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Research Article

High-sensitivity staining of proteins for one- and two-dimensional gel electrophoresis using post migration covalent staining with a ruthenium fluorophore

This paper describes the use of a ruthenium complex ((bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium-*N*-succidimyl ester-bis(hexafluorophosphate), abbreviated below as ASCQ_Ru) commercially available and chemically pure. This new ruthenium complex ASCQ_Ru brings an activated ester, allowing the selective acylation of amino acid side chain amines for the post migration staining of proteins separated in 1-DE and 2-DE. The protocol used is a simple three-step protocol fixing the proteins in the gel, staining and then washing, as no lengthy destaining step is required. First the critical staining step was optimized. Although in solution the best described pH for acylating proteins with this reagent is phosphate buffer at pH 7.0, we found that best medium for in-gel staining is unbuffered ACN/water solution (20/80 v/v). The two other steps are less critical and classical conditions are satisfactory: fixing with 7% acetic acid/10% ethanol solution and washing four times for 10 min with water. Sensitivity tests were performed using 1-DE on protein molecular weight markers. We obtained a higher sensitivity than SYPRO® Ruby with a detection limit of 80 pg of protein *per* well. However, contrary to SYPRO Ruby, ASCQ_Ru exhibits a logarithmic dependency on the amount of protein. The dynamic range is similar to SYPRO Ruby and is estimated between three and four orders of magnitude. Finally, the efficiency of the post migration ASCQ_Ru staining for 2-D gel separation is demonstrated on the whole protein extract from human colon carcinoma cells lines HCT 116. ASCQ_Ru gave the highest number of spot detected compared to other common stains Colloidal CBB, SYPRO Ruby and Deep Purple™.

1 Introduction

2-D PAGE is one of the most important tools in protein separation where thousands of protein spots can be separated, resulting in a global view of the state of a proteome. Radioactive detection offers the best sensitivity allowing the visualization of approximately 10 000 protein spots. However, limitations in safety regulation confine this method to equipped laboratories. Among the various standard protein detection methods, CBB dyes [1, 2] that reveal <1000 protein spots have been the most frequently used in laboratories due to their simplicity of use. Nevertheless, the increase of MS detection sensitivity allows analyzing few femtomoles of proteins, creating a race on high-sensitivity protein detection method discovery. So, sensitive staining methods such as silver staining [3–5] have been improved to allow protein identification by MS. But the compatibility of this method with MS is often discussed [6] due to oxidation side-reactions. Recently, fluorescent dyes have gained popularity, since they met the maximum needs of protein detection: sensitivity and broad linear dynamic range. Actually, the most sensitive stain remains the SYPRO Ruby that detects

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Abbreviation: ASCQ_Ru, bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium-*N*-succidimyl ester-bis(hexafluorophosphate)

Table 1. Summary of physical and spectral properties of the common dyes and of the new ruthenium complex ASCQ_Ru used in this study

Stains	Wavelengths	Theoretical sensitivities	Linear dynamic ranges	References
Conventional CBB	$\lambda = 595$ nm	30–100 ng	10–30	[2]
Colloidal CBB	$\lambda = 595$ nm	8–10 ng	10–30	[1]
Silver nitrate	$\lambda = 490$ nm	2–4 ng	10	[3–6]
SYPRO Ruby	$\lambda_{excitation} = 280$ nm and 450 nm, $\lambda_{emission} = 610$ nm	$<$1–2 ng	10 000	[9–11, 13, 26]
Deep Purple	$\lambda_{excitation} = 395$ nm and 520 nm, $\lambda_{emission} = 610$ nm	65 pg	10 000	[14]
ASCQ_Ru	$\lambda_{excitation} = 458$ nm, $\lambda_{emission} = 628$ nm, [17–19]	80 pg	10 000	This work

$<$1 ng/band of proteins from gel electrophoresis [7] and exhibits three orders of dynamic range [8–13]. However, despite its high sensitivity and its broad dynamic range, the use of SYPRO Ruby is still limited as it is sold only as a formulated solution, which does not allow optimizing it for the various electrophoresis protocols and nature of proteins.

This study describes the development of a new ruthenium complex ASCQ_Ru commercially available as a pure chemical for 1-DE and 2-DE staining, which presents a higher sensitivity than SYPRO Ruby. Furthermore, the new ruthenium complex bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium-*N*-succinimidyl ester-bis(hexafluorophosphate) (ASCQ_Ru) has a lower cost compared to SYPRO Ruby. Staining a large 2-D gel with 2 mg of ASCQ_Ru in a 500 mL solution costs approximately 48 euros (#120 euros *per* 5 mg), although this volume of SYPRO Ruby costs 144 euros (#1440 euros *per* 5 L). The developed methodology is evaluated both on 1-DE and 2-DE and compared to common stains presented in Table 1 (colloidal CBB, SYPRO Ruby) and also to the Deep Purple fluorophore [14, 15] commercialized by Amersham Biosciences.

