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Research Article

Performance of a novel sieving matrix of poly(vinyl alcohol)/acrylamide copolymer in electrophoretic separations of high molecular weight proteins from red cell membrane

The analysis of high molecular weight (HMW) proteins from complex mixtures is still a challenge in proteomics. This work introduces a novel hydrogel obtained by the copolymerization of an allyl-PVA derivative with acrylamide and bisacrylamide and applies this matrix to the electrophoretic separation of HMW proteins. By inducing gelation of polyacrylamide in the presence of variable amounts of allyl-PVA, it is possible to control and vary the average gel porosity. This gel is easy to produce and handle and offers the advantage of being highly mechanically resistant and macroporous. The new matrix was tested in mono-dimensional separations of complex protein mixtures extracted from red cell membranes with different detergents. The improved performance of this macroporous matrix allowed to identify new proteins by MS and immunoblot analysis using specific antibodies. In particular, the resolution of proteins ranging in size between 97 and 279 kDa was greatly improved here compared to standard polyacrylamide gels, suggesting that this matrix can be a useful tool in routine analysis of HMW proteins in cell biology.

Keywords:
High molecular weight proteins / Macroporous sieving matrix / Poly(vinyl alcohol) / Polyacrylamide / Proteomics DOI 10.1002/elps.201300529

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1 Introduction

The analysis of high molecular size proteins is still only partially covered by the methodological strategies presently available in proteomics as demonstrated by the small number of studies on high molecular weight (HMW) proteins in various cell models [1–3].

In the past 20 years, there have been many attempts to optimize the composition of protein sieving matrices to improve the electrophoretic resolution of HMW proteins using classical polyacrylamide (PA) or agarose and agarose-related polymers [1–4].

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Abbreviations: Cymal-6, 6-cyclohexyl-1-hexyl-β-D-maltoside; **DS**, destaining solution; **HMW**, high molecular weight; **PA**, polyacrylamide

Although PA pore size can be increased reducing bisacrylamide concentration, this is not the best approach for separating HMW proteins [5, 6] because poorly cross-linked gels are mechanically unstable.

Agarose and related polymers have shown some advantages over PA in the resolution of HMW proteins such as myosin heavy chains (200 kDa), dystrophin (400 kDa), thyroglobulin (330 kDa), spectrins (250 kDa), or von Willebrand factor [3, 7–10]. However, it is difficult to optimize the simultaneous separation of high and low molecular weight proteins in agarose. In a previous work we have synthesized and characterized covalently cross-linked, mixed-bed agarose-PA gels for electrophoresis formed by copolymerization of allyl-modified agarose with acrylamide [3]. The cross-linking mechanism enabled the formation of gels with higher porosity and elasticity. However, being weekly cross-linked, these gels tend to considerably overswell in buffer and, as a consequence, their application as SDS-sieving matrices is hampered.

This work reports on the development of a hydrogel for electrophoretic separation of HMW proteins characterized by high mechanical stability, optimal swelling properties, and soft rubberlike behavior. This hydrogel is formed by a

combination of physical entanglement and covalent crosslinking of PVA, bearing olefinic moieties, with PA and bisacrylamide. The presence of allyl groups allows the crosslinking of the two polymers thus reinforcing the gel mechanical strength.

The features of the new gel were tested in the SDS separation of HMW proteins from red cells using different strategies to extract membrane proteins. The gel proved to be an excellent, easy to handle matrix to study HMW proteins, suitable for application in classical proteomic techniques such as tryptic digestion, peptide extraction and identification, or immunoblot analysis with specific antibodies.

2 Materials and methods

2.1 Chemicals

NaCl, KCl, Na_2HPO_4, Na_3VO_4, KH_2PO_4, $MgCl_2$, NH_4HCO_3, MOPS, Tris, choline chloride, benzamidine, β-mercaptoethanol, glycine, bromophenol blue, trypsin, SDS, 6-cyclohexylhexyl β-D-maltoside (Cymal-6), nonylphenol ethoxylate (Tergitol-type NP40), PVA (99% hydrolyzed, Mw ranging from 89 000 to 98 000 Da), NaOH, sodium borohydride, *N*-ethylmaleimide (NEM), allylglycidyl ether, and glycerol were purchased from Sigma/Aldrich (St. Louis, MO, USA); urea, thiourea, DTT, iodoacetamide, tri-*n*-butylphosphate, TFA, and α-cyano-4-hydroxycynnamic acid (CHCA) were from Fluka (Buchs, Switzerland); acetone, methanol, and ACN were from Baker (Deventer, the Netherlands); protease inhibitor cocktail tablets were from Roche (Basel, Switzerland), 40% acrylamide solution and 40% acrylamide/Bis solution, 37.5:1 were from Bio-Rad (Hercules, CA, USA); Immobilon Western Chemiluminescent HRP Substrate was from Millipore (Billerica, MA, USA); HiMark™ unstained and prestained protein standards were from Life Technologies (Carlsbad, CA, USA).

