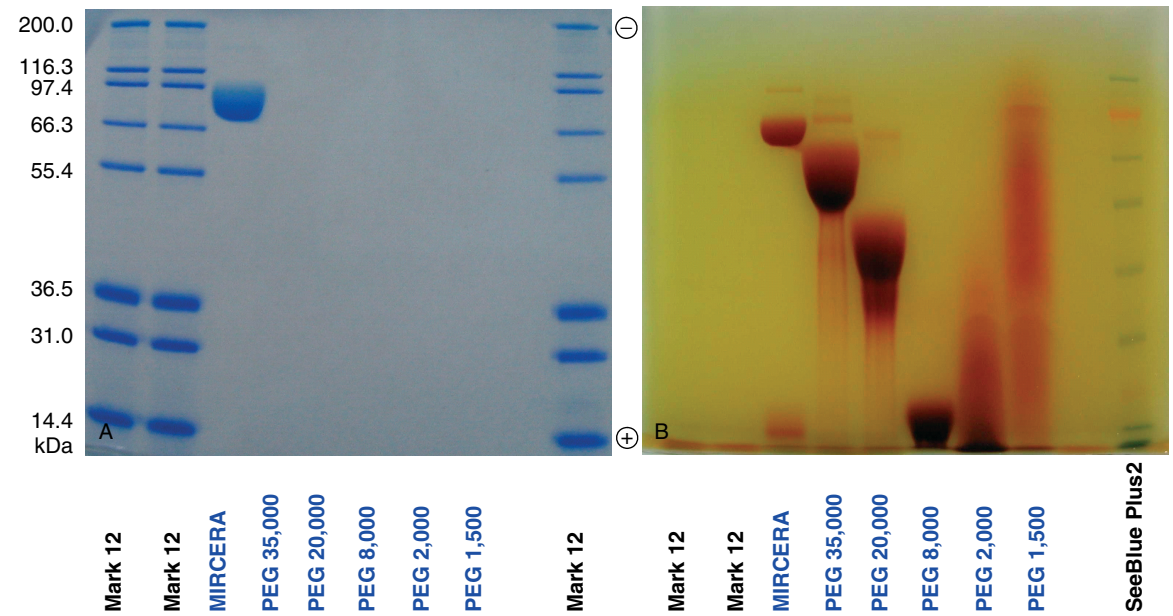


Figure 2. SDS-PAGE of MIRCERA and PEGs of different average molecular masses after Coomassie R-250 (Figure 2A) and PEG-specific (Figure 2B) stain. Note the different specificity of both stains for proteins (Mark12; molecular weight marker proteins) and PEGs. Only MIRCERA was stained by both methods. Applied amounts on gel: Erypo (approximately 1.0 µg), NeoRecormon (approximately 1.7 µg), Dynepo (approximately 1.0 µg), NESP (approximately 1.3 µg), MIRCERA (approximately 2.2 µg), NIBSC (approximately 26 µg; a crude uhEPO fraction); PEGs (approximately 5 µg each).