2 Materials and methods

2.1 Reagents

High-molecular-weight Calibration Kits, Immobiline Dry-Strip™ and Deep Purple Total Protein Stain™ were obtained from Amersham Biosciences (Uppsala, Sweden). SYPRO Ruby was purchased from Molecular Probes (Interchim, Monluçon, France). Duracryl™ and immobilized pH gradient (IPG) rehydratation/loading buffers were supplied from Genomic Solutions (Steinheim, Germany). The proteinase-inhibitor Complete™ tablets were obtained from Roche Diagnostics (Meylan, France). The ASCQ_Ru and all other reagents used were obtained from Sigma Aldrich/Fluka (St-Quentin Fallavier, France).

2.2 Cell culture

Human colon carcinoma cell lines HCT 116 (ATCC CCL 247) were cultured in McCoy's 5 A modified medium (Life Technologies) supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM), 100 U/mL penicillin, and 100 g/mL streptomycin. The cells were maintained at 37°C in a 5% CO_2 atmosphere.

2.3 Protein extraction

The HCT 116 cells were washed three times with ice-cold PBS. The cells were lysate with a buffer containing 50 mM Tris base pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 7 M urea, 4% CHAPS, 65 mM DTT, proteinase-inhibitor tablet for 30 min at −20°C. The samples were centrifuged at $20\,000 \times g$, at 4°C for 15 min. The proteins were precipitated with ethanol and solubilized in a sample containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.04 M Tris base and 65 mM DTT. Proteins samples were stored at −20°C until the protein amounts were quantified using the commercial kit from Amersham Biosciences PlusOne 2-D Quant Kit (Uppsala, Sweden).

2.4 1-DE and 2-DE

2.4.1 SDS-PAGE gel electrophoresis

SDS-PAGE was performed by the standard method [16] with a 4.0% stacking gel and a 8.0% separating gel using ready-to-use Duracryl solution of acrylamide and cross-linker. The high-molecular-weight calibration kit used for dilution series contains myosin (220 kDa, 25 μg), α_2-macroglobulin (170 kDa, 100 μg), β-galactosidase (116 kDa, 16 μg), transferrin (76 kDa, 17 μg), and glutamate dehydrogenase (53 kDa, 18 μg). The protein mix was dissolved in a denaturing buffer containing 3.75 M Tris base, 20% v/v glycerol, 4% w/v SDS, and 3% w/v DTT, diluted 3:5 at each step range from 166 to 1.6 ng deposited *per* well. Electrophoresis was carried out using a Hoefer SE 600

Ruby™ system (Amersham Biosciences) with running buffer containing 25 mM Tris base and 192 mM glycine, at 100 mA *per* gel until the bromophenol blue front had reached the bottom of the gel.

2.4.2 2-DE

Protein samples (400 μg of total proteins) were mixed with 225 μL of loading buffer for IPG strips (Genomic Solutions) and 225 μL of urea solubilization/rehydratation buffer for IPG strips (Genomic Solutions) to obtain a final volume of 450 μL. The mixture was applied in-gel for reswelling with a dry IPG 240 mm, pH 3–10 linear gradient (Immobiline DryStrip, Amersham Pharmacia Biotech) on an Ettan IPGphor system (Amersham Pharmacia). Complete sample uptake into the strips was achieved after 9 h at 20°C with a voltage of 50 V. Focusing was performed at 200 V for 1 h, at 1000 V for 1 h, and at 8000 V for 13 h. The current was limited to 50 μA *per* strip, and the temperature maintained at 20°C for all IEF steps. For SDS-PAGE, the IPG strips were incubated in equilibration buffer containing 37.5 mM Tris-HCl (pH 8.8), 6 M urea, 2% w/v SDS, 30% v/v glycerol, and 2% w/v DTT for 15 min, and then incubated for 15 min in equilibration buffer supplemented with 2.5% w/v iodoacetamide. The equilibrated IPG strips were transferred for the second dimension (SDS-PAGE) onto 10% Duracryl (Genomic solutions) gels ($255 \times 205 \times 1.5$ mm). Electrophoresis was carried out at 20°C using a Ettan Daltsix system (Amersham Pharmacia) with 25 mM Tris as the running buffer, 192 mM glycine containing 0.1% w/v SDS, at 15 mA *per* gel for 16 h, until the bromophenol blue had reached the bottom of the gel.

2.5 Staining

2.5.1 Staining with CBB

CBB staining of the preparative gels was performed according to Neuhoff *et al.* [1]. Gels were fixed in 50% v/v ethanol/water containing 2% w/v orthophosphoric acid for at least 2 h, and rinsed three times in ultra pure water. Gels were then incubated for 3 days in 34% v/v methanol containing 17% ammonium sulfate, 2% w/v orthophosphoric acid, and 1 g of CBB G-250 for 2 L of solution. Gels were destained by multiple washing with water for 1 day.

2.5.2 Staining with SYPRO Ruby

The staining method used was described previously by Berggren *et al*. [9]. SYPRO Ruby is available only as a commercial and ready-to-use solution. Gels were fixed after electrophoresis using 7% acetic acid and 10% etha-

nol for 1 h. Gels were then placed into SYPRO Ruby protein gel stain for a night with continuous gentle agitation. The gels were destained three times using 7% acetic acid and 10% ethanol for 20–30 min. Finally, the gels were rinsed three times with water before the image acquisition.