2.2 Synthesis of allyl-PVA

Three grams of PVA were dissolved in 70 mL of water at boiling temperature. To the solution, cooled at room temperature, 30 mL of 1 M NaOH were added, followed by the addition of 66 mg of sodium borohydride and of 3.2 mL of allylglycidyl ether (Fig. 1). The reaction mixture was stirred overnight at room temperature, diluted 1:1 with MilliQ water and neutralized with 1 N hydrochloric acid; finally it was dialyzed against water and freeze-dried to recover the polymer.

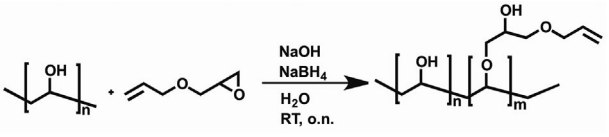

Figure 1. Allyl-PVA synthesis: schematic representation of allyl-PVA synthesis.

The degree of substitution of PVA was assessed by means of ^{13}C NMR spectroscopy. The proton-decoupled ^{13}C NMR spectrum shows the typical resonance of the PVA methylene carbons (44.5 and 45.8 ppm) and methine carbons (66 and 69 ppm) in agreement with previously reported assignments [11]. In the allyl-PVA spectrum, the two characteristics signals of $CH_2=$ and $=CH-$ of the double bond are present in a separated region: 116.05 and 135.27 ppm, respectively. The ratio between the integrals of methylene carbons of the backbone and allyl signal of the substituent was used to quantitatively determine the degree of allyl groups insertion, which was found to be 1.3%.

2.3 NMR analysis of allyl-PVA

^{13}C NMR spectra were obtained using a Bruker DPX 400 MHz spectrometer with DMSO-d^6 as solvent. Allyl-PVA was dissolved in DMSO-d^6 at room temperature.

2.4 Gel preparation

Stock solutions of allyl-PVA and PVA (5% w/v) were prepared by dissolving the polymer powder in water at boiling temperature. The stock solution, stable for at least 6 months, was diluted to form a final solution 1% w/v allyl-PVA, 7% T, 0.5% C PA/BIS in 375 mM Tris, pH 8.9, 0.1% SDS. TEMED (0.05% w/v) and ammonium persulfate (0.015% w/v) were added to initiate the polymerization. PA control gels (7% T, 2.6% C or 7% T, 0.5% C) were cast using a classical procedure [3,12–15]. In both gels, monomer solutions were polymerized for 2 h at room temperature before overlaying the stacking gel (4% T, 1.3% C in 0.125 M Tris-HCl buffer, pH 6.8).

2.5 Red cell membranes preparation

Whole blood was centrifuged at 2500 × *g* at 4°C in order to remove plasma, filtered through cotton to remove white cells, and rinsed three times with choline washing solution (150 mM choline, 1 mM $MgCl_2$, 10 mM Tris-MOPS pH 7.4 at 4°C). Packed red cells were lysed in ice-cold phosphate lysis buffer (5 mM Na_2HPO_4 pH 8.0, containing: protease inhibitor cocktail tablets, 3 mM benzamidine, 1 mM Na_3VO_4) [14, 15]. Red cell ghosts were washed several times in lysis buffer to obtain white membranes and used for monodimensional electrophoresis. The use of human samples was approved by the Ethical Committee of Verona University and the informed consent of all healthy participating subjects was obtained.