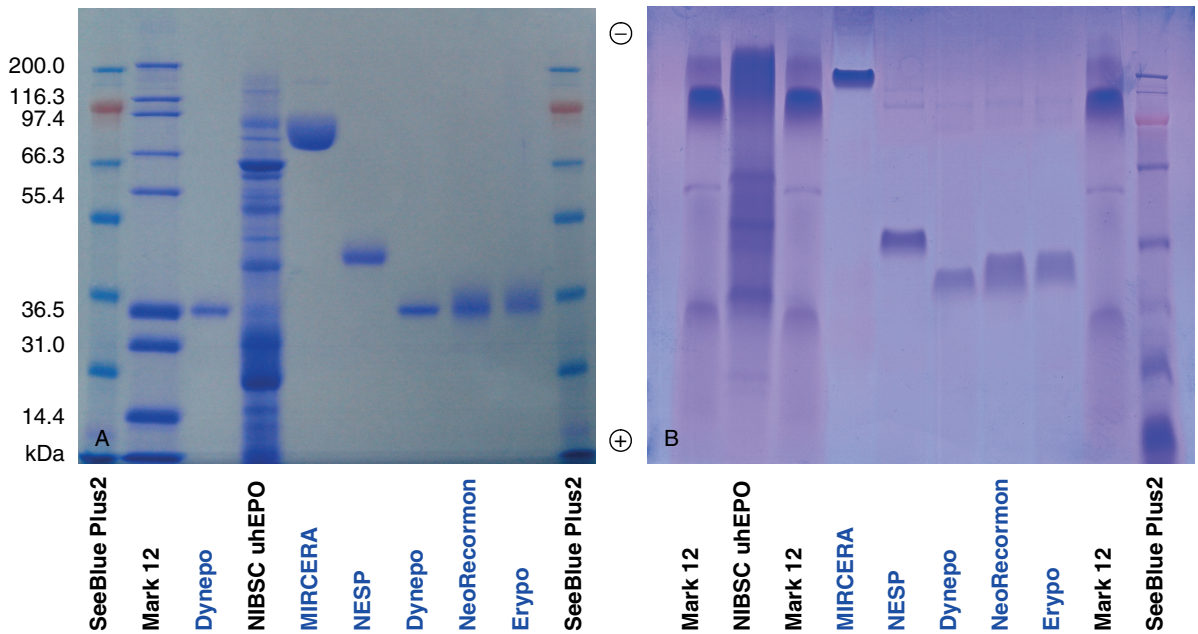


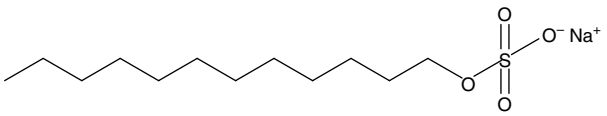
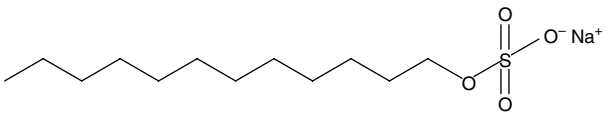
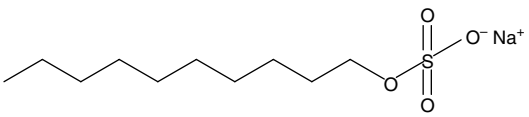
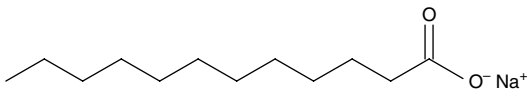
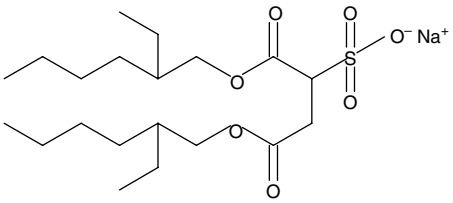
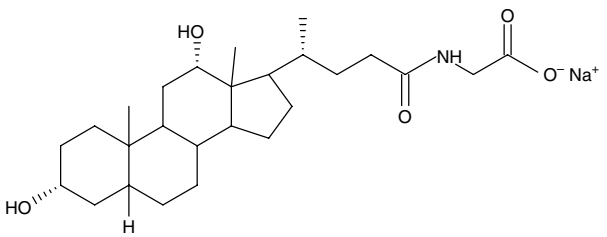
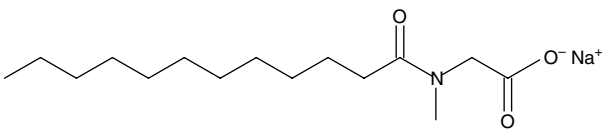
Figure 3. Comparison of SDS-PAGE (0.2% (w/v) SDS; Figure 3A and SDecS-PAGE (0.1% (w/v) SDecS; Figure 3B. Applied sample amounts were as in Figure 2.

with different physicochemical properties than SDS but without changing the electrophoretic system. The ultimate goal was the compatibility with long shelf-life pre-cast gels, i.e. the BisTris buffer system as used for standard SDS-PAGE separations, and standard Western blotting conditions (see experimental section). Table 1 summarizes the anionic surfactants that were evaluated with BisTris-gels and the MOPS running buffer system and the results obtained.

Replacing SDS by sodium decyl sulfate led to an improvement in the resolution of MIRCERA but to a loss in solubilizing power for all other rhEPOs (Figure 3). The idea was to use a detergent similar to SDS (i.e. an alkyl sulfate) but with less hydrophobic properties (shorter chain length) – assuming reduced interaction with the PEG-part of MIRCERA. Due to its non-denaturing properties SDecS-PAGE was already used by Akins *et al.*[33] for studying interactions of proteins with nucleic acids.

Switching from SDS to sodium dodecanoate, a detergent with a less acidic polar group (i.e. a carboxyl group instead of a sulfate group) but similar chain length, resulted in complete incompatibility with the selected electrophoretic system. Sodium

Table 1. Evaluation of anionic surfactants regarding their electrophoretic performance characteristics for MIRCERA and rhEPOs