2.5.3 Staining with deep purple total protein stain

The protocol [14] used for the staining with Deep Purple is precisely the one commercially recommended by Amersham Biosciences. Gels were fixed overnight in a solution containing 7.5% v/v acetic acid and 10% v/v methanol. The concentrated stock stain was diluted at 1:2000 ratio with ultra pure water, and gels were stained by a simple incubation for 1 h in a dark environment. Then the stain was poured off and replaced with 7.5% acetic acid. The gels were rinsed three times for 20 min with water before the image acquisition.

2.5.4 Optimization of the staining with the new ruthenium complex ASCQ_Ru

Gels were fixed after electrophoresis using a 7% acetic acid/10% ethanol solution for 1 h. The bis-(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium-*N*-succinimidyl ester-bis(hexafluorophosphate) was used at different proportions between 200 μg and 4 mg (discussed in Section 3.1). For each quantity, the stain was dissolved in 500 mL of a range of solutions (Section 3.1), including a 0.1 M phosphate buffer (pH 7.0 and 9.0), a 0.1 M ammonium bicarbonate buffer (pH 7.0 and 9.0), a 1.5 M Tris base buffer/ACN 20% (pH 8.0), a Tris base buffer-ethanol 20% (pH 8.0), an unbuffered 20% ethanol/water v/v solution (measured pH 4.5), and a 20% ACN/water solution (measured pH 4.0). Different times of incubation were evaluated varying from 1 h to a night. The gels were placed into the solutions containing the stain for various times of incubation from 1 h to overnight (Section 3.1). Prior to the image acquisition, the stained gels were rinsed four times with deionized water for 10 min.

2.6 Optimized staining protocol with the new ruthenium complex ASCQ_Ru

The fixation step is done for 1 h with a 7% acetic acid/10% ethanol solution v/v. Each gel is rinsed twice for 10 min with fresh water. Then each gel is placed in the staining solution containing 2.03 mg of dye in 500 mL of unbuffered 20/80% ACN/water solution v/v at room temperature for a night. Prior to the image acquisition, the stained gels were rinsed four times with deionized water for 10 min.

2.7 Detection of proteins

Fluorescent stained-1-D and 2-D gels were digitized at 200 dpi resolution using a Typhoon® 9000 scanner (Amersham Biosciences). Image acquisition of gels stained with SYPRO Ruby and with the new ruthenium complex ASCQ_Ru were performed at 550 V with the blue 1 laser (λ_{exc} = 457 nm/λ_{em} = 610 nm BP30). The gels stained with Deep Purple were performed at 550 V with the green laser (λ_{exc} = 532 nm/λ_{em} = 610 nm BP 30 nm). Colloidal blue stained–2-D gel was digitized at 200 dpi resolution using an Imagescanner® (Amersham Biosciences). A calibration filter using different shades of gray was applied to transform pixel intensities into optical density units. The images were exported in TIF format and imported for analysis into ImageQuantTL (Amersham Biosciences) for 1-D gels and Progenesis® V2003–01 2-D gel image analysis software (Nonlinear Dynamics) for 2-D gels. Briefly, after automatic spot detection, the background was removed from each gel and the images were edited manually, adding, splitting, and removing spots if the program missed spots or if it did not define the spots properly.

3 Results and discussion

The stain evaluated in this paper, the bis (2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium-*N*-succinimidyl ester-bis(hexafluorophosphate) (Fig. 1), is commercially available from Fluka. This molecule contains an activated ester of ruthenium complex that induces an acylation reaction of amino side chain amines. The first part of this paper describes stain parameters optimization including fixing step, buffer pH and composition, dye quantity, staining incubation time and washing. The next one shows staining performance on standard proteins separated on 1-DE. Finally, the last part shows results obtained with ASCQ_Ru on a biological sample HCT 116 total protein extract separated on 2-DE and the comparison with other classical stains (colloidal CBB, SYPRO Ruby and Deep Purple). The presented work demonstrates a detection limit of 80 pg of stained proteins and a dynamic range of 10 000.
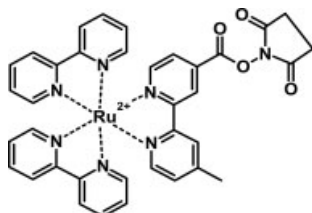
Figure 1. Chemical formula of ASCQ_Ru, bis-(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium-*N*-succinimidyl ester-bis(hexafluorophosphate).

3.1 Optimization of stain parameters

The protocol used is a simple three-step protocol (fixing with 7% acetic acid/10% ethanol solution, staining, and washing with water) and contrasts with some complex staining procedures as silver staining.