2.6 Mono-dimensional (1DE) and immunoblot analysis

Red cell ghosts were solubilized in the standard sample buffer (SB: 50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 100 mM β-mercaptoethanol, few grains of bromophenol blue) either

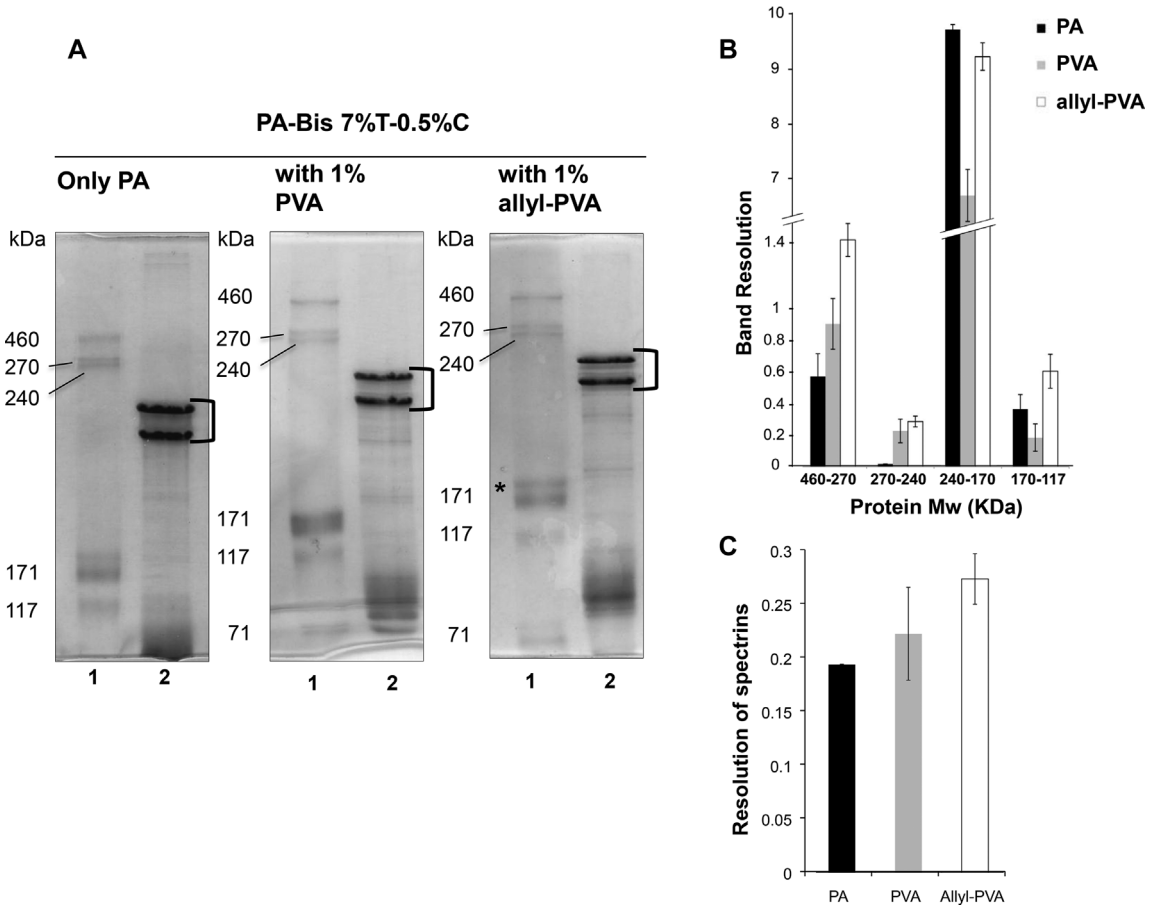

Figure 2. Evaluation of the resolution of HMW protein separations. (A) Separation profile of HMW proteins (lane 1: HMW markers; lane 2: proteins from red cell membranes solubilized in the sample buffer described in Section 2.6) in cross-linked PA (left panel) compared to mixed-bed gels containing PVA (middle panel) and allyl-PVA (right panel) in addition to PA and BIS (see Section 2.4 for gel preparation and Section 2.6 for 1DE conditions). (B) Histogram of the resolved bands from PA, PVA, and allyl-PVA gels run as in Fig. 3A. Mean values ± SD ($n = 4$) are reported. We considered gel areas corresponding to four molecular weight ranges: 460–270 kDa, 270–240 kDa, 240–170 kDa, 170–117 kDa. (C) Comparison of the resolution of the spectrins referred to gels in Fig. 2A. Data are shown as mean ± SD ($n = 4$). One representative gel of four with similar results.

alone or with different detergents (see Section 3 and Supporting Information Fig. 1).