Detergent	Chemical structure	Result
Sodium dodecyl sulfate (SDS), 0.1% (w/v)		Limited solubilizing power for PEGs Broad PEG-dominated MIRCERA band Decreased sensitivity on Western blots
Sodium dodecyl sulfate (SDS), 0.2% (w/v)		Limited solubilizing power for PEGs Broad PEG-dominated MIRCERA band Decreased sensitivity on Western blots
Sodium decyl sulfate (SDecS), 0.1% (w/v)		Improved band shape for MIRCERA Poor solubilizing power for rhEPOs (Erypo, NeoRecormon, NESP, Dynepo)
Sodium dodecanoate (SDA, sodium laurate), 0.1% (w/v)		Complete incompatibility with BisTris gel and running buffer system
Sodium bis(2-ethylhexyl) sulfosuccinate (AOT, sodium docusate), 0.1% (w/v)		Excellent solubilizing power for MIRCERA and rhEPOs Difficult to dissolve in sample and running buffers Poor results on standard Western blots
Sodium glycodeoxycholate (GDOC), 0.1% (w/v)		Excellent solubilizing power for MIRCERA and rhEPOs Poor results on standard Western blots
Sodium N-Lauroylsarcosinate (SARCOSYL, N-Dodecanoyl-N-methylglycine sodium salt), 0.1% (w/v)		Excellent solubilizing power for MIRCERA and rhEPOs Easy to dissolve in sample and running buffers Excellent results on standard Western blots Excellent overall compatibility with many electrophoretic buffer systems

dodecanoate precipitated within the first third of the gel matrix next to the cathode (data not shown), thus no satisfying separation was obtained. Next, sodium bis(2-ethylhexyl) sulfosuccinate was tested. The surfactant contains two hydrophobic chains linked via ester bonds to sulfobutanedioic acid. AOT-PAGE was described by Lu *et al.*[34] as being especially suited for the separation of small peptides with resolving capabilities down to 0.8 kDa. They used 6 M urea containing gels with the standard running buffer of Towbin (pH 8.3) but with 0.1% AOT instead of SDS. However, in the MOPS running buffer system (pH 7.7) AOT proved to be difficult to dissolve (75 °C, 60 min), and even more difficult to dissolve while preparing the fourfold concentrated sample buffer (pH 8.5).

After heat denaturation in a onefold AOT sample buffer and under reducing conditions samples were quite viscous and difficult to load on the gels. However, excellent electrophoretic resolution was obtained for MIRCERA and rhEPOs (Coomassie stained gels; Figure 4A) but only poor results on Western blots (data not shown).

Several investigators used bile salt-related detergents (for example, sodium taurodeoxycholate, sodium deoxycholate) instead of SDS for PAGE applications, especially for solubilizing hydrophobic membrane proteins or lipopolysaccharides.[35–38] Typically, aggregation numbers of these detergents (the number of detergent molecules necessary for micelle formation) are much lower than the aggregation number of SDS (62), for example, 2 to 4 for sodium cholate or 4 to 10 for sodium deoxycholate, while their critical micellar concentrations (CMC) are comparable to SDS (typically in the low mM range).[39] Their HLB number (hydrophile-lipophile balance), which is indicative for the solubilizing strength (hydrophilic character) of a detergent, is also lower than for SDS (for example, 16 for sodium deoxycholate, 40 for SDS). Out of the list of possible bile-salt based surfactants sodium glycodeoxycholate was chosen. Sodium glycodeoxycholate possesses a polar headgroup (in addition to the two polar hydroxyl groups on the steroid core structure), which combines the headgroup characteristics of taurodeoxycholate (2-aminoethanesulfonic acid) and those of amino acid-based surfactants (for example, N-methylglycine (sarcosine) derived compounds).[40] sodium glycodeoxycholate performed very well in respect to the resolution of MIRCERA and rhEPOs (Figure 4B) and was quite comparable to the performance of AOT. Unfortunately, behaviour on Western blots was poor – resembling the lower solubilizing power of GDOC compared to SDS (data not shown). So far, the obtained results supported the theory that the resolution of MIRCERA on PAGE could be improved by using detergents with lower hydrophobicity (for example, SDecS) and lower solubilizing properties (for instance, GDOC) than SDS.

Sodium N-lauroyl sarosinate (SARCOSYL) was tested next. It is an amino acid-based detergent (N-dodecanoyl-N-methylglycine sodium salt, an acyl sarcosinate), which has been typically used in molecular biology (for example, in guanidine-containing RNA purification buffers)[41] and protein chemistry for solubilizing membrane proteins.[42–43] Acyl sarcosinates have been described as 'interrupted soaps'. They contain a carboxylate (head) group and an additional amide function, which greatly enhances the surfactant properties (a structural element also found in GDOC).[40,44] The molecular weight (MW) and CMC of N-lauroyl sarosinate are similar to SDS (SAR: MW 293.4, CMC 14.6 mM; SDS: MW 288.4, CMC 7–10 mM; supplier information), while the aggregation number is much lower (2 instead of 62) and comparable to the aggregation number of bile salt-related detergents. SARCOSYL-PAGE performed excellent on Coomassie R-250 stained gels and Western blots by resolving MIRCERA in a sharp band and simultaneously keeping the resolution of rhEPOs and uhEPO on the performance level of SDS-PAGE (Figure 5A).

