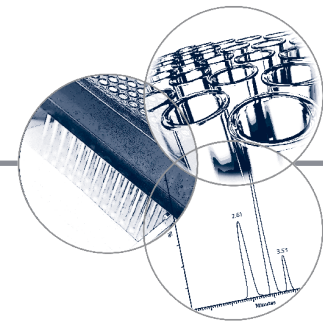

Gel electrophoretic methods for the analysis of biosimilar pharmaceuticals using the example of recombinant erythropoietin

Due to their versatility and cost–effectiveness, gel electrophoretic methods provide an important set of tools for the analysis of therapeutic proteins. As an increasing number of biosimilar pharmaceuticals are entering the market, techniques are required that allow reliable demonstration of comparability of these products with the reference products. Isoelectric focusing, SDS-PAGE, native PAGE and 2D electrophoresis (2D-PAGE) have been frequently applied for this purpose. Supplementary techniques are fluorophore-assisted carbohydrate electrophoresis and sarcosyl-PAGE. Of additional importance is the comparison of recombinant with endogenously synthesized glycoproteins. Reagent array analysis combined with SDS-PAGE and western blotting proved especially useful for this purpose. As an example for the application of these methods, the analysis of recombinant originator erythropoietins and some of their biosimilar counterparts is described.

Recombinant human erythropoietin (EPO) is one of the best-selling protein drugs marketed worldwide [1,2,101]. It was originally developed for treatment of anemia caused by chronic kidney disease, but has also been used for treating anemia related to, for example, chemotherapy or HIV-infection [3,4]. EPO is a **glycoprotein**, which is primarily produced by the kidneys in adults and stimulates the maturation of bone marrow stem cells to erythrocytes [5,6]. The first EPO pharmaceutical (epoetin alfa) was approved by the US FDA in 1989. Other epoetins followed in 1997 (epoetin beta), 2001 (darbepoetin alfa), 2002 (epoetin delta) and 2007 (methoxy polyethylene glycol-epoetin beta) [102,103]. All originator epoetins were protected by patents. With expiration of the patents for epoetin alfa and -β, the first EPO biosimilars appeared on the European market in 2007 (e.g., epoetin alfa Hexal®, Binocrit®, Abseamed®, Silapo® and Retacrit®) [1]. Before that, copy products were already sold in other countries (e.g., Argentina, China, India and Korea). In 2010, the first darbepoetin alfa biosimilar (CRESP®) appeared on the Indian market [7]. Since glycoproteins are difficult to reproduce unless completely identical host cells, cell culture conditions and purification protocols are used, slightly different final products are obtained. Hence, the term 'biosimilar' was introduced and the term 'generics' reserved for small-molecule pharmaceutical reproductions [8–10]. However, since biosimilars may not be completely identical with their reference compounds, biological activity and immunogenic potential may be different [11]. Among the array of analytical methods used for comparing biosimilars with originator products, gel electrophoretic techniques have been frequently applied [12,13]. They are relatively cost–effective and are particularly sensitive when combined with immunochemical detection methods.

PAGE

Common to all electrophoretic methods is the migration of charged molecules (e.g., proteins, peptides, carbohydrates and nucleic acids) in an electric field. Gel electrophoretic methods use gel-forming polymers as supporting matrices for sample application, separation and detection. Frequently applied polymers are either based on carbohydrates (e.g., starch, dextran and agarose) or acrylamide [14,15]. In protein chemistry, the majority of gel electrophoretic separations are performed in polyacrylamide gels (e.g., **SDS**-PAGE, isoelectric focusing [IEF]-PAGE; *vide infra*) [16]. Depending on the concentration of total acrylamide (% T) and the ratio of crosslinker (% C), gels with well-defined pore-sizes can be prepared (Figure 1) [17].

For applications, which are best performed in high-porosity gels (e.g., IEF), polyacrylamide gels with low total acrylamide amounts are used (e.g., 5% T and 3% C). If an additional sieving effect is required (e.g., for separating proteins

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Key Terms

Glycoprotein: Glycosylated protein. Many human proteins are glycoproteins (e.g., transferrin and erythropoietin).

SDS: Negatively charged detergent frequently used for solubilizing proteins. Due to its excellent cleaning properties SDS is also present in many cosmetics (e.g., shampoos).

according to their size), supporting matrices with higher acrylamide concentrations are employed (e.g., 10% T and 2.6% C). Separations can be additionally performed under native (i.e., nondenaturing) or denaturing conditions [18]. Denaturation of proteins is achieved by heating samples before electrophoretic separation and/or by addition of denaturing agents, such as **detergents** (e.g., SDS), chaotropes (e.g., urea) or strongly reducing compounds (e.g., dithiotreitol [DTT]). Not all detergents denature proteins, hence some detergents can be used in native **electrophoresis** [19]. Additional diversity is obtained by the electrolyte system applied (i.e., carrier ampholytes, tris-glycine, tris-tricine or tris-acetate) and, in case ionic detergents are used, whether they are negatively (e.g., SDS) or positively (e.g., cetyltrimethyl ammonium bromide) charged [20,21]. PAGE-separations can be performed in both horizontal and vertical orientations. Horizontal separations are preferred if strictly controlled cooling of the gel during electrophoresis is required (e.g., IEF-PAGE). Finally, different PAGE methods can be combined with 2D methods for enhancing protein resolution (2D-PAGE) [22–25].

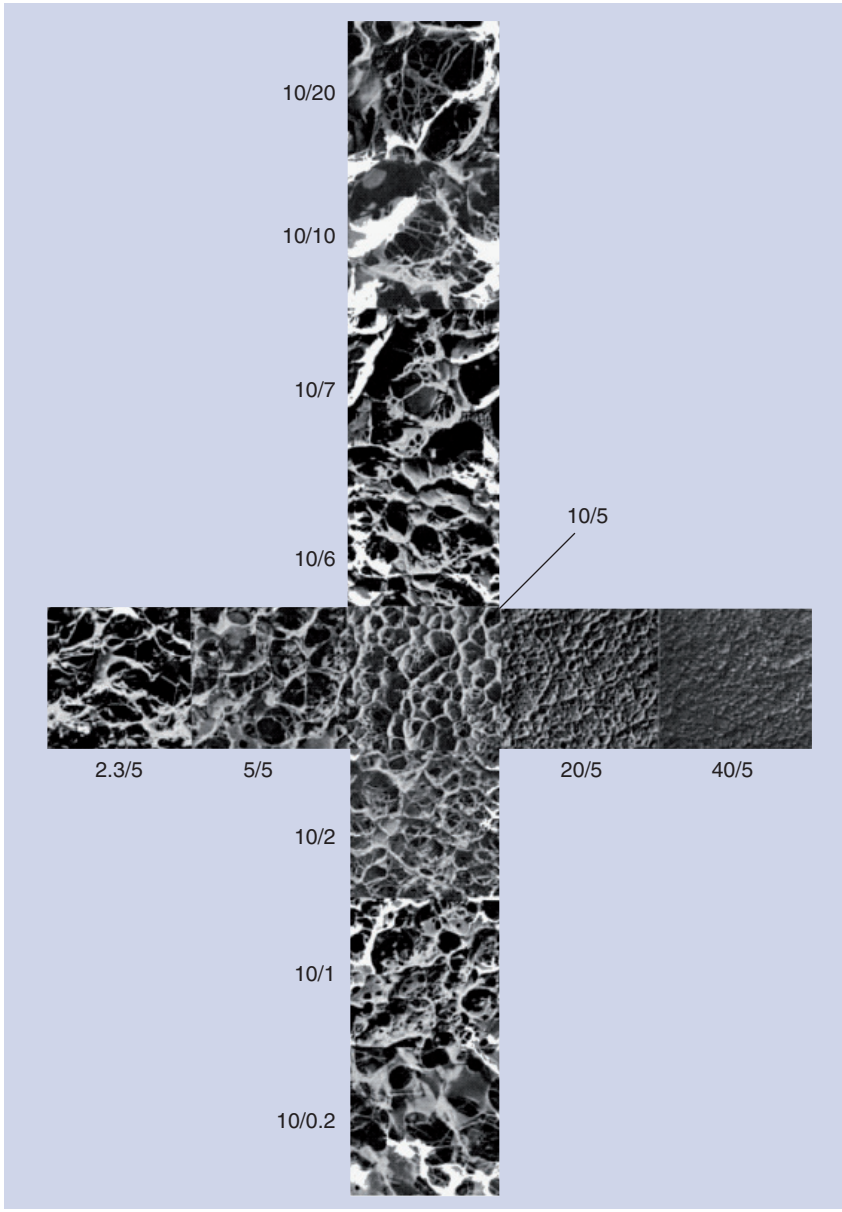

Figure 1. Dependence of polyacrylamide pore size on %T and %C.
Transmission-electron microscope images of freeze-edged gels.
Reproduced with permission from [17].

After electrophoresis, proteins are either directly detected in the electrophoretic gel by various staining methods (e.g., Coomassie®, silver or fluorescent stains) or after transfer ('blotting') to a membrane (e.g., nitrocellulose or polyvinylidene fluoride). Highest sensitivity is usually achieved, if antibodies are used for targeted detection of proteins on membranes (i.e., western blot or immunoblotting) [26]. Disadvantages are analysis time (typically in the range of hours to days) and lack of automation possibilities.