Different buffers were used for the dilution of the ASCQ_Ru. The first buffer evaluated was the one recommended by Fluka, the 0.1 M phosphate buffer (pH 7.0). The results obtained with this buffer were not satisfactory. Only bands corresponding to the most concentrated protein (α_2-macroglobulin) of the standard mixture were visualized. Buffer concentration variations do not give any improvement in results. The use of other buffers adjusted at pH 7.0 (ammonium bicarbonate buffer) does not increase the protein visualization. So, other types of buffers were evaluated at basic pH in order to stay close to the Fluka pH recommendations. Phosphate buffer, ammonium bicarbonate buffer and solutions based on the use of Tris base buffer (1.5 M Tris base buffer with 20% of ACN (pH 8.0) and 1.5 M Tris base buffer with 20% of ethanol (pH 8.0)) were evaluated without any protein visualization on gels. According to the work of Geisser *et al.* [17] on the pH dependence of the maximum emission intensities of different ruthenium complexes, solutions with more acidic pH were evaluated for the stain dilution. An unbuffered 20% ethanol solution, which led to a measured pH 4.5, allows the visualization of proteins, but very slightly. This result is probably due to the fact that the ruthenium complex is not fully solvable in the ethanol solution and a part of the dye is probably lost during the dilution step, leading to this low spot intensity observed on the gel. Some works on the evaluation of the excited state absorption of different ruthenium complexes [18–20] propose the ACN mixed with water as a good solvent, so we evaluated the capacity of a 20% ACN solution which gave a measured pH of 4.0. The result obtained with this condition (Fig. 2a) leads to the conclusion that this type of solvent is appropriate for stain dilution and, in consequence, for protein visualization.

The dye amount and the stain incubation were optimized in order to obtain the best response in term of spot intensity. The experiments on different stain incubation time show that protein bands are less intense after 1 h of incubation than a night, showing that stain incubation duration is crucial for protein-sensitive revelation. More than a night of staining does not increase the intensity of protein detection. The dye quantity was evaluated over a range varying between 200 nmol and 4 μmol. The fluorophore quantity variation effect on the fluorescence intensity of α_2-macroglobulin (in quantity of 94 680 pg) is fitted by the
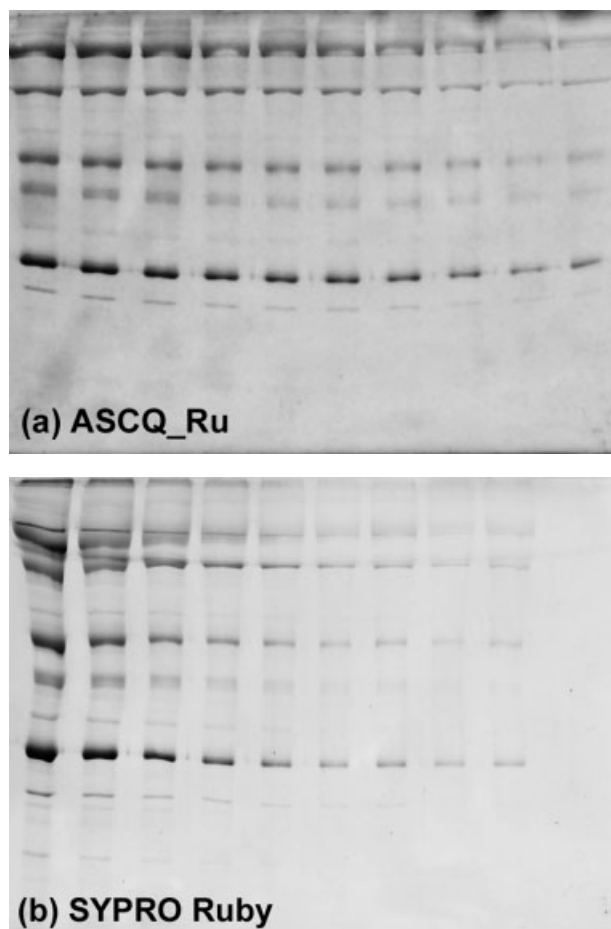
Figure 2. (a) 1-D gel (180 mm × 160 mm, 4% stacking gel and 8% separating gel) of the high-molecular weight calibration kit stained with 2 μmol of the new ruthenium complex ASCQ_Ru in unbuffered 20% ACN/water solution. Dilution series of protein standards by 3:5 at each step ranges from 166 to 1.6 ng deposited *per* well. (b) 1-D gel (180 mm × 160 mm, 4% stacking gel and 8% separating gel) of the high-molecular weight calibration kit stained with SYPRO Ruby. Dilution series of protein standards by 3:5 at each step ranges from 166 to 1.6 ng deposited *per* well.

equation I_{max}(1-exp(ASCQ_Ru –0,4)/0.5), where ASCQ_Ru is the complex quantity expressed in micromole. This fit shows that the best result is obtained with 2 μmol of dye. The fully optimized protocol is described in Section 2.6.