Performance characteristics of SARCOSYL-PAGE

In order to identify the reason for the excellent performance characteristics of SAR-PAGE for MIRCERA, PEGs of different molecular sizes (from 1500 to 35000 Da) were applied on gels and subsequently PEG stained. Amazingly, PEGs did not migrate on SAR-PAGE; hence they stayed at the place of application and did not produce the streaked bands (lanes) as observed on SDS-PAGE. SARCOSYL was obviously not binding to polyethylene glycol and was thus leaving PEGs in their native uncharged state. Consequently, and due to the fact that SARCOSYL was only binding to the protein part of MIRCERA, the obtained band sharpness of MIRCERA on SAR-PAGE could be explained (Figure 5B). The strong solubilizing characteristics of SDS for PEGs were the reason for the poor resolution of MIRCERA on SDS-PAGE.

Next, the sensitivity of SAR-PAGE for MIRCERA on Western blots was investigated. Surprisingly, the sharpness of the MIRCERA-band also led to a dramatic increase in the sensitivity of the method. Overlaying the images of the Coomassie stained gels and antibody stained membranes indicated a perfect match between both bands (Figures 1D to 1F). On SAR-PAGE, the monoclonal EPO-antibody (clone AE7A5) was binding to MIRCERA with similar characteristics as to rhEPOs and uhEPO, and hence similar sensitivity. Besides, the sensitivity for rhEPOs and uhEPO was not affected by SARCOSYL, i.e. it was identical on SDS-PAGE and SAR-PAGE. The employed enhanced chemiluminescence detection system used a medium sensitivity substrate (West Pico; Pierce), nevertheless 3 pg of MIRCERA (approximately 50 amol), 0.5 pg (approximately 13 amol) of NESP and 0.7 pg (approximately 22 amol) of Dynepo were still detectable (Figure 6). Higher sensitivity substrates (for example, West Femto; Pierce) might even lower the current detection limits. A recently developed ELISA-kit for the detection of MIRCERA in serum reported a limit of detection (LOD) of 10.8 pg/mL[21], and according to Lamon *et al*.[20] the lower limit of quantification (LLOQ) of this ELISA was defined to be 30 pg/mL. However, SAR-PAGE has the benefit of being a *direct detection method* that reveals *structural information of the antibody-bound molecules* (apparent molecular mass and molecular mass distribution) and which requires no additional cutoff limit as the ELISA-kit (for example, 100 pg/mL as suggested)[20]. All SAR-PAGE gels were blotted using an SDS-containing blotting buffer (Bjerrum-buffer). Exchanging the SDS for 0.1% (w/v) SARCOSYL did not further enhance the sensitivity of Western blots.

In order to further evaluate whether the entire (broad) MIRCERA-band as obtained after SDS-PAGE and Coomassie R-250 stain was actually transferred to the blotting-membrane or only that part which gave the sharp band after Western blotting and chemiluminescence detection, the PVDF-membrane was stained after the blot with Coomassie R-250. The band on the membrane exactly resembled the band on the gel, indicating a complete transfer of the PEGylated protein to the membrane (data not shown).

However, it remained unclear why the increase in band sharpness on SAR-PAGE also increased the antibody binding efficiency. On SDS-PAGE the monoclonal antibody was binding only to the part of the band that contained the lowest amount of MIRCERA, but no binding occurred to the region of the highest MIRCERA concentration. As demonstrated by isoelectric focusing of MIRCERA, PEGyaltion did not significantly decrease the affinity of the antibody to its N-terminal epitope, i.e. the sensitivity for MIRCERA on IEF-PAGE was comparable to the sensitivity for non-PEGylated epoetins.[1] Hence, it is hypothesized that the formation of micelles containing the detergent-solubilized PEG-part as well as the detergent-solubilized protein-part hinder the accessibility of the N-terminal epitope for the antibody (clone AE7A5; directed against a linear epitope consisting of the first 26 amino acids of the erythropoietin N-terminus). If anionic detergent-based micelles are formed that consist of only the solubilized protein-part of MIRCERA then no interference occurs – the resolution of the band is not disturbed and antigen-antibody interactions are not hindered. Consequently, the less an anionic detergent is able to interact with the PEG-group of the protein (as is the case for SARCOSYL)