IEF-PAGE

IEF is an electrophoretic method that is capable of separating proteins according to their isoelectric point – the pH value at which the net-charge of a protein is zero [27]. For establishing the pH gradient within a gel matrix either carrier ampholytes or acrylamido buffers are used. Carrier ampholytes are complex mixtures of polyamino polycarboxylic acids or polyamino polysulfonic/polyphosphonic acids (trade names: Ampholine™, Bio-Lyte™, Resolyte™, Servalyte™ and Pharmalyte™) [28]. Usually they are mixed into the gel solution at a concentration of 2–4% (v/v) and before initiating polymerization. Alternatively, a polyacrylamide gel can be cast without the carrier ampholytes. After washing and drying, the gel is rehydrated with the ampholyte solution [29]. The latter technique has the advantage that nonreacted acrylamide monomers and polymerization starters (e.g., *N,N,N′,N′*-letramethylethylenediamine, ammonium persulfate) are removed, which may give rise to interferences during the focusing step. In-gel rehydration also allows the incorporation of compounds into the gel matrix, which are usually incompatible with in-solution polymerization (e.g., detergents). Both horizontal and vertical slab gels can be used for IEF-PAGE. However, horizontal systems are preferred as

they permit efficient and uniform cooling of the gel during electrophoresis, and also allow higher sample throughput [30]. Nonuniform cooling leads to lane distortions and band broadening. While carrier ampholytes establish the pH gradient during electrophoresis, acrylamide buffers (trade name: Immobiline™) are used for generating a fixed ('immobilized') pH gradient (IPG) during gel-casting. IPG-gels are mainly applied in 2D-electrophoresis (*vide supra*) [28].

Carrier ampholyte-based separation of EPO isoforms has been frequently utilized for characterizing recombinant and endogenous EPOs. A typical protocol uses ampholytes in the pH 2–6 range and a gel with a high concentration of urea (e.g., 7 M) for protein denaturation [31]. Under these conditions human endogenous EPO (serum/plasma, urine) separates into a mixture of 14–15 isoforms. Contrary to that, EPO pharmaceuticals contain a lower number of isoforms (e.g., 6–9), which are in general more 'basic' and focus within approximately pH 3.5–6 [31]. An exception is darbepoetin α (Ananesp®, novel erythropoiesis stimulating protein [NESP®]), a biotechnologically modified EPO that contains two additional *N*-glycans and more sialic acids [32]. Hence, it focuses in the acidic pH region of the gel (Figure 2; lane 3) [33,34,104].

The clinical potency of EPO isoforms increases with the amount of terminal sialic acids. EPO contains up to 14, and darbepoetin alfa up to 22, of these sialic acids [35]. The more sialic acids the EPO contains, the longer serum half-life and, hence, higher biological activity [36]. Thus, production of recombinant EPOs enriched in the content of highly sialylated isoforms is an important goal of the pharmaceutical industry.

Compared with their originator substances, EPO biosimilars partly differ from the originals in the number of isoforms and/or relative isoform distribution. One of the first studies on the isoform distribution of EPO biosimilars was published in 2004 [7]. The tested biosimilars varied in relative isoform concentration and the content of additional more basic or acidic isoforms. Additionally, batch-to-batch variation was observed for some products and *in vivo* bioactivity varied from 71–226%. Similar studies were performed later and confirmed the results [37–40]. In 2009, Macdougall and Ashenden estimated that approximately 80 biosimilar epoetins were available worldwide [41]. Since then, the number has continuously increased.

Figure 3 shows the isoform profiles of various biosimilar EPO pharmaceuticals, in comparison with the reference preparations for epoetin alfa/β (BRP-EPO; 1:1 mixture of both EPOs; European Directorate for the Quality of Medicines, Strasbourg, France), darbepoetin α (Aranesp®) and human urinary EPO (National Institute for Biological Standards and Control, Hertfordshire, UK). Some of these preparations contain more intense α bands (e.g., Hemax™, Erythrostim™, Shanpoietin™ and Wepox™), others a higher amount of more basic isoforms (e.g., Epocrin™ and Vintor™).

However, all of these epoetins are produced in Chinese hamster ovary (CHO) cells. Due to differences in cell-culture conditions (e.g., temperature, pH and composition of culture medium) and the subsequent purification process, different final products are obtained [42]. Yang and Butler, for instance, investigated the effect of ammonium ions on the **glycosylation** properties of CHO cells [43]. They were able to show that NH_4^+ leads to a decrease in terminal sialic acids and consequently a shift of the EPO IEF profile to higher pI values. Likewise, Crowell *et al.* demonstrated that manganese

Key Terms

Detergents: Compounds with hydrophilic (water loving) and hydrophobic (water repelling) properties. Due to their dual nature, detergents are capable of dissolving hydrophobic substances in water.

Electrophoresis: Charge-based separation method frequently used in protein biochemistry.

Glycosylation: Chemical modification of compounds (e.g., proteins and lipids) with carbohydrates.

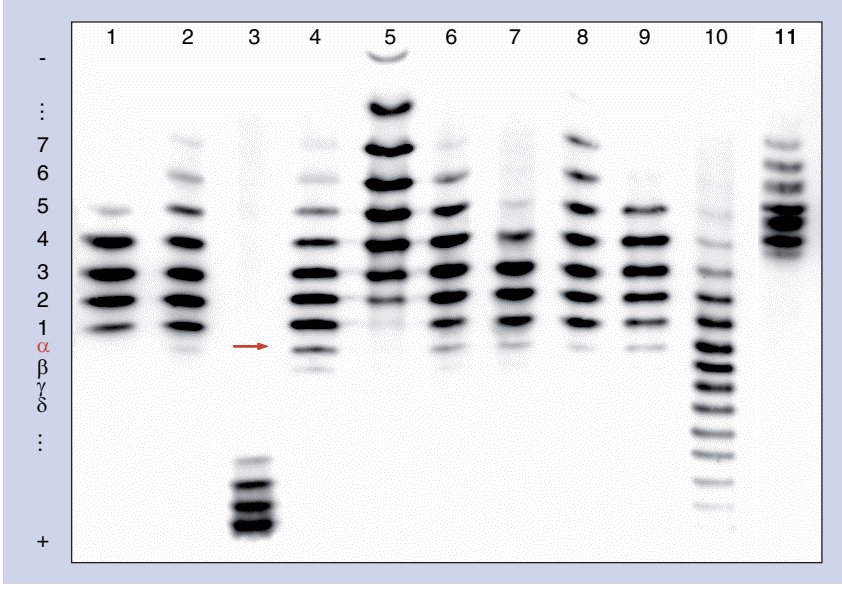

Figure 2. Isoelectric focusing PAGE (pH 2–6) of originator (Erypo®, NeoRecormon®, Dynepo®, Aranesp® and Mircera®) and biosimilar human recombinant erythropoietins in comparison with endogenous urinary erythropoietin. Note the presence of additional basic and acidic isoforms in the two originator epoetins (NeoRecormon and Dynepo), respectively. Due to additional modifications, isoform-profiles of Novel Erythropoiesis Stimulating Protein® and Mircera are significantly different. Lanes from left (1) to right (11): Erypo, NeoRecormon, Aranesp, Dynepo, Repotin®, Hemax™, Alfaepoetina™, Epocrin™, Erythrostim™, human endogenous erythropoietin standard (National Institute for Biological Standards and Control, Hertfordshire, UK) and Mircera. Position of the α band is indicated by the arrow. Detection method: western blot with monoclonal antibody against human erythropoietin (clone AE7A5). Reproduced with permission from [34].