3.2 Stain performance characteristics on 1-DE

To conduct studies on the intensity of the new ruthenium stain, a dilution series of molecular mass markers was assessed by determining the relative intensity of five proteins at different concentrations. Samples were serially diluted by a factor of 3/5 giving concentrations ranging from 166 600 to 1600 pg of the total protein extract. However, each protein is more or less concentrated according to the initial quantity in the "high-molecular-weight" calibration kit as described in Section 2. Protein-to-protein variations in staining intensity were measured by photodensitometry as described in Section 2.7. These experiments were performed on both gels stained with the new ruthenium-based complex (Fig. 2a) and gels stained with SYPRO Ruby (Fig. 2b) to establish a comparison between these two dyes in term of sensitivity. Graphs of the fluorescence intensity against the logarithm of the protein amount for the five standard proteins (as for example the Fig. 3a for the glutamate dehydrogenase) were plotted for both the new ruthenium stain ASCQ_Ru and SYPRO Ruby. The total amount of protein *per* band varies from 0.15 to 15 pg. So, the low-range weight of individual proteins varies from 0.015 pg for the less concentrated protein (β-galactosidase, transferrin, glutamate dehydrogenase) to 0.022 pg for myosin and 0.084 pg for the most concentrated protein α_2-macroglobulin whereas the high range quantity is 100 times higher. First, it should be pointed out that for the lower amounts of proteins ASCQ_Ru is always more sensitive than SYPRO Ruby. In the high weight range, the fluorescence intensity data of the new ruthenium stain ASCQ_Ru are well fitted by a straight line, which demonstrates a logarithmic dependence on the protein amount in the studied mass range. In contrast, at very low concentration, Fig. 3b shows that the fluorescence of the ASCQ_Ru varies linearly with the protein amount at the low weight range. SYPRO behaves very similarly and shows the same logarithmic dependence at upper protein amount but the curving of the dependence occurs at a much higher value (data not shown).

Table 2 shows that SYPRO is not very sensitive to the structure of the protein and that the observed slope in the dilution series is proportional to the amount of the protein in the high molecular weight calibration kit. In contrast, the new ruthenium stain ASCQ_Ru is much more sensitive to the protein structure. As this reagent reacts specifically with amino acid bearing an amino group, we tried to correlate the fluorescence intensity with the amino acid composition of the protein. Indeed, the observed slope is proportional to the amount of protein multiplied by the fraction of the reactive amino acid lysine and arginine in the protein (Fig. 4) except for glutamate dehydrogenase which is by far less responsive than expected. The observed fit is very good ($y = 89.488x + 12.448$, $R^2 = 0.9894$).

The detection limit of β-galactosidase typically observed with the new ruthenium complex ASCQ_Ru is 140 pg whereas the detection limit in-gels stained with SYPRO Ruby is 250 pg, as suggested by other studies [9, 11, 21,
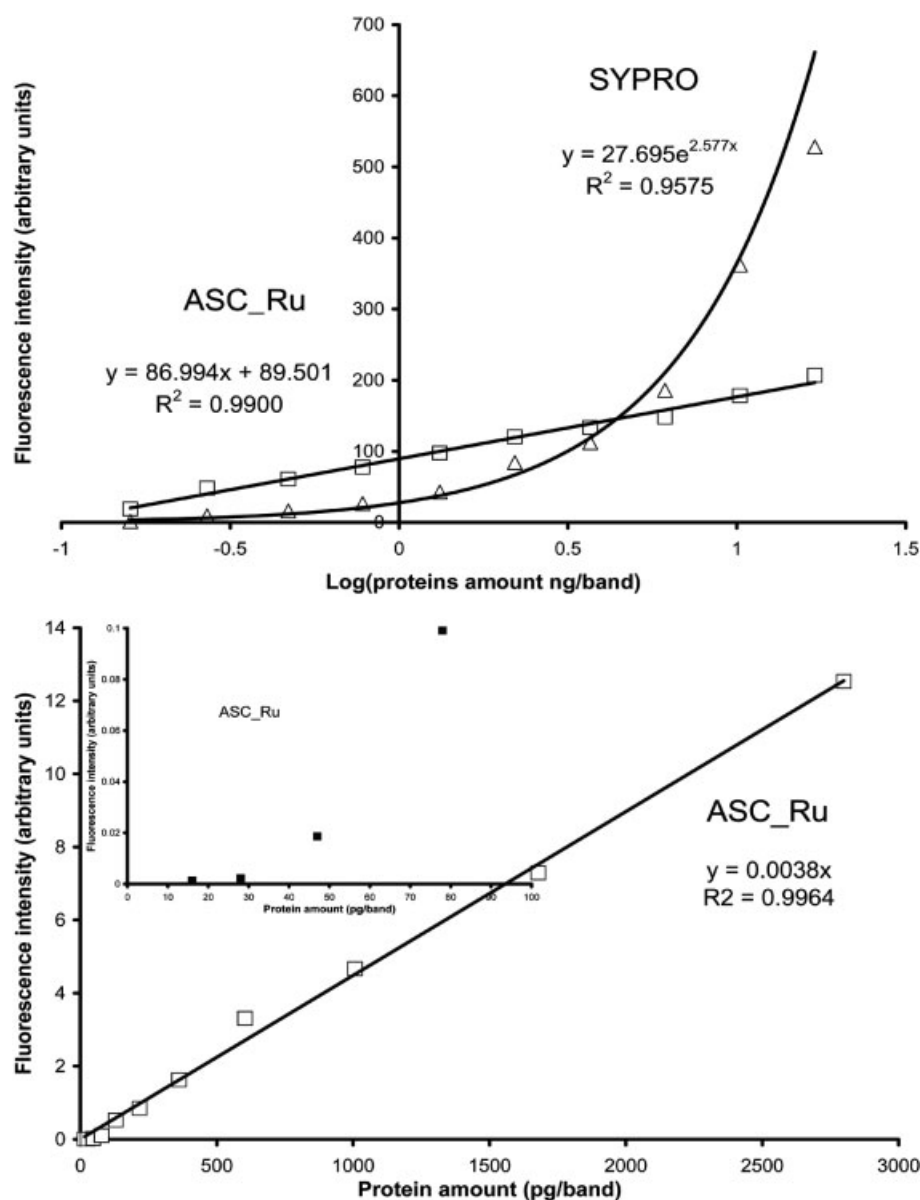
Figure 3. (a) Quantitation of dilution series of glutamate dehydrogenase visualized by both the new ruthenium complex ASCQ_Ru and SYPRO Ruby. The square marks refer to ASCQ_Ru, and the triangle depicts SYPRO Ruby. (b) Quantitation of dilution series of glutamate dehydrogenase visualized by the new ruthenium complex ASCQ_Ru. The zoom of the low mass range in insert shows that ASCQ_Ru allows quantification of glutamate dehydrogenase down to 80 pg and detect down to 50 pg.