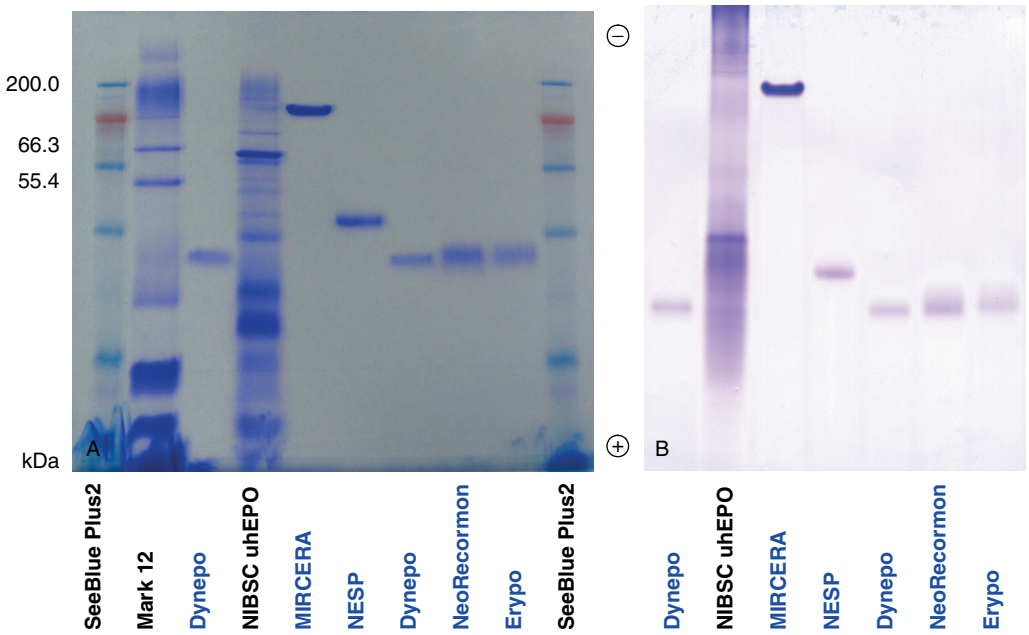


Figure 4. Performance characteristics of MIRCERA and rhEPOs on AOT-PAGE (0.1%; Figure 4A) and GDOC-PAGE (0.1%; Figure 4B). Gels were stained with Coomassie R-250. Further details are as in Figure 2. Note the poor solubilizing power of GDOC for urinary proteins in general (NIBSC uhEPO lane).

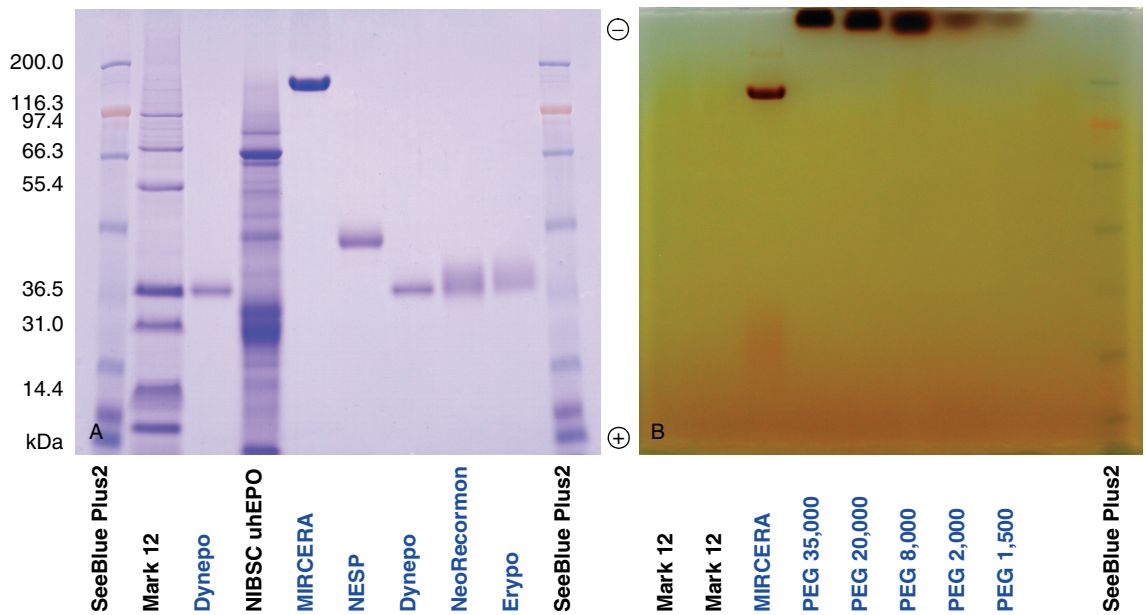


Figure 5. SARCOSYL-PAGE of MIRCERA, rhEPOs, and PEGs of various chain lengths. Applied sample amounts were identical to those in Figure 2 with the exception of PEGs (approximately 20 µg each).

the more unaltered the binding behaviour of the monoclonal antibody becomes and the more sensitively the MIRCERA can be detected.