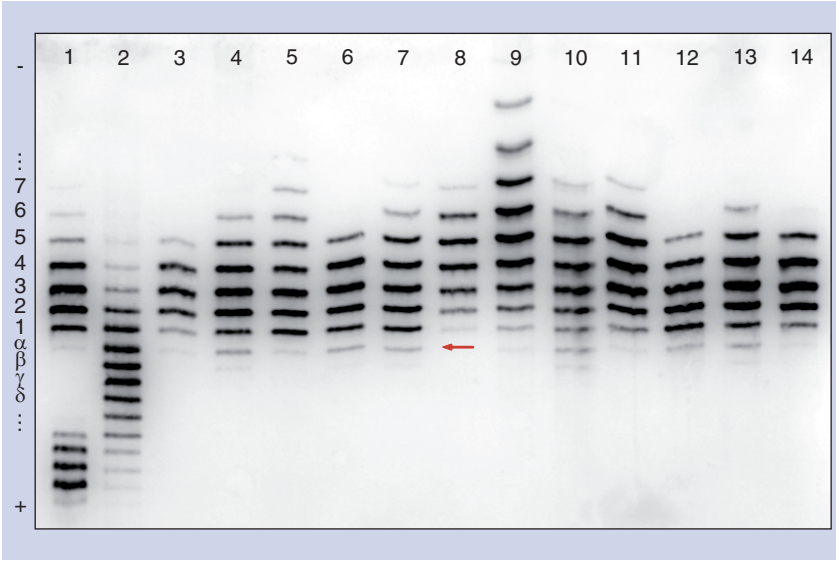

Figure 3. Isoelectric focusing PAGE profiles (pH 2–6) of various biosimilar epoetins. Significant differences in the isoform distribution are observed for some biosimilars. Note the general difference between endogenous urinary erythropoietin (EPO) (National Institute for Biological Standards and Control standard, Hertfordshire, UK) and the recombinant pharmaceuticals. Lanes from left (1) to right (14): Mix of BRP-EPO and Aranesp®, human endogenous EPO standard (National Institute for Biological Standards and Control), Alfaepoetina™, Hemax™, Epocrin™, Erythrostim™, Shanpoietin™, Beijing 4 Rings®, Vintor™, Wepox™, Eposure™, Espogen™, Zyrop™ and Retacrit®. The arrow shows the position of the α band. Western blot in combination with clone AE7A5 monoclonal EPO antibody.

ions (Mn^{2+}) are capable of enhancing galactosylation and sialylation of EPO glycans [44]. In perfusion culture, an increase in cell-culture temperature, for example from 25 to 37°C, leads to synthesis of more acidic isoforms, but cell viability and EPO quality drop at 37°C [45]. The authors also showed that below 32°C the amount of teta-antennary *N*-glycans significantly decreases. Additionally, a shift from mannose-5-phosphate-containing oligomannosidic *N*-glycans towards mannose-6-phosphate structures was observed. Due to possible effects on the conformation of EPO, the extent of phosphate-modified mannoses is kept as low as possible.

In conclusion, IEF-PAGE is an excellent tool for monitoring both the quality of EPO pharmaceuticals and their production processes. In combination with western blotting (*vide supra*), IEF-PAGE also allows the comparison of human recombinant epoetins with human endogenous (e.g., serum/plasma, urinary) EPOs. Interestingly, all studies conducted so far demonstrated a profound difference between recombinant and endogenous EPOs, which has not been exploited by the pharmaceutical industry until now [46].

SDS-PAGE

Contrary to IEF-PAGE, which separates proteins according to their intrinsic charge, SDS-PAGE is an electrophoretic method, which allows studying of the molecular mass of proteins. It combines the denaturing characteristics of an anionic detergent (SDS), with the sieving properties of the polyacrylamide matrix. SDS strongly solubilizes proteins and imposes a uniformly negative charge density on them. Consequently, SDS-solubilized proteins migrate towards the anode during electrophoresis and their migration velocity is a direct function of their molecular mass. Despite having the same molecular masses, glycoproteins usually appear at higher apparent molecular masses on SDS-PAGE than nonglycosylated proteins. The decreased mobility is caused by the glycan-part, which reduces interaction with SDS [47]. Hence, glycoprotein masses measured by MS are lower than those determined by SDS-PAGE. Typically, total acrylamide concentrations between 3 and 20% T, and crosslinker ratios between 0.9 and 6.25% C, are used for generating gels with defined pore sizes [47]. The method employs a discontinuous buffer system consisting of a 'stacking' and a separating ('running') gel. The stacking gel is a high-porosity polyacrylamide gel (e.g., 3.1% T, 2.6% C), which contains two types of anions – a fast-moving ('leading', e.g., chloride, acetate or sulfate) and a slow-moving ('trailing', e.g., glycine, 2-[*N*-morpholino]ethanesulfonic acid [MES], 3-[*N*-morpholino]propanesulfonic acid [MOPS] or tricine) one. The latter is continuously supplied with the sample and/or running buffers together with the cationic counter ion (e.g., tris[hydroxymethyl] aminomethane [tris], bis[2-hydroxyethyl] amino-tris[hydroxymethyl]methane [bis-tris]) (Figure 4) [105]. Within the two fronts of moving ions, SDS-solubilized proteins are concentrated in a narrow zone and subsequently enter the separating gel (e.g., 4–20% T, 2.6% C) at approximately the same time. Consequently, discontinuous gel systems lead to sharper protein bands than continuous systems.

The so-called Laemmli method is one of the most frequently employed discontinuous systems in protein chemistry. It uses chloride/glycine as the anion and tris as the cation. One drawback is the pH of the separating gel, which is basic (pH 8.3). Since polyacrylamide gels degrade under alkaline conditions, the shelf-life of Laemmli gels is limited to only a few weeks [14]. Hence, tris-acetate and bis-tris gels

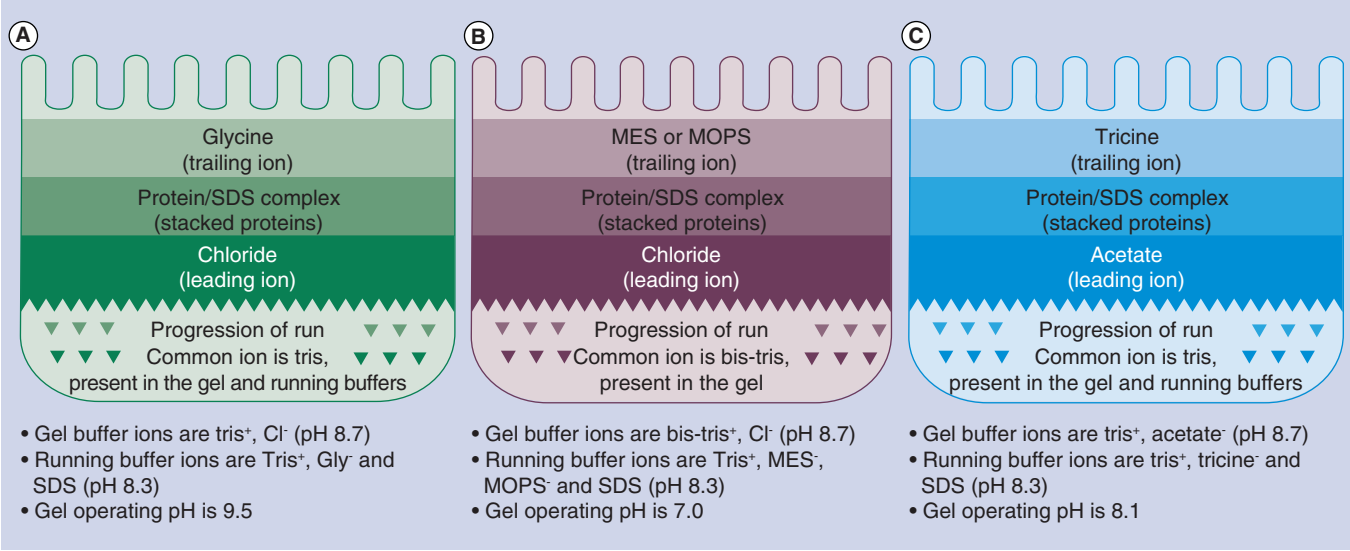

Figure 4. Comparison of three SDS-PAGE buffer systems. The Laemmli system uses chloride and glycine as leading and trailing ions and separates proteins under **(A)** basic pH conditions. **(B)** Bis-tris and **(C)** tris-acetate gels operate at neutral pH and, hence, provide longer shelf-lives than tris-glycine gels.
MES: 2-[*N*-morpholino]ethanesulfonic acid; MOPS: 3-[*N*-morpholino]propanesulfonic acid.
Reproduced with permission from [105].

were introduced, which operate at neutral pH and provide prolonged shelf-lives of up to 12 or more months.

For SDS-PAGE of EPOs, polyacrylamide gels with 8–12% T have been frequently used [34,48,49]. Originator recombinant human EPOs of the epoetin alfa and -β type display average apparent molecular masses in the range of 36–38 kDa. NESP and Mircera®, two biotechnologically or chemically engineered epoetins, migrate with apparent masses of approximately 44–45 and 66–97 kDa, respectively [34,50]. Since EPO is a glycoprotein, which typically consists of a heterogenous mixture of bi-, tri-, and tetra-antennary glycoforms with variable degrees of sialylation, the broadness of the EPO-band on SDS-PAGE directly reflects the mass distribution of its glycoforms. The comparison of Dynepo® (epoetin delta) with epoetin alfa and -β shows that Dynepo generates a much narrower ('sharper') band (~36 kDa; Figure 5). This rather atypical behavior among recombinant epoetins can be explained by the presence of mainly one type of *N*-glycan (i.e., tetra-antennary glycan) [51,52].