Table 2. Correlation of fluorescence intensity of the new ruthenium complex ASCQ_Ru and SYPRO Ruby with the amount of protein in the high-molecular-weight calibration kit and with their reactive amino acid composition

Proteins	MW, kDa	Quantity, μg	SYPRO, linear fit[a]	Number of reactive amino acids, lysine+arginine	Quantity *x*, relative amount of reactive amino acids	ASCQ_Ru, logarithmic fit[b]
Myosin	220	25	57.8, $R^2 = 0.97$	314	4.05	334.9, $R^2 = 0.98$
α_2-Macroglobulin	170	100	49.0, $R^2 = 0.99$	140	9.51	878.1, $R^2 = 0.98$
β-Galactosidase	116	16	37.6, $R^2 = 0.97$	86	1.35	171.7, $R^2 = 0.99$
Transferring	76	17	38.3, $R^2 = 0.98$	85	2.07	184.7, $R^2 = 0.99$
Glutamate dehydrogenase	53	18	32.0, $R^2 = 0.99$	63	2.26	86.9, $R^2 = 0.99$

a) Correlation of fluorescence intensity of SYPRO Ruby with the amount of protein *per* band.
b) Correlation of fluorescence intensity of the new ruthenium complex ASCQ_Ru with the logarithmic amount of protein *per* band.
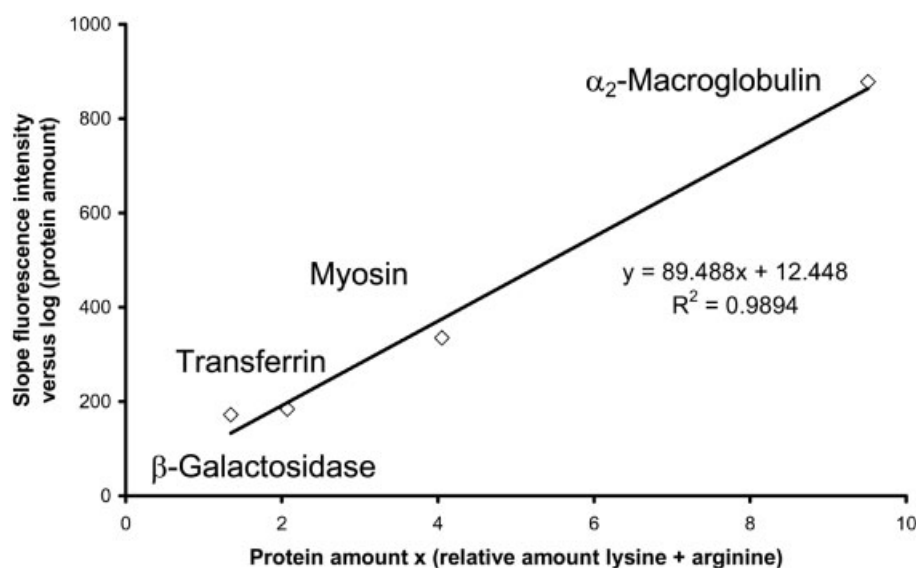
Figure 4. Correlation of the slope of the fluorescence intensity *versus* the log of the protein amount of the new ruthenium complex ASCQ_Ru with the fraction of protein multiplied by reactive amino acid fraction.

22]. In order to have more information on the lowest protein quantity detectable, we performed an experiment with the new fluorophore with a total protein amount dilution ranging from 30 789 to 176 pg. Figure 3b shows the graph of the fluorescence intensity against protein amount of the minor protein present in the mix, β-galactosidase, ranging from 2799 to 16 pg. The new ruthenium stain allows protein visualization at the level of 80 pg with a good confidence, and allows detection of as few as 50 pg of proteins, a quantity that is not detected with SYPRO Ruby [23]. This last consideration is very important because the challenge in the protein detection field is to gain sensitivity in low protein quantities.