HMW protein standards and the solubilized proteins from red cell membrane ghosts were separated by gel electrophoresis using a Mini-Protean II dual-slab gel cell (Hercules, CA, USA), 7 cm long, 5.5–6.5 cm wide 1.5 mm thick. The gels (PA, PVA, and ally-PVA matrix) used for the analysis of proteins were run at constant voltage to reach 250 V/h total. The Gels were either stained with colloidal Coomassie or transferred to nitrocellulose membranes for immunoblot analysis with specific antibodies raised against β-spectrin (Acris, Herford, Germany) or band-3 (clone IVF12, DSHB, IA, USA), as previously described [15].

2.7 Image analyses and protein identification

1DE gels underwent image analysis using Quantity One (Bio-Rad). Images were acquired using the ImageQuant LAS 4000 mini Instrument (GE Healthcare, Little Chalfont, UK) and analyzed with the ImageQuant TL software (GE Healthcare). The following equation was used to analyze the resolution between contiguous peaks:

$$R = 2 \times Dx/(W1 + W2), \quad (1)$$

where $W1$ and $W2$ are bandwidths and Dx is the distance between bands, as previously described [3].

Based on the image analyses, the selected bands (see numbered bands in Fig. 3) were excised from the colloidal Coomassie stained gels for MS and were destained in destaining solution (DS; 50% ACN, 5 mM NH_4HCO_3), dehydrated in 100% ACN, and digested overnight at 37°C with 20 μL of trypsin solution (0.01 mg/mL trypsin, 5 mM NH_4HCO_3) [12, 15]. MS was performed using a Tofspec SE (Micromass, Manchester, UK) equipped with a delayed extraction unit. Peptide desorption was achieved using a laser wavelength of 337 nm, and mass spectra were obtained in the reflectron mode in the mass range 800–4000 Da.

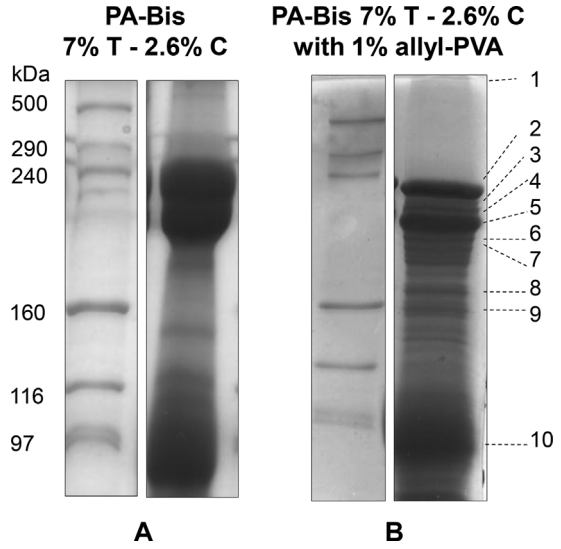

Figure 3. Separation of red cell membrane proteins, solubilized in standard sample buffer (see Section 2.6 for buffer composition), by 1DE in PA gel (A) and allyl-PVA gel (B). Red cell membrane proteins were solubilized with Triton X-100 and Cymal-6 in addition to standard sample buffer. The gels were stained with colloidal Coomassie to evaluate protein separation and analyzed by image analysis software (Section 2.7). The selected bands for spectrometric analysis are indicated by numbers from 1 to 10 (see Table 1). One representative gel of other six with similar results.

Peptide solutions were mixed 1:1 v/v with a saturated CHCA solution containing 40% ACN and 0.1% TFA v/v. External calibration was performed using fragment ions from standard peptides: adrenocorticotropic hormone 18–39 and angiotensin I. Each mass spectrum was generated by accumulating data from 100 to 120 laser pulses. Database searches of peptide masses were performed using the search program "Mascot, Peptide Mass Fingerprint" (available at http://www.matrixscience.com) using the following search criteria: taxa *Homo sapiens* protein molecular mass range from 10 to 300 kDa, trypsin digest, monoisotopic peptide masses, one missed cleavage by trypsin, and a mass deviation of 100 ppm allowed in the NCBI database searches [12,13,15].