It is further hypothesized that the MIRCERA concentration gradient as observed within the MIRCERA band on SDS-PAGE (see above) also resembles a concentration gradient of SDS bound to the PEG-chain of MIRCERA. The hypothesis is supported by Odom *et al.*[32] who observed that PEGs migrate in running buffers containing 0.07% (w/v) SDS but not in buffers containing 0.05% (w/v) SDS, while standard proteins still migrated under the reduced SDS-concentration. This denotes that at a low concentration SDS is preferentially binding to the amino acid-chain but also starts binding to PEG-chains when the SDS-concentration is increasing. However, simply reducing the SDS-amount in the running buffer to 0.05% (w/v) proved unsuccessful for MIRCERA and rhEPOs, probably due to MIRCERA's long PEG-chain (approximately 30 kDa) and the highly glycosylated nature of mammalian epoetins in general (data not shown). Consequently, the sharp band of MIRCERA as observed on SDS-PAGE after Western blotting and subsequent incubation with the monoclonal anti-EPO antibody might resemble MIRCERA with SDS bound only to its protein-part. Due to the constant supply of SDS from the catholyte, more and more SDS-molecules (micelles) also start binding to the PEG-part of MIRCERA. Thus, more and more MIRCERA-molecules containing

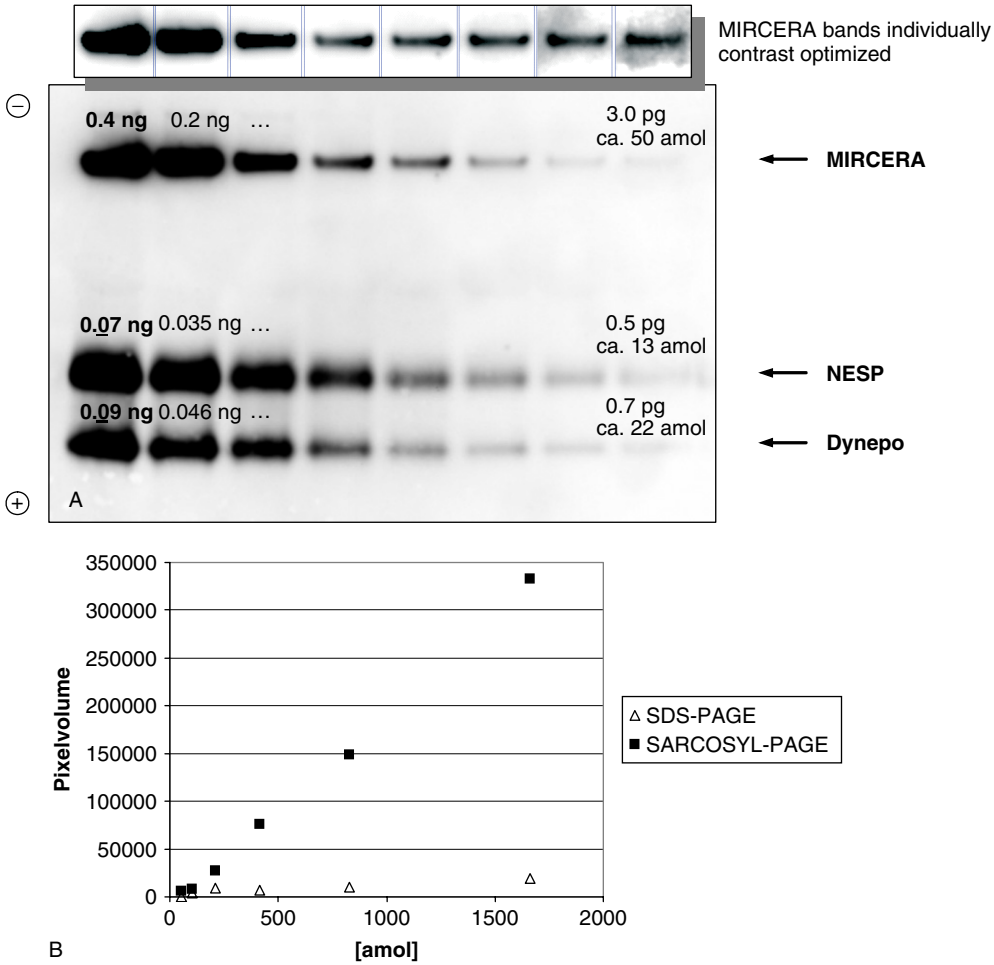


Figure 6. SAR-PAGE specifically enhances the Western blot sensitivity for MIRCERA (Figure 6A; twofold serial dilution). The sensitivity for other epoetins was unaltered compared to SDS-PAGE (Figure 6B; quantitative evaluation by densitometry).