### 3.3 Detecting proteins on 2-DE using the new ruthenium complex ASCQ_Ru

The new stain was extensively investigated on proteins separated by 2-DE. Proteins from human colon carcinoma cells lines HCT 116 were separated on 2-DE as previously described [24]. Gels were stained with Colloidal CBB (Fig. 5a), Deep Purple, SYPRO Ruby (Fig. 5b) and with the new ruthenium complex ASCQ_Ru (Fig. 5c). The small spikes observed on the gel stained with the ASCQ_Ru (Fig. 5c) are due to precipitated particles in the staining either from the stain itself or from chemical impurities. They can be reduced by multiple washing with deionized water. In order to compare the sensitivity of each stain, Progenenesis software (Nonlinear Dynamics) was used to find and count protein spot numbers of the 2-D gels. After an automatic spot detection, the images were edited manually in order to verify the automatic spot detection (missed spots, merged spot). A number of 1537 spots was detected using the new ruthenium complex staining *versus* 1354 detected with SYPRO Ruby, 554 with Deep Purple, and 697 with colloidal CBB staining. The new ruthenium complex detects more than 10% spots than the SYPRO Ruby, demonstrating its higher sensitivity.

However, whereas the spot intensities stained with the SYPRO Ruby (Fig. 5b) and the ASCQ_Ru (Fig. 5c) are very similar in the acidic and neutral regions, it is obvious that the new ruthenium stain is less sensitive in the alkaline region. This is surprising, as basic proteins which thus contain more lysine and arginine amino acids should be better detected by the new procedure. This observation can be correlated to the results obtained on standard proteins (Fig. 4) which shows that glutamate dehydrogenase is less responsive than the other proteins. In fact, glutamate dehydrogenase presents a slightly more basic p*I* than the other standard proteins used in the mass maker kit (p*I* = 7.3 compared to 5.4 for myosin, 6.6 for α_2-macroglobulin, 6.2 for transferrin, and 5.3 for β-galactosidase) and contains a little higher percentage of lysines and arginines (except for myosin). It is well known that there is a strong quenching effect when two ruthenium fluorophores are closed together. So, this reduced intensity can be, as suggested by one of the referees, a result of the quenching effect due to Ru-complexes in close proximity [25].