In addition to evaluating the molecular mass distribution of glycoproteins, SDS-PAGE also allows the detection of degradation products and covalently linked aggregates, which might act as immunogens and result in the formation of EPO autoantibodies [53]. In the study by Park *et al.* (*vide supra*), various EPO biosimilars

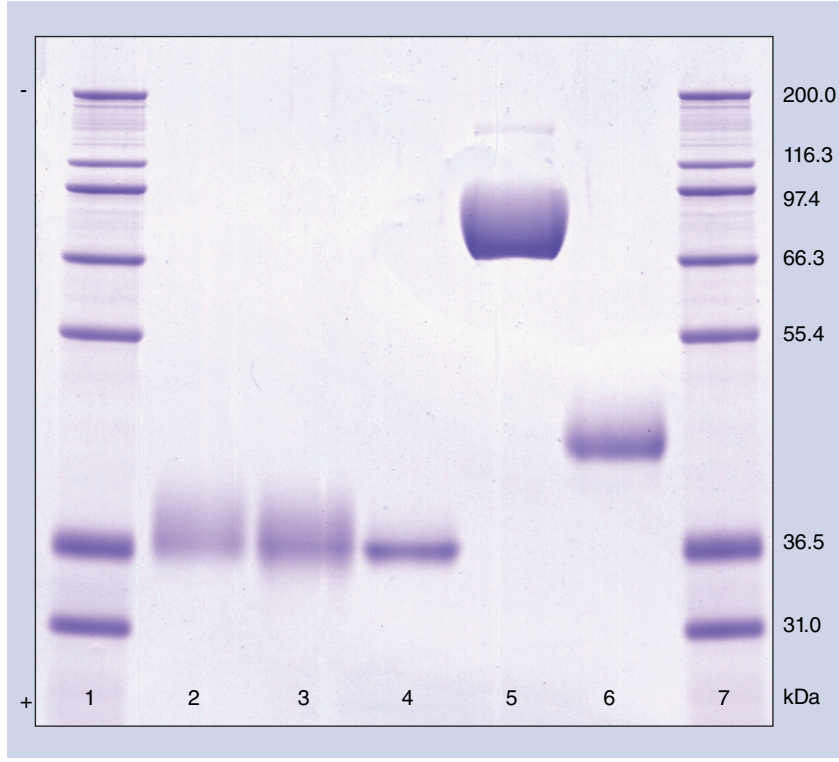

Figure 5. SDS-PAGE (bis-tris, 10% T) of five originator epoetins. Characteristic differences in molecular mass and glycoform distribution (band broadness) are seen. PEGylated erythropoietin (Mircera®) performs with a significant smear in the higher molecular mass region of the gel due to the interaction of polyethylene glycol with SDS. Lanes from left (1) to right (7): Mark 12 (molecular weight standard), Erypo®, NeoRecormon®, Dynepo®, Mircera, Aranesp® and Mark 12. Detection method: Coomassie R-250 stain.
Reproduced with permission from [34].

Key Term

Glycosidases: Enzymes that cleave carbohydrates. Exoglycosidases degrade carbohydrate structures from the end, endoglycosidases starting from the center.

from Korea, India and China were investigated by SDS-PAGE [37]. Some preparations showed smears in the higher molecular mass range, others showed one or more additional bands above the EPO band, which were all indicative for the formation of aggregates, dimers and/or oligomers.

Figure 6 shows SDS-PAGE profiles of biosimilars received from Brasil (Alfaepoetina™ and Hemax), Russia (Epocrin™ and Erythrostim), China (Beijing 4 Rings®), India (Shanpoietin, Wepox, Espogen™, Eposure™, Vintor and Zyrop™) and Europe (Retacrit). Several pharmaceuticals (Hemax, Beijing 4 Rings, Shanpoietin, Retacrit, Zyrop and Vintor) had a slightly lower median molecular masses than the originator products (Erypo® and NeoRecormon®). Wepox gave the broadest band of the tested EPOs, ranging from masses slightly below that of human urinary EPO to masses well above Erypo/NeoRecormon [54].

Similar studies were performed specifically for EPO biosimilars from Japan [39], India [40], Korea [38] and Europe [53]. Taken together, all of these results demonstrate that SDS-PAGE is a powerful tool for rapidly classifying epoetins based on their glycoform mass distribution. Additionally, SDS-PAGE in combination with western blotting enables molecular masses of recombinant and endogenous EPOs (serum/plasma, urinary EPO) to be compared. Interestingly, most recombinant epoetins possess higher glycoform masses than endogenous EPOs – another fact that might be of interest for future exploitation by the pharmaceutical industry (also see IEF-PAGE). On the other hand, the masses of human serum/plasma and urinary EPO are quite identical (Figures 6 & 7) [48,54].

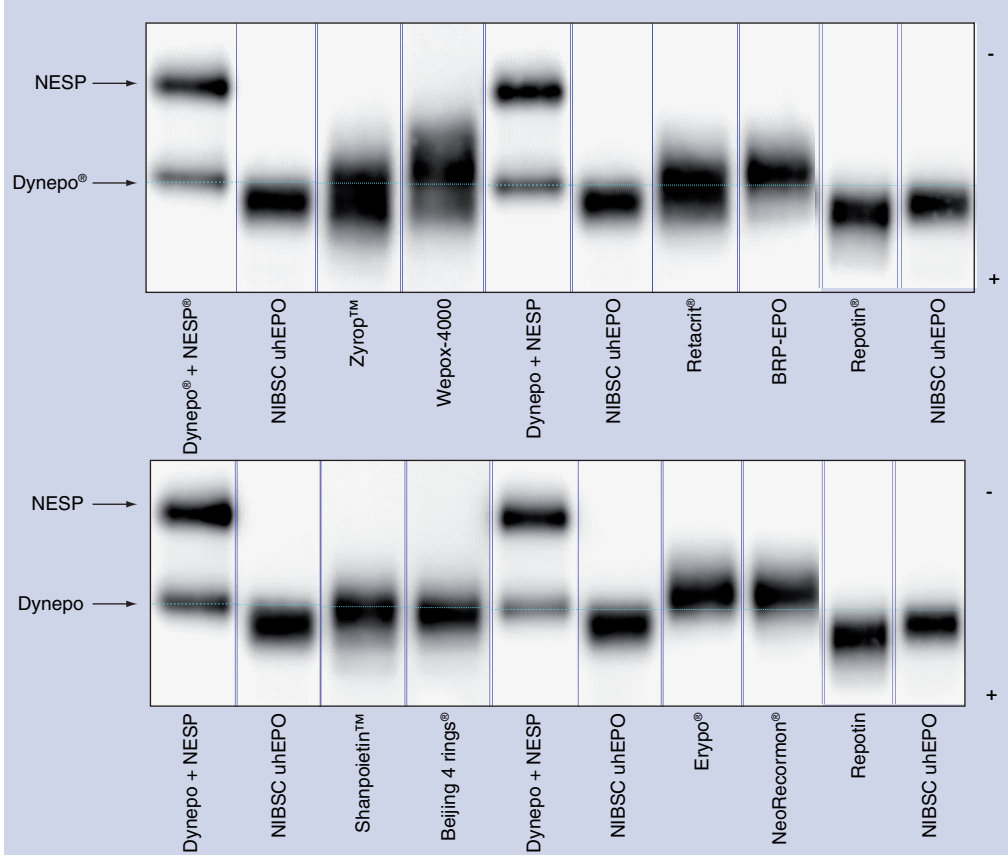

Figure 6. Performance of various erythropoietin biosimilars on SDS-PAGE (bis-tris, 3-(*N*-morpholino)propanesulfonic acid running buffer, 10% T). Many products show differences in glycoform composition (band broadness) and molecular mass compared with the originator pharmaceuticals (Erypo®, NeoRecormon®, Aranesp® and Dynepo®) and endogenous urinary EPO (NIBSC standard, Hertfordshire, UK). Detected by western blotting and clone AE7A5 EPO antibody.
EPO: Erythropoietin; NESP®: Novel erythropoiesis-stimulating protein; NIBSC: National Institute for Biological Standards and Control; uhEPO: Human endogenous erythropoietin.
Reproduced with permission from [54].

SDS-PAGE can also be used for structural glycan analysis of epoetins by monitoring mass changes after treatment with **glycosidases** (*vide infra*).

2D-PAGE

2D-PAGE methods combine two different electrophoretic separation techniques for enhancement of resolution. Typically, proteins are first separated by IEF-PAGE based on their net charge (1D) and then by SDS-PAGE according to their molecular mass (2D). Other combinations are, for example, 16-BAC/SDS-PAGE and blue native/SDS-PAGE. 16-BAC-PAGE uses a cationic detergent (benzylhexadecyldimethylammonium chloride) together with an acidic discontinuous buffer system and has been successfully applied together with SDS-PAGE in the 2D for the separation of integral membrane proteins [24]. Likewise, 1D separation with blue native PAGE proved useful for investigating multiprotein complexes [25]. However, IEF/SDS-PAGE is the most frequently employed combination. Before the development of IPG gels, carrier ampholyte-based tube gels were used for IEF-PAGE. After focusing, the tube gels were transferred on top of an SDS-PAGE gel. IPG-gels ('IPG-strips') are commercially available in many different pH ranges and gel lengths. They are either actively or passively rehydrated with a defined volume of buffer containing the sample or with buffer only. Usually, the buffer contains urea (e.g., 9 M) or a combination of urea (7 M) plus thiourea (2 M), nonionic and/or zwitterionic detergents (e.g., 1–4% CHAPS), a reducing agent (e.g., 15 mM DTT) and carrier ampholytes (e.g., 0.8%) [55–57]. After rehydration and sample entry, proteins are focused for several hours under high voltage gradients (typically, up to 5000 and 10,000 V, for 7 and 24 cm strips, respectively) [106]. Since such high-voltage gradients are not achieved with carrier ampholyte gels, IPG-gels are preferably used when high resolution is required or otherwise difficult-to-focus proteins have to be separated in their isoforms [58]. One drawback of the IPG-technology is that preparation of IPG-gels is more complicated than casting carrier ampholyte gels. A reproducibly working mixer is required for generating a gradient of Immobiline™ monomer solution. After polymerization, gels have to be washed and dried. Moreover, IPG-strips below pH 3.0 and above pH 11.0 are currently not commercially available, which is of importance if highly acidic (e.g., NESP) or basic proteins have to be focused. However, these gels can be manually prepared. Görg *et al.* demonstrated that focusing of NESP is possible with a pH 2.5–5.0 IPG gel [55]. After isoelectric focusing, strips have to be equilibrated in a buffer containing SDS (2%), urea (6 M), glycerol (30%) and Tris-HCl buffer (50 mM, pH 8.8). For protein reduction and alkylation, the buffer has to be supplemented with DTT and iodoacetamide, and afterwards the strip is transferred to the SDS-PAGE gel. In total, 2D-PAGE is more time-consuming than running IEF- and/or SDS-PAGE alone.