SDS bound to both the protein- and PEG-chains migrate out of the zone of pure protein-bound SDS – creating a mixed band with an increasing amount of SDS-solubilized PEG-groups towards the lower apparent molecular mass region (anode oriented end) of the band. Hence, the proposed *dynamic model of SDS-binding* to a long PEG-chain modified protein (MIRCERA) also helps to explain the paradox of being able to solve a solubility problem (broad band) by actually reducing the amount of surfactant instead of increasing it.

SARCOSYL-PAGE: application in doping control

The applicability of SAR-PAGE for the detection of doping with MIRCERA and other recombinant epoetins (NeoRecormon, Dynepo) was tested using serum samples of controlled (single dose) excretion studies. Due to limited excretion of MIRCERA in urine and its prolonged serum half life (approximately 130 h) blood is the preferred matrix for detecting the abuse of MIRCERA.[1] However, due to the high protein content of serum samples (approximately 60–80 mg/mL)[23] epoetins have to be first affinity purified before they can be analysed via SAR-PAGE, SDS-PAGE, or IEF-PAGE. Typically, this can be achieved by immunoaffinity purification, for example by a one-step procedure employing a reusable anti-EPO immunoaffinity column or bead-based immunoprecipitation step or by a two-step method, which combines a reusable protein depletion column (for example, MARS system; Agilent) followed by an anti-EPO ELISA (single usage) immunoaffinity extraction step.[3,23] Samples were purified according to the latter method (see the experimental section for further details) and were separated after elution from the ELISA-wells on SAR-PAGE. Only 200 µL of serum were used per sample – which was sufficient even for detecting the low amount of endogenous shEPO in all samples (typically in the range of 30–170 pg/mL).[23] For the two administration studies performed with Dynepo (epoetin delta; 3000 IU, 35 IU/kg) and NeoRecormon (epoetin beta; 5000 IU, 66 IU/kg) the band of the recombinant erythropoietin could be detected at least until day 5 (Dynepo study) and day 7 (NeoRecormon study) after the application (Figures 7B and 7C). In case of the two MIRCERA excretion studies (50 µg, 0.67 and 0.58 µg/kg) detection was possible roughly until day 14 after the application (Figure 7A). Again, it has to be emphasized that in all cases *only* 200 µL *of serum* were used and only 50 µg of MIRCERA were applied. Higher sample volumes and higher sensitivity substrates (for example, West Femto; Pierce) might broaden the detection window, as will higher applied MIRCERA doses. The results of the MIRCERA studies also demonstrate that the capture antibody of the ELISA-kit (clone 26G9C10; StemCell Technologies)[45] is capable of binding the PEGylated protein. According to Wognum *et al.*[46] clone 26G9C10 does not recognize the same epitope as the antibody used for the