3 Results and discussion

3.1 PVA PA gel

The pore size of a PA gel is determined by the total amount of acrylamide and cross-linker it contains: the lower the total monomer and cross-linker concentration, the higher the pore size. However, gels with low cross-linking density are soft and prone to change their swelling degree. By inducing gelation of PA in the presence of variable amounts of a preformed polymer, it is possible to control the average gel porosity and to vary pore size in a wide range. Righetti et al. have exploited this concept to produce "macroporous" gels back in 1995 [16]. According to their view, if constraints to chain motion are imposed during gel polymerization, large pores are formed. It was suggested that the presence of preformed polymers in the gelling solution forces the growing chains to "laterally aggregate" via interchain hydrogen bond formation. Upon consumption of pendant double bonds, such bundles chains are frozen in the 3D space by permanent cross-links.

In a previous work, we suggested that polymerization of acrylamide in the presence of allyl-modified agarose induces formation of a macroporous gel due to combination of hydrogen bonds between agarose chains and cross-linking of acrylamide through a macromolecular cross-linker. The gel obtained was successfully used in 2D electrophoresis to separate large proteins. However, several drawbacks have hampered its widespread use: first of all, the agarose/PA mixed bed must be cast above the agarose gelling temperature to prevent gelation. In addition, the absence of bisacrylamide leads to significant changes in gel swelling during staining and post processing. In an effort to overcome such drawbacks, a new system is proposed here, based on the copolymerization of acrylamide and bisacrylamide with a derivative of PVA which can be considered as a macromonomer with allyl groups pending from its backbone. This new gel has void regions separated by highly cross-linked chains, it is easy to produce and handle and offers the advantage of being highly mechanically resistant and macroporous. Its unique structure results from the physical entanglement of two polymer networks stabilized by their covalent cross-linking. In fact, the insertion of allyl moieties pending from PVA to the growing chains of PA together with interchain hydrogen bonds established immediately before and during acrylamide gelation is responsible for the formation of thicker gel fibers. This mechanism of chain aggregation makes the gel pore size insensitive to temperature and urea, allowing the use of the system in a broad range of conditions.

3.2 Mono-dimensional electrophoresis with PVA-acrylamide mixed-bed matrix and HMW protein resolution

In preliminary experiments, we evaluated the resolution of HMW proteins using commercial HMW standard (see Section 2.1) in gels with different concentrations of acrylamide (7 or 10% T, 0.5% C) and variable amounts of allyl-PVA ranging from 0.5 to 1% w/v. The optimal concentration of allyl-PVA for the best separation of HMW standards was found to be 1% w/v (data not shown) therefore, all subsequent experiments were carried out at this PVA concentration. In Fig. 2A (lane 1), a typical separation of HMW protein markers in 7% w/v PA cross-linked with bisacrylamide (0.5% w/v) is shown. The separation profile was compared with that of mixed-bed gels containing either PVA or allyl-modified PVA both at 1% concentration in addition to acrylamide and BIS. Gels, run side by side in the same conditions (same buffer, running time, and applied voltage), were stained with colloidal Coomassie and the images processed as reported in Section 2.7. The conventional bisacrylamide gels were extremely soft and difficult to handle due to the low amount of

cross-linker used to increase the matrix pore size. The resolution was higher in allyl-PVA gels than in either un-derivatized PVA or PA gels. The higher resolution in allyl-PVA gels results from the increased efficiency observed in the separation of a broad range of molecular weight proteins in this matrix (Fig. 2B). It is interesting to note that only allyl-PVA allows to separate an additional band around 171 kDa, which is only visible in this gel (Fig. 2A, asterisk). The absence of allyl groups on PVA markedly reduced the separation efficiency in all the molecular weight ranges. However, the resolution in the underivatized PVA mixed-bed matrix was still higher than in PA (Fig. 2B). This is most likely related to the higher average pore size of PVA mixed-bed gels that allows the HMW proteins to penetrate in the gel matrix independently from the presence of allyl moieties on PVA. These results suggest that the derivatization of PVA with an allyl moiety plays an important role in the stabilization of the 3D gel structure, leading to reduced band broadening. The resolution of proteins ranging in size from 71 to 36 kDa is preserved in allyl-PVA mixed-bed matrix (Supporting Information Fig. 1), indicating that this gel extends the range of protein sizes that can be resolved at constant monomer concentration leading to results similar to those typically obtained in concentration gradient gels that are far more difficult to produce.