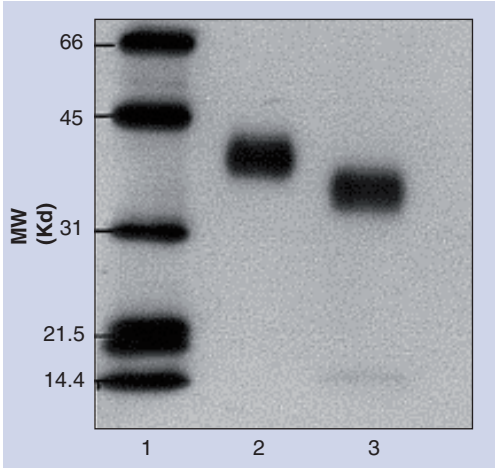

Figure 7. SDS-PAGE also reveals a significant molecular mass difference between recombinant erythropoietin (lane 2; Eprex®) and human serum erythropoietin (lane 3). Masses of serum and urinary erythropoietin are approximately the same. Lane 1: molecular weight marker; western blot with a monoclonal erythropoietin antibody.
Reproduced with permission from [48].

The benefit of 2D-PAGE for the analysis of EPOs is that, in addition to information about isoform distribution (IEF-PAGE) and total mass range (SDS-PAGE), data regarding the mass distribution of each individual isoform are obtained and can be monitored. Figure 8 shows how, for example, the EPO biosimilar Vintor performs on IEF-, SDS- and 2D-PAGE, and how broadly the masses of each of the nine isoforms are distributed. Contrary to that, the mass ranges of Dynepo and human endogenous epoetin (serum/plasma or urinary EPO) isoforms are rather narrow and uniform [48,51]. 2D-PAGE has also been used for optimizing biotechnological EPO production [43,59,60]. However, while 1D-methods have been frequently applied for

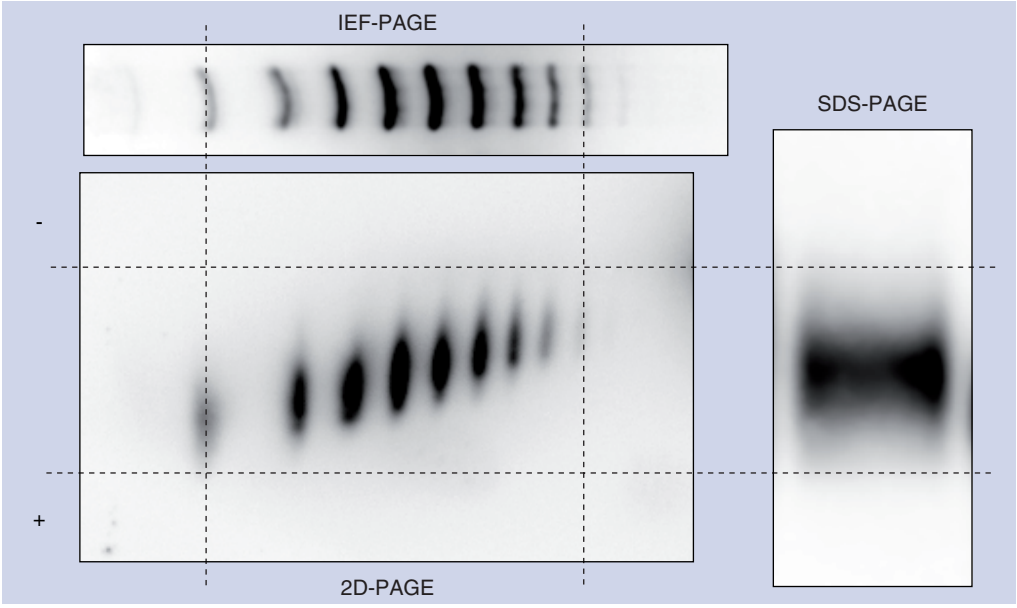

Figure 8. 2D separation of erythropoietin by combination of isoelectric focusing- and SDS-PAGE. Erythropoietin is first charge-separated by isoelectric focusing, and then the isoforms are further separated by mass on SDS-PAGE. Thus, 2D-PAGE provides additional information on the molecular mass distribution of the erythropoietin isoforms. This information is not obtained if only 1D separations are performed. Shown is erythropoietin biosimilar Vintor™. Detection method: western blot (clone AE7A5 antibody).
IEF-PAGE: Isoelectric focusing-PAGE.

characterizing EPO biosimilars, 2D-PAGE data are largely missing.

Nondenaturing PAGE (native PAGE)

SDS-PAGE is a denaturing electrophoretic method as it applies a detergent (SDS) that acts to denature proteins. Additionally, SDS-PAGE is frequently run under reducing conditions, which means that intra-molecular disulphide bridges of proteins are cleaved. The overall effect is that the 3D protein structure is destroyed, which enhances band sharpness during electrophoresis. Native PAGE does not use denaturing compounds and, thus, proteins are separated only on the basis of their net charge at the pH of the electrophoretic buffer system [18]. Hence, native PAGE is used to study proteins in their native conformation. Proteins with pI values below the buffer pH will be negatively charged and migrate towards the anode. Since many proteins are negatively charged at pH 8.3, the Laemmli system can be directly used for performing native PAGE, if SDS is omitted in all samples, gels and running buffers [16]. It allows the detection of protein aggregates and the determination of conditions under which no formation of these aggregates is observed. Since aggregate formation is a critical factor in the formulation of therapeutic proteins, native PAGE has also been used for the characterization of recombinant erythropietins [13,61]. Ionic strength of storage buffers, as well as types of buffer ions, pH and temperature, contribute to the stability of protein pharmaceuticals. Arakawa *et al.* demonstrated that heating of EPO in phosphate and citrate buffers led to aggregate formation, while no aggregates were observed when EPO was heated in buffers containing glycine, histidine or Tris-HCl, or was not heated all (Figure 9) [13]. However, aggregates can also be detected by nonelectrophoretic methods, such as size-exclusion chromatography, analytical ultracentrifugation or dynamic light scattering [13]. Currently, size-exclusion chromatography is the most frequently applied method for aggregation analysis.

Sarcosyl-PAGE

In order to prolong serum half-life of proteins and peptides, PEGylation gained interest in the pharmaceutical industry. PEGylation is the covalent modification of a target compound with PEG [62]. PEGylation not only increases the molecular mass of the compound but also its hydrodynamic volume, by binding approximately three to five water molecules per ethylene glycol unit. Additionally, PEGylated

peptides and proteins are less prone to enzymatic degradation and show decreased immunogenicity [63]. Molecules with masses below 60 kDa are easily excreted by the kidneys. Since many potential protein pharmaceuticals (e.g., cytokines) have molecular masses within the range of 15–30 kDa, chemical modification by PEGylation gained increased interest in pharmaceutical development [63]. Examples of PEGylated proteins, which received FDA-approval between 2000 and 2002, are PEG-IFN-α2b (PEG-INTRON®, 2000), PEG-IFN-α2a (PEGASYS®, 2001) and PEG-granulocyte-colony-stimulating factor (G-CSF; Neulasta®, 2002). In 2007, the first PEGylated recombinant EPO (Mircera) was approved for the European market (*vide supra*) and is also known as continuous EPO receptor activator [63]. Mircera is a PEGylated epoetin beta (methoxy polyethylene glycol-epoetin beta) that contains one 30 kDa PEG-unit attached to either the amino group of the N-terminus or the ε-amino group of a lysine-residue (mainly Lys 52 or 45). Thus, the nominal mass of epoetin beta is increased from approximately 30 kDa to approximately 60 kDa, and the serum half-life from approximately 8/24 h (intravenous/subcutaneous application) to over 130 h [64,65,107]. Hence, patients can reduce dosing of the drug to once per month.