4 Concluding remarks

As proteomics moves toward high-throughput and more intensive studies of protein regulations, more sensitive detection methods are required. Protein visualization
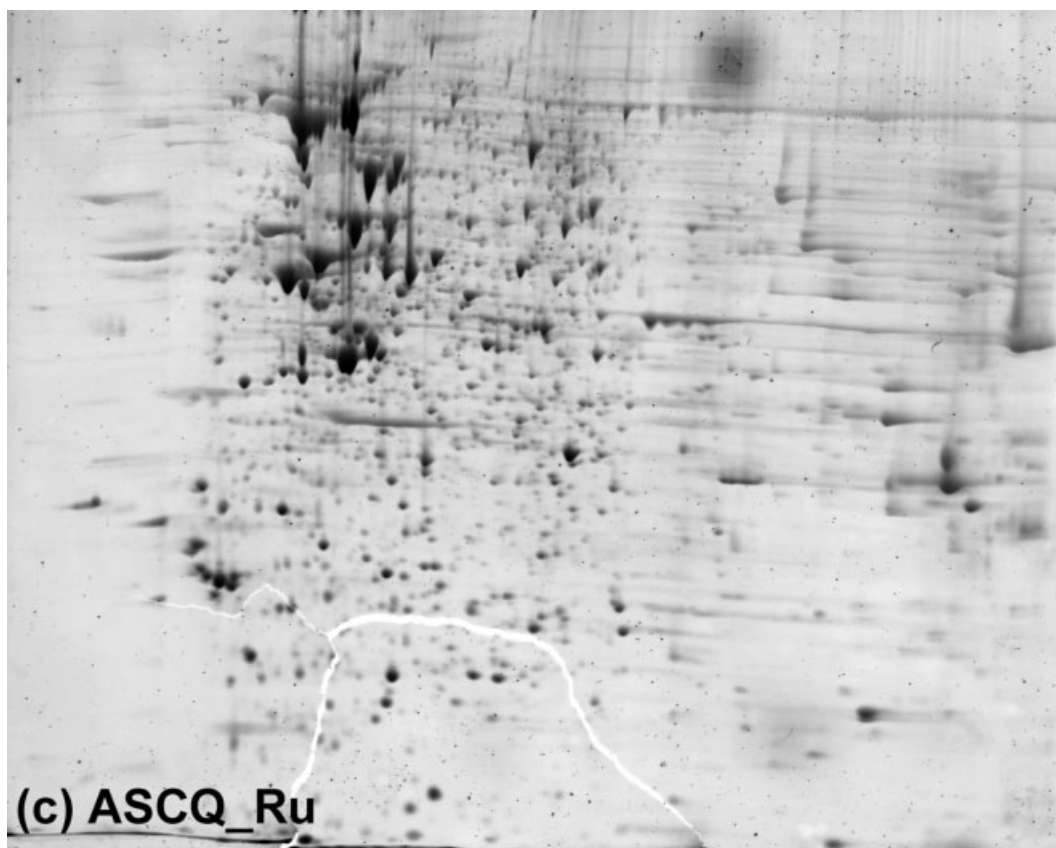
(b) SYPRO Ruby
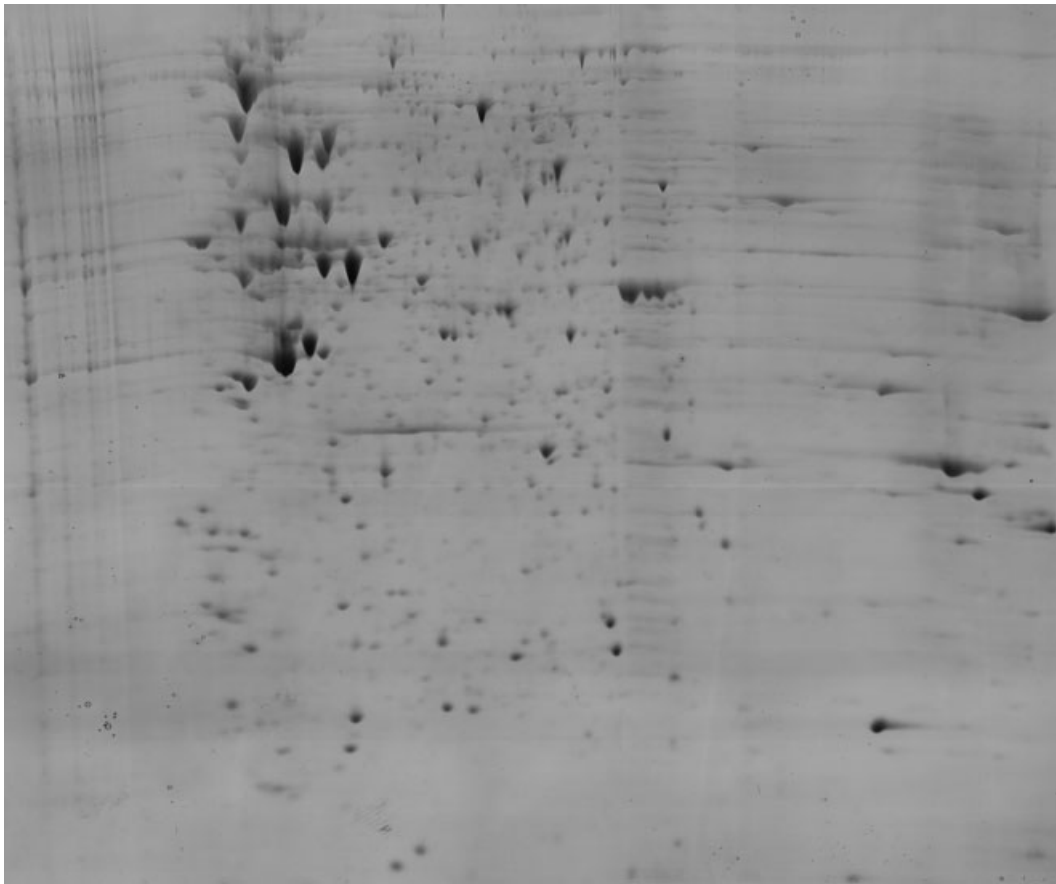
Figure 5. (a) Two-dimensional gel (240 mm × 200 mm, pH 3–10 linear gradient) of 400 µg of proteins from human colon carcinoma cells lines HCT 116 stained with Colloidal CBB. (b) Two-dimensional gel (240 mm × 200 mm, pH 3–10 linear gradient) of 400 µg of proteins from human colon carcinoma cells lines HCT 116 stained with SYPRO Ruby. (c) Two-dimensional gel (240 mm × 200 mm, pH 3–10 linear gradient) of 400 µg of proteins from human colon carcinoma cells lines HCT 116 stained with the new ruthenium complex ASCQ_Ru.

methods developed some years ago, such as Colloidal CBB, have limited sensitivities. Fluorescent stains, such as SYPRO Ruby, allow higher detection sensitivity but remain very expensive. This study demonstrates that the new ruthenium stain ASCQ_Ru introduced in this paper is more sensitive than SYPRO Ruby allowing the visualization of individual proteins over the quantity of 80 pg. This stain has also the advantage to be available in chemically pure form which allows a full control of the protocol and its optimization for various conditions. This new stain less expensive than the commercially available SYPRO Ruby: staining a large 2-D gel with 500 mL of ASCQ_Ru (2 mg) costs approximately 48 € (120 € *per* 5 mg), whereas this volume of SYPRO Ruby costs approximately 144 € (1440 € *per* 5 L). Because the evaluated stain is not fully compatible with the tryptic hydrolysis, other enzymes are currently under evaluation, as well as the compatibility of the generated peptides to MS analysis and protein identification by peptide mass fingerprint.

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