Then, we evaluated the performance of allyl-PVA mixed-bed gels in the separation of red cells membrane proteins. These proteins are well characterized [17–20] and have been previously used as reference samples in the development of new protein separation strategies [21,22]. As shown in Fig. 2A (lane 2), red cell membrane proteins were solubilized in the sample buffer (SB) and separated by gel electrophoresis in either PA, PVA, or allyl-PVA matrices. The resolution of spectrins (black square brackets) well-known proteins belonging to the cytoskeleton network of red cells [21–23] was found to be higher in the allyl-PVA gel compared to either PVA or PA gels (Fig. 2C). All together, these results suggest that allyl-PVA can be useful in the analysis of complex mixtures of proteins containing HMW proteins.

3.3 Allyl-PVA matrix and identification of proteins from red cell membranes

In order to demonstrate the applicability of this new matrix to the electrophoretic separation of challenging mixtures of proteins, we analyzed HMW proteins from red cell membrane ghosts. In particular, the influence of sieving matrix and solubilization strategy was investigated. In Fig. 3, the separation of red cell membranes obtained in a conventional PA gel (7% T, 2.6% C) is compared to that in allyl-PVA copolymer gel (1% PVA, 7% T, 0.5% C). The gels were run side by side and analyzed to assess the resolution of bands covering a wide interval of molecular weights. The SB contained Triton X-100 (1% v/v) and Cymal-6 (1% v/v). Among several detergents tested, this combination provided the best resolution possibly, due to optimal denaturation of HMW proteins. Since sticking might occur, when partially unfolded proteins are in contact with hydrophobic surfaces, we have also evaluated the possible synergic effect of detergents and chaotropes to facilitate HMW protein unfolding. However, addition of 2 or 8 M urea to the Triton X-100 (1%) and Cymal-6 (1%) solubilization buffers did not significantly modify mobility and resolution (Supporting Information Fig. 2), indicating that a complete denaturation was already obtained in the presence of the detergents alone. These results are in agreement with previous studies showing how the use of nonionic detergents such as Triton X-100 [24], NP40 as well as other glycol-based detergents (Brij-56 and Brij-96 detergents) [22, 25–29] favors the separation between membrane proteins and cytoskeleton network (formed by α-spectrin, β-spectrin and actins).

Figure 3 shows that the resolution of proteins in the 97–240 kDa range is higher in allyl-PVA and well-defined bands of proteins, not visible in PA, ranging from 160 to 240 kDa, are clearly detected. The increased pore size of allyl-PVA gels allowed to improve the separation of the HMW proteins while maintaining acceptable gel handling properties.

In order to demonstrate that the new matrix is compatible with the most commonly used identification strategies, proteins separated as in Fig. 3 were identified, both by MS and immunoblot analysis after electrophoresis. Ten bands, excised from allyl-PVA gel (Fig. 3B), were destained (in DS of 50% v/v ACN, 5 mM NH_4HCO_3), dehydrated in 100% ACN, digested overnight at 37°C with 20 μL of trypsin solution (0.01 mg/mL trypsin, 5 mM NH_4HCO_3), and analyzed by MS. As shown in Table 1, various HMW proteins were identified, such as the inositol triphosphate receptor type 3 (Mw 304 kDa) or the α-spectrin chain (SPTA1, Mw 280 kDa). The cystic fibrosis transmembrane conductors regulator (CFTR), previously detected in red cells by a functional test (immunoblot analysis with a specific antibody and atomic force microscopy with quantum-dot-labeled CFTR antibodies), was also detected here [30–32]. Palladin, an interesting and still partially unknown protein in red cells, involved in the organization of actin-based cytoskeleton structure [33–35], as well as ARMC4, a protein containing the armadillo repeat domain, were identified thanks to this new matrix. Assessing the presence of these proteins in red cell membrane ghosts might lead to new interesting discoveries. For instance, it is known that the interaction between ARMC4 and proteins with a proline-rich domain promotes proteasomal-dependent degradation of cytoskeletal components in certain cell types [36, 37]. α-Spectrin, a component of cytoskeleton contained in red cells, which has a proline-rich domain, could interact with ARCM4 and participate in ubiquitination processes. Since red cells survive long time in circulation without the possibility of new protein synthesis in response to different stresses (such as oxidative or shear stress in microcirculation), the "ubiquitin/proteasome-dependent" or the "ubiquitin/proteasome-independent" proteolysis might represent a very interesting and still unknown element in red cell homeostasis.