PEGylated EPO can be studied by the described electrophoretic methods. On IEF-PAGE, at least seven isoforms are observed in the basic region of the pH 2–6 gel (Figure 2, lane 11). The rather broad ('smeared') band on SDS-PAGE corresponds to an apparent molecular mass of 66–97 kDa (*vide supra*). However, if the protein was detected by immunoblotting, binding of the detection antibody (clone AE7A5, mouse monoclonal anti-EPO antibody directed against the first 26 amino acids of the *N*-terminus) to Mircera occurred with decreased affinity, compared with non-PEGylated epoetins [66]. It could be demonstrated that the strong solubilization properties of SDS were the reason for the lowered detection sensitivity. SDS binds to both the PEG- and protein-parts of Mircera and the SDS-solubilized PEG-chain was shown to be the prime reason for the smeared band on SDS-PAGE [67]. Experiments with polyethylene glycols of various chain lengths and subsequent PEG- and protein-specific gel stains helped clarify this behavior (Figure 10A–D).

By replacing SDS with a detergent, which does not bind to PEG, the band shape of Mircera could be significantly narrowed. A detergent with these properties is sodium *N*-lauroylsarcosinate (sarcosyl), a methyl glycine-based anionic surfactant, which has been frequently employed in molecular biology for preparation of cell lysis buffers. In analogy to SDS-PAGE, the electrophoretic method was named sarcosyl-PAGE [66]. Sarcosyl-PAGE specifically improves the electrophoretic performance of PEGylated EPO, but leaves the performance characteristics of other epoetins unaltered (Figure 10C & D). So far, no approved PEGylated EPO biosimilars have entered the international market.

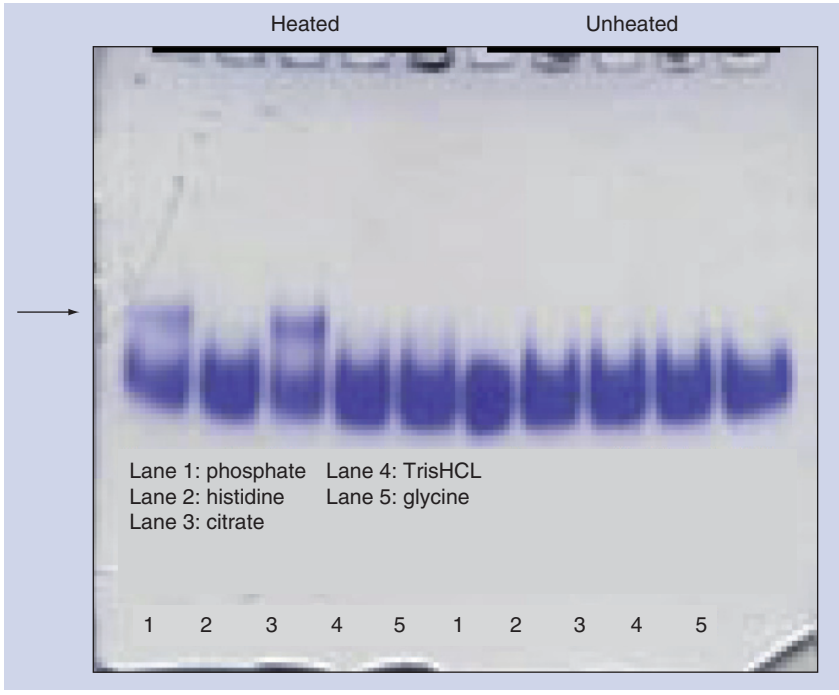

Figure 9. Native PAGE of recombinant erythropoietin. Depending on the composition of the formulation, erythropoietin may form aggregates upon heat exposure. Oligomerization and aggregate formation are seen by additional bands with higher molecular mass (e.g., lanes 1 and 3). Detection method: Coomassie stain.
Reproduced with permission from [13].

Reagent array analysis method in combination with SDS- & IEF-PAGE methods

Glycan analysis plays an important role in the characterization of therapeutic glycoproteins. Protein glycosylation is a post-translational modification, which is cell- and organism-specific and, consequently, also characteristic for the host cell-line and culture conditions employed in the production of pharmaceuticals [68]. Glycans can be either analyzed on the level of the intact glycoprotein, its glycopeptides, or after removal of the protein chain. LC, GC, CE, MS and NMR

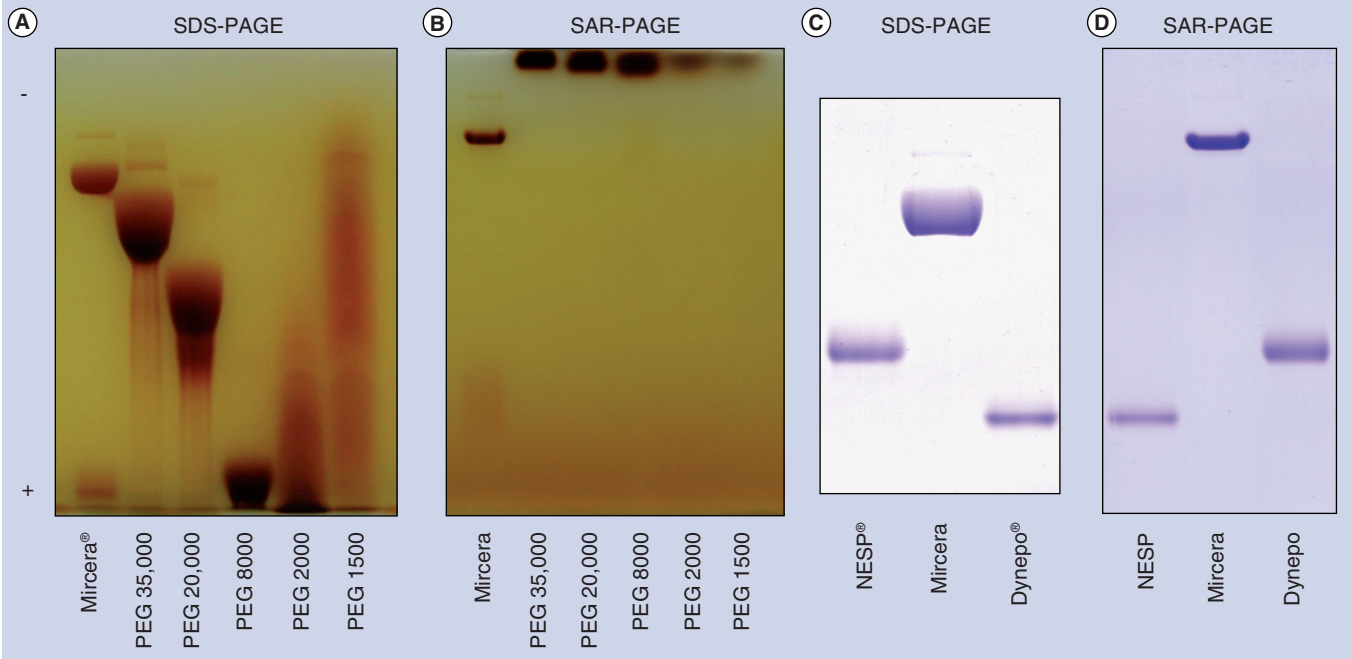

Figure 10. Comparison of SDS- and sarcosyl-PAGE regarding performance characteristics for epoetins. Due to the interaction of SDS with polyethylene glycols **(A)** PEGylated erythropoietin (Mircera®) leads to a smeared band on SDS-PAGE **(C)**. No such interaction is seen for the detergent sarcosyl (SAR-PAGE; **B & D**). Iodine-based PEG-specific **(A & B)** and protein-specific Coomassie R-250 **(C & D)** stains are shown.
NESP®: Novel erythropoiesis stimulating protein; SAR-PAGE: Sarcosyl-PAGE.
Reproduced with permission from [66].

spectroscopy, or a combination of them (e.g., LC–MS, GC–MS or CE–MS), are the main methods used for this purpose [12,69]. Glycans can be either enzymatically (exo- and endoglycosidases) or chemically (e.g., hydrazinolysis and β-elimination) removed from glycoproteins. Peptide-*N*-glycosidase F, an endoglycosidase, is frequently applied for removal of *N*-glycans. Depending on their linkage-specificity, some exoglycosidases may provide information on the monosaccharide composition and the linkage types present in glycans. The so-called reagent array analysis method (RAAM) sequentially deglycosylates oligosaccharide structures by combining different exoglycosidases. After each cleavage step, the remaining glycan-fragments are analyzed and more detailed information regarding the intact glycan structure is derived.