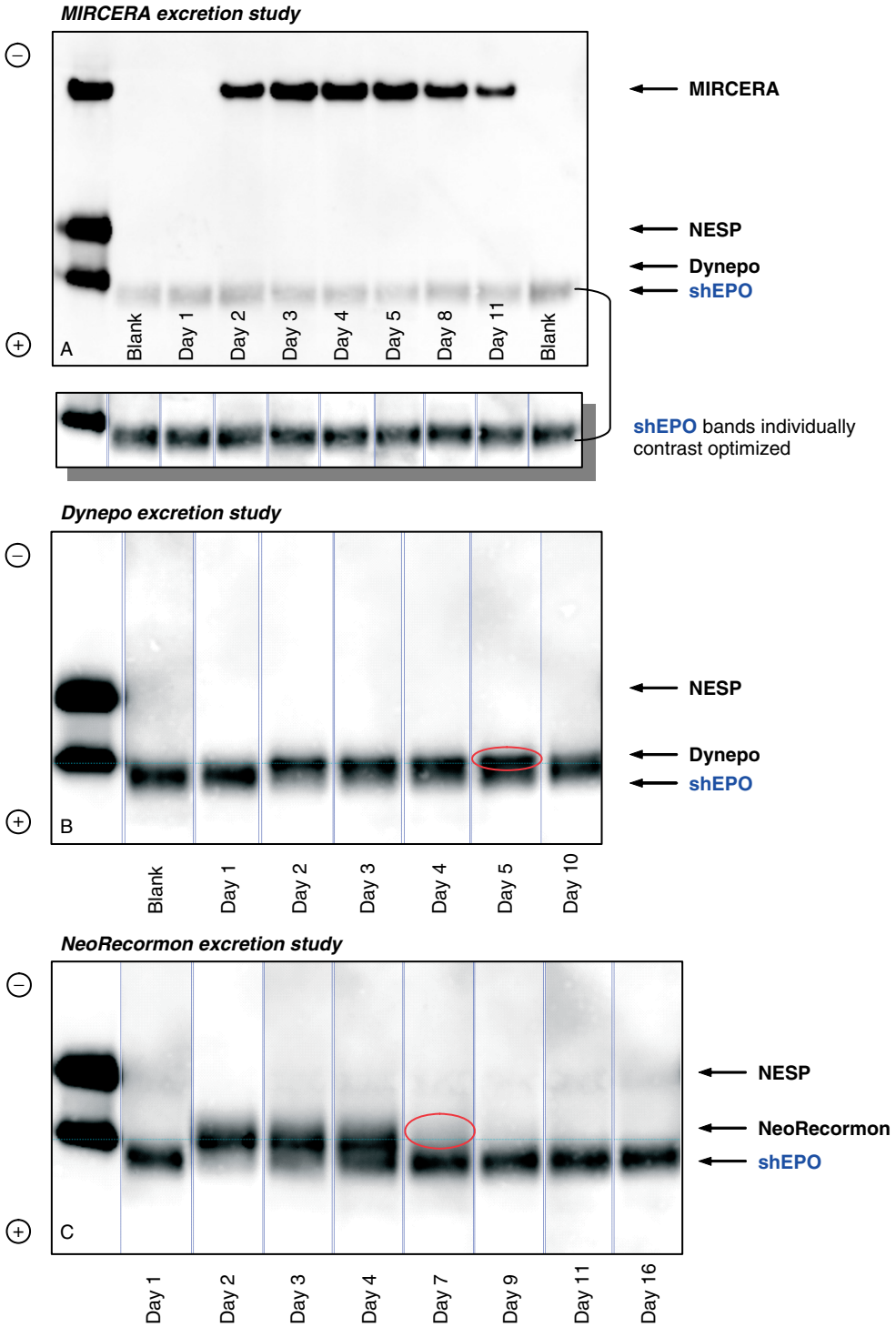


Figure 7. Detection of Dynepo (Figure 7A), NeoRecormon (Figure 7B), and MIRCERA (Figure 7C) by SARCOSYL-PAGE in 200 µL of serum and after subcutaneous application of a single dose.

subsequent Western blotting procedure (clone AE7A5), i.e. it is not directed against the first 26 amino acids of the N-terminus.

Conclusion

Recombinant erythropoietins perform with different sensitivity on SDS-PAGE after Western blotting. While the sensitivity of the majority of epoetins (for example, epoetins alfa, beta, delta, omega; darbepoetin alfa) is similar on SDS-PAGE, the sensitivity of MIRCERA (PEGylated epoetin beta) is drastically reduced. Redesigning SDS-PAGE by exchanging the SDS for SARCOSYL in the sample and running buffers specifically enhanced the sensitivity for MIRCERA. SARCOSYL, a methyl glycine-based anionic surfactant with slightly higher CMC but much lower aggregation number than SDS, is not capable of solubilizing PEGs under

PAGE-conditions – regardless of their polymerization degree (PEGs 1500 to 35000 were tested). Instead, SARCOSYL is only binding to the protein-part of MIRCERA leading to a sharp band on SAR-PAGE. Sodium dodecyl sulfate, on the other hand, is binding to both the PEG- and protein-chains of MIRCERA, which leads to band broadening on SDS-PAGE. As a result, the monoclonal anti-EPO antibody (clone AE7A5) is no longer binding to the fully – i.e. PEG- and protein-chain – solubilized MIRCERA-molecules, but only to those molecules that contain only SDS bound to the protein chain. Naturally, these molecules are located on top of the band because their charge density is reduced and their migration behaviour decreased. These molecules resemble only a small fraction of the MIRCERA-molecules originally loaded on the gel, so a decrease in sensitivity is observed. SARCOSYL, on the other hand, leads to a sharp MIRCERA-band, since no solubilization of PEG-chains occurs. Consequently, the antibody is able to bind to all MIRCERA molecules and no loss in sensitivity is observed after Western blotting. Besides, SARCOSYL-PAGE detects non-PEGylated epoetins with the same sensitivity and resolution as SDS-PAGE. The applicability of SAR-PAGE for detecting MIRCERA, recombinant epoetins and endogenous EPO in blood and with high sensitivity could be demonstrated by performing single-dose excretion studies. Besides, SAR-PAGE is not restricted to electrophoretic separations using the BisTris buffer system (for example, MOPS-chloride boundary) but is fully compatible with other discontinuous buffer systems, namely the standard Laemmli (glycine-chloride boundary),[47] Neville (borate-sulfate boundary)[48], and Allen-Moore (for example, borate-citrate boundary)[49] stacking systems – also indicating that the net charge of the SARCOSYL protein (erythropoietin, MIRCERA) micelles is stable within the pH range of approximately 7 – 10.

Acknowledgements

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