β-spectrin that together with α-spectrin belongs to the red cell cytoskeleton network has been identified at lower molecular weight than the theoretical one. The bands 5 and 8

Table 1. Identified proteins from red cell membrane separated by 1DE on allyl-PVA gel

Band no.	AC	Protein name	Theoretical Mw (kDa)	Coverage (%)	Matching peptide
1	Q14573	Inositol 1,4,5-trisphosphate receptor type 3_ITPR3	304 106	5	10
2	P02549	Spectrin alpha chain, erythrocyte_SPTA1	280 014	14	26
3	P13533	Myosin-6_MYH6	223 735	5	6
4	Q5R372	Rab GTPase-activating protein 1-like_RBG1L	92 513	9	8
5	P11277	Spectrin beta chain, erythrocyte_SPTB1	246 468	11	19
6	P13569	Cystic fibrosis transmembrane conductors regulator_CFTR	168 142	6	6
7	Q8WX93	Palladin_PALLD	150 564	6	7
8	P11277	Spectrin Beta chain SPTB1	246 468	9	19
9	Q5T2S8	Armadillo ARMC4	115 679	4	8
10	P02730	Band-3 anion transport protein_B3AT	101 792	14	10

Identifications referred to bands excised from gel in Fig. 1A.
Mw, molecular weight; AC, accession number.

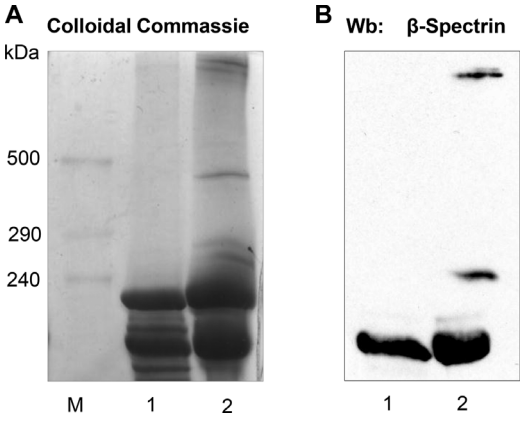

Figure 4. Immunoblot analysis with specific anti-β-spectrin antibody of red cell membrane proteins separated in ally-PVA gel. Twin allyl-PVA gels (1% PVA, 7% T, 0.5% C) were run 5 h at 20 mA: one was stained with colloidal Coomassie (A) and the other transferred to an NC membrane for immunoblot analysis (B). The red cell membrane ghosts (75 μg) were solubilized with 1% Triton X-100 and 1% Cymal-6 added to the standard sample buffer (see also Section 2.6) in the presence (lane 1) or absence of β-mercaptoethanol (lane 2), M: high molecular weight commercial marker (HiMark™ Life Technologies; Carlsbad, CA, USA). One representative gel of four with similar results.

in Fig. 5 could be fragments of β-spectrin [23], which are not well resolved in conventional PA gels.

Finally, we identified membrane-associated protein, such as Rab GTPase, which has been recently described to participate in red cell membrane–cytoskeleton organization [38]. The discrepancy between the theoretical and experimental molecular weight of Rab GTPase protein might be related to its possible functional association with still unidentified membrane protein.

The transfer of proteins, separated in allyl-PVA gels to an NC membrane for immunoblot analysis, was also investigated. We first verified the efficiency of protein transfer by staining with Ponceau the blotted membrane (see Supporting Information Fig. 3). Then, the immunoblot analysis of β-spectrin was performed. Spectrins were chosen as a model in this study, since they represent 25–30% of the total red cell membrane proteins and are organized in tetramers and high-order oligomers. Samples of red cell membrane proteins were run in the two gels using both reducing (Fig. 4, lane 1) and non reducing (Fig. 4, lane 2) conditions to detect β-spectrin monomers and high-order oligomers. One gel was stained with colloidal Coomassie (Fig. 4A) and a second gel was used to transfer the proteins to an NC membrane with a buffer containing 5% w/v of methanol (Fig. 4B). As shown in Fig. 4B, the spectrins were efficiently transferred to NC membrane and detected by a specific anti-β-spectrin antibody.

The results of Figs. 2–4 indicate that the allyl-PVA gel allows an efficient electrophoretic separation of HMW proteins, compatible with MS and immunoblot analysis.