RAAM analysis can be also combined with gel electrophoretic methods. The combination is particularly useful when very low-abundant endogenous glycoproteins have to be compared with their recombinant counterparts. 'Higher' amounts of human EPO (8 mg) were first isolated in 1977 [70]. A total of 2550 l of urine were needed and the urine was from patients suffering from aplastic anemia, who excrete much higher amounts of EPO than nondiseased persons. This EPO was the basis for cloning the EPO gene in 1985 and the first production of recombinant EPO [71,72]. However, due to its low abundance, highly purified endogenous EPO is unavailable today and no detailed structural analysis with state-of-the-art techniques could be performed. In 2011, a RAAM analysis on EPO pharmaceuticals, including many biosimilars, was published [54]. The aim of this study was to compare endogenous EPO (urine or serum/plasma) with therapeutic products. For sensitivity reasons, RAAM was combined with SDS-PAGE and western blotting. Up to five exoglycosidases were used: a broadly specific α-sialidase (*Arthrobacter ureafaciens*) for removal of terminal sialic acids; a β(1–4) linkage-specific galactosidase (*Steptococcus pneumonia*); an *N*-acetyl-β-D-glucosaminidase from *Steptococcus pneumonia*, which can be blocked by steric hindrance; a broadly specific α-mannosidase (Jack bean); and a β-mannosidase from *Helix pomatia*. The experiment was first performed with BRP-EPO, the reference preparation for human recombinant epoetin alfa and -β and the reference for human urinary EPO (National Institute for Biological Standards and Control, Hertfordshire, UK),

which contains approximately 80 ng of human endogenous (uh)EPO in 2 mg urinary extract. While both EPOs behaved similarly upon treatment with α-sialidase and β-D-galactosidase (as seen by a concordant decrease in molecular mass on SDS-PAGE), the action of *N*-acetyl-β-D-glucosaminidase was partly blocked by endogenous EPO. According to what was known then regarding glycan structure of uhEPO, both EPOs should have resulted in a cleavage product with the same molecular mass, that is the pentasaccharide core structure consisting of three mannoses linked to a disaccharide of two *N*-acetylglucosamins. Instead, the mass of BRP-EPO decreased more than the mass of uhEPO, suggesting significant structural differences between their glycans (FIGURE 11). Additional treatment with α- and β-mannosidase further decreased the mass of recombinant EPO, in agreement with the known glycan structure, but no such decrease was observed for uhEPO. The resistance was obviously caused by blocking of α-mannosidase due to the incomplete action of *N*-acetyl-β-D-glucosaminidase.

The experiment was repeated with 25 EPO biosimilars (Abseamed, Alfaepoetina, Beijing 4 Rings, Epo alfa Hexal, Binocrit, Epiao, Epocrin, Epofer, Epofit, Eposino, Eposis, Eposure, Epotop, Epotrust, Erythrostim, Espogen, Hemax, Jimaixin, Repotin®, Retacrit, Shanpoietin, unknown [China], Vintor, Wepox and Zyrop) and three originator substances (Erypo, NeoRecormon and Dynepo). All of these preparations behaved similarly, that is they were more or less completely cleaved by the exoglyosidases, while uhEPO displayed the described resistance (FIGURE 12) [54].

The behavior was independent of the cell-lines used for the production of the recombinant epoetins (CHO, baby hamster kidney or human fibrosarcoma HT-1080). In order to rule out that structural changes during the excretion process of endogenous EPO in urine were responsible for exoglycosidase blockage, endogenous EPO isolated from human serum was studied with RAAM. A similar blocking effect was observed, which demonstrated close structural identity between the two forms of endogenous EPO [54].

Instead of SDS-PAGE, RAAM experiments can also be performed in combination with IEF-PAGE. In the study described above, treatment with α-sialidase resulted in almost complete loss

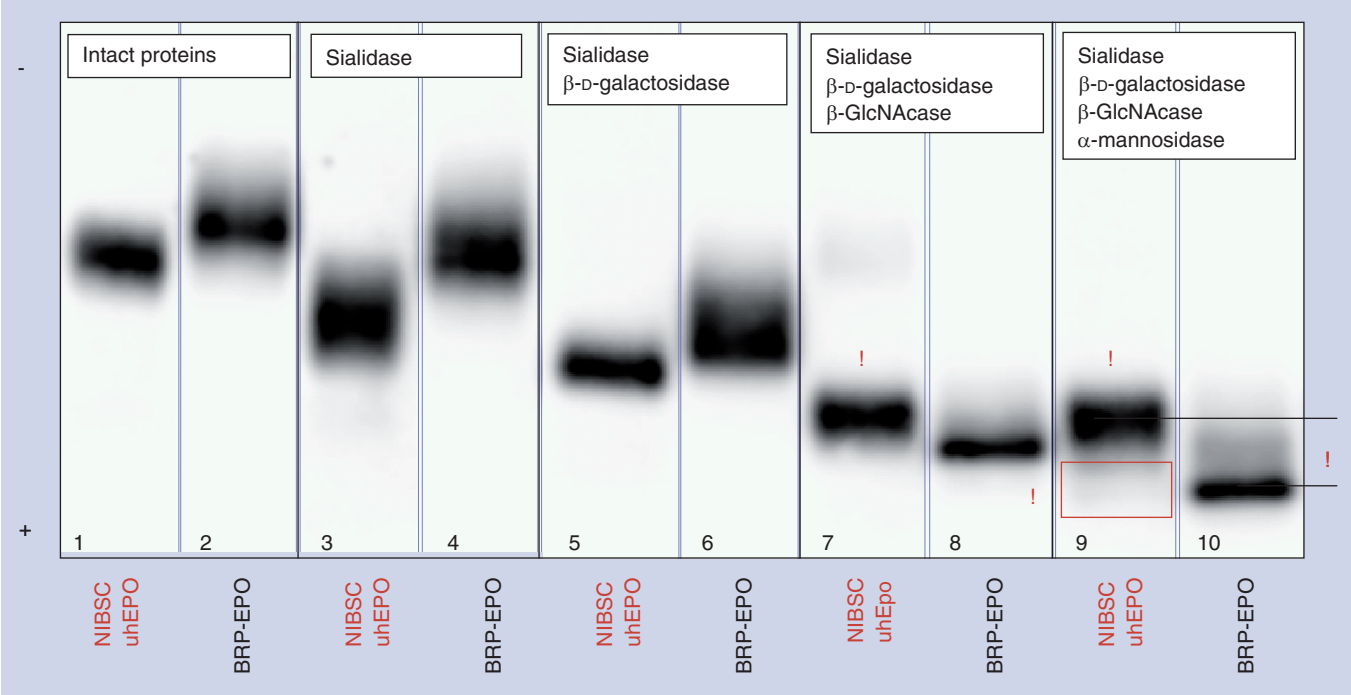

Figure 11. Reagent array analysis method combined with SDS-PAGE. Due to partial blocking of *N*-acetyl-β-D-glucosaminidase (*Steptococcus pneumonia*), human endogenous EPO (NIBSC [Hertfordshire, UK] human endogenous EPO standard) cannot be further degraded by α-mannosidase (Jack bean). No such behaviour is observed for the recombinant standard (BRP-EPO), suggesting profound structural differences. Western blot with monoclonal EPO antibody (clone AE7A5).
EPO: Erythropoietin; NIBSC: National Institute for Biological Standards and Control; uhEPO: Human endogenous erythropoietin.
Reproduced with permission from [54].

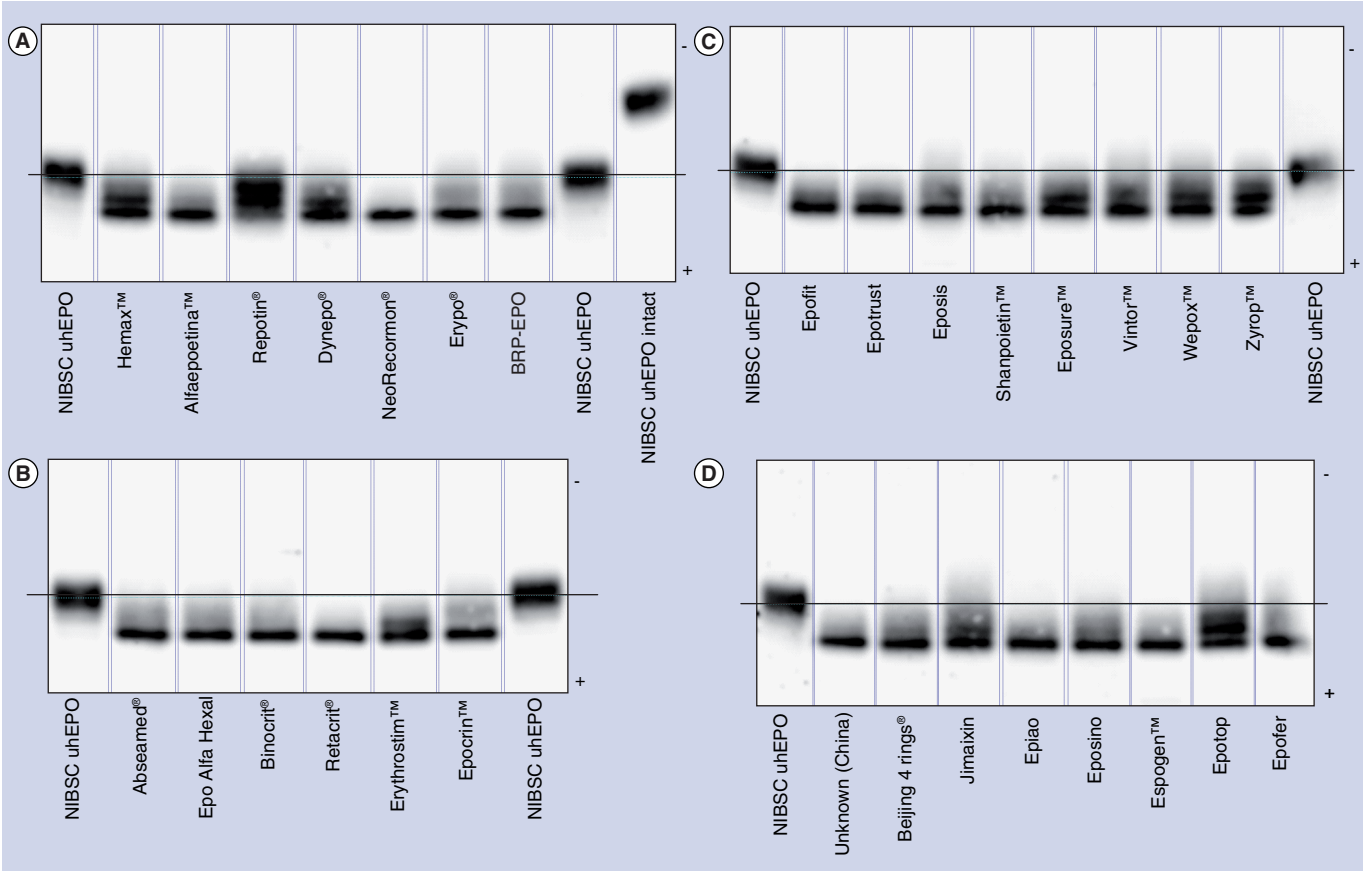

Figure 12. Reagent array analysis method/SDS-PAGE analysis of 25 originator and biosimilar epoetins. Independent of the cell line used for production, all recombinant proteins were cleaved by the four exoglycosidases (α-sialidase, β-galactosidase, *N*-acetyl-β-D-glucosaminidase and α-mannosidase). Contrary to that, uhEPO partly resisted degradation by blocking two of the four enzymes. Shown is the cleavage behavior of various EPOs after simultaneous treatment with the four enzymes **(A–D)**. Immunoblot with clone AE7A5 antibody.
EPO: Erythropoietin; NIBSC: National Institute for Biological Standards and Control; uhEPO: Human endogenous erythropoietin.
Reproduced with permission from [54].

of charged sugars on recombinant EPO, but not on uhEPO. Additional treatment with the other four exoenzymes confirmed this observation [54].

Fluorophore-assisted carbohydrate electrophoresis (PAGE of fluorophore-labeled saccharides)

Fluorophore-assisted carbohydrate electrophoresis (FACE) was developed in 1990 and is also known under the name 'PAGE of fluorophore-labeled saccharides' [73,74]. The method requires oligosaccharides with a reducing terminal sugar for derivatization with a suitable fluorophore (e.g., 8-aminonaphthalene-1,3,6-trisulphonic acid) [73]. The fluorophore adds negative charges to the oligosaccharides and ensures that even noncharged oligosaccharides can be separated by gel electrophoresis. Additionally, the fluorophore enables highly sensitive fluorescence detection of the carbohydrate after UV illumination. FACE can be combined with RAAM for structural analysis of glycans released from glycoproteins. Typically, microgram-amounts of highly purified glycoproteins are required.

FACE analyses were also performed with recombinant EPO, and confirmed the presence of bi-, tri- and tetra-antennary *N*-glycans composed of α(2,3)-linked terminal sialic acids, β(1,4)-linked D-galactoses, β(1,2/1,4)-linked *N*-acetyl-D-glucosamins, as well as α(1,3/1,6)- and β(1,4)-linked D-mannoses and α(1–6)-linked L-fucose [75–77]. Figure 13 shows data obtained with glycans released from recombinant EPO [77]. However, so far no EPO biosimilars have been analyzed with FACE. Due to the fact that no highly purified endogenous human EPO is available, FACE has also not been applied for direct comparison of endogenous and recombinant epoetins.

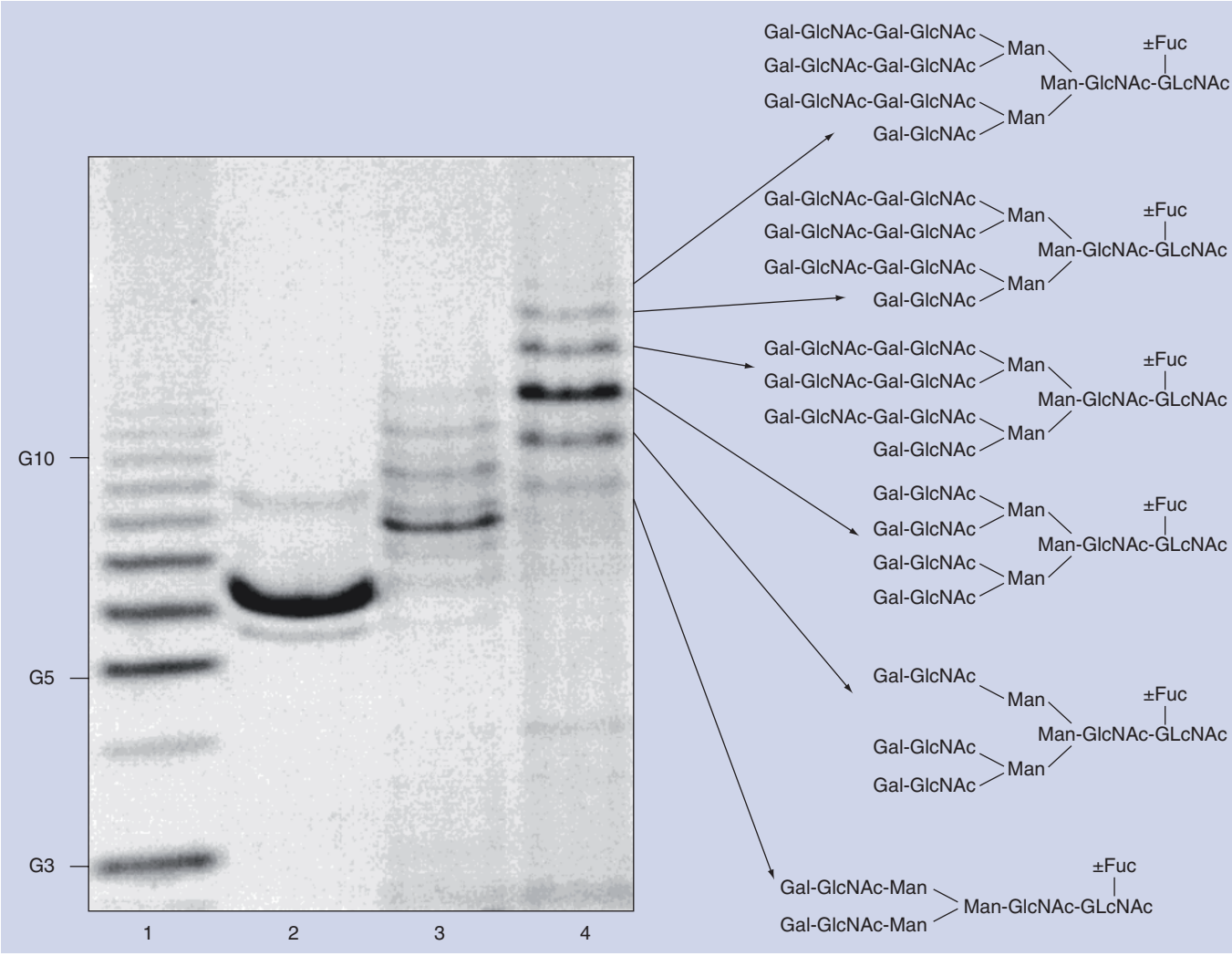

Figure 13. Fluorophore-assisted carbohydrate electrophoresis analysis of recombinant erythropoietin *N*-glycans.
By combining fluorophore-assisted carbohydrate electrophoresis with reagent array analysis, details about glycan structures are obtained.
Reproduced with permission from [77].

Conclusion & future perspective

Gel electrophoretic methods have been frequently applied for the analysis of recombinant EPOs and in particular those using polyacrylamide as matrix. The majority of studies used IEF- and SDS-PAGE. Both methods are relatively inexpensive, where costs of the analytical setup and daily operation are concerned. They allow fast comparisons of pharmaceutical products on the molecular level (distribution of isoforms and mass distribution of glycoforms) and, hence, have proven useful for the analysis of EPO biosimilars. Unlike other techniques used for characterization of therapeutic proteins, only the combination of gel electrophoretic methods with immunological methods (western blotting) provide the necessary sensitivity for comparing recombinant epoetins with their extremely low abundant endogenous counterparts (e.g., serum/plasma EPO). Sequential exoglycosidase treatment (RAAM) followed by SDS-PAGE analyses revealed significant structural differences between endogenous EPO and all available EPO pharmaceuticals. Data such as these may be useful for the generation of new EPO pharmaceuticals with closer structural identity to human endogenous EPO. In addition, gel electrophoretic techniques will also provide valuable data in case fully synthetic EPOs enter the pharmaceutical market [78,79].

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