3.4 Separation of membrane proteins from red cells exposed to oxidative stress

The new matrix proved to be extremely useful to study red cells oxidative damage, a phenomenon that plays an important role in shortening red cell survival in the peripheral circulation. Normal red cells were exposed to 20 mM diamide, a cysteine-specific thiol oxidizer, in a buffer containing: 10 mM NaCl, 140 mM KCl, 1 mM MgCl2, 10 mM glucose, 2.5 mM K-phosphate) [13, 39, 40]. As shown in Fig. 5, the resolution of diamide-oxidized red cell membranes was limited in standard PA gels, while allyl-PVA gels allowed a better separation of proteins located between 121 and 279 kDa. When the diamide-oxidized red cell ghosts were solubilized in presence of the thiol-direct reagent NEM, an alkylating reagent that blocks cysteine residues in their reduced state, the separation quality in allyl-PVA gels was further improved.

Finally, the allyl-PVA matrix was applied to the separation of HMW clusters of band-3 generated by diamide treatment [13, 39, 40] (Fig. 6A) and detected by immunoblot analysis (Fig. 6B). In red cells, band-3 is the most abundant integral membrane protein and is affected in different hereditary red cell disorders [41]. Band-3 is a hydrophobic

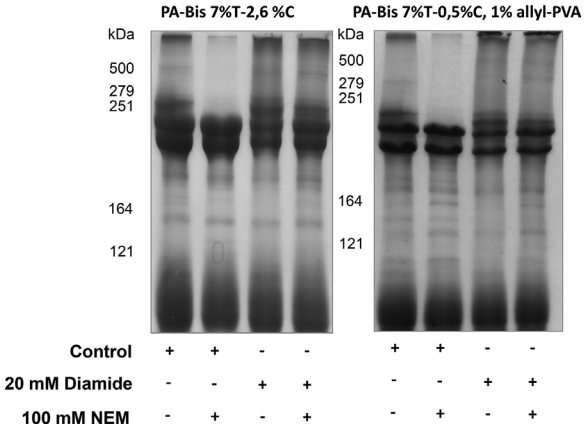

Figure 5. (A) Analysis of diamide-oxidized red cell membrane proteins by 1DE. Red cells were incubated with and without diamide (20 mM) and the red cell membrane ghost were prepared as detailed in Section 2.6 with and without NEM (100 mM). The resolution of diamide-oxidized red cell membranes was limited in standard PA gels, while allyl-PVA gels allowed a better separation of proteins located between 121 and 279 kDa; in the presence of the thiol-direct reagent NEM (100 mM), the separation quality in allyl-PVA gels was further improved. One representative gel of five with similar results.

membrane protein and is generally organized in dimers; when red cells are oxidized by diamide, band-3 forms clusters, generating large protein complexes [39, 42]. As shown in Fig. 6, allyl-PVA allowed a good separation of HMW band-3 clusters from diamide-treated red cells. This proves that allyl-PVA is extremely useful in the analysis of complex mixture of proteins even in difficult experimental conditions such as those required for separation of large aggregates of hydrophobic-oxidized proteins.

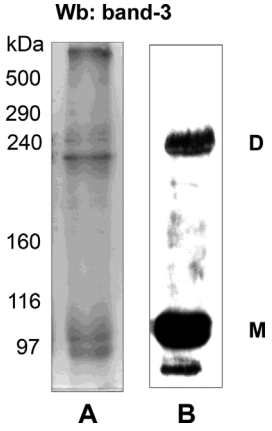

Figure 6. Immunoblot analysis with specific antiband 3 antibody of membrane proteins from diamide-treated red cells separated in ally-PVA gel. Red cells were incubated with and without diamide (20 mM) and the red cell membrane ghost were prepared as detailed in Section 2.6. Twin allyl-PVA gels (1% PVA, 7% T, 0.5% C) were run at constant voltage to reach 250 Vh total: one was stained with colloidal Coomassie (A) and the other transferred to an NC membrane for immunoblot analysis; M: band 3 monomers; D: band 3 dimers (B). One representative gel of five with similar results.

4 Concluding remarks

A new mixed-bed gel matrix obtained by copolymerizing acrylamide and bisacrylamide with PVA chemically modified with allyl glycidyl ether was used as sieving matrix in SDS gel electrophoresis. It has been demonstrated that the allyl-PVA-containing gel is easy to handle in gel electrophoresis and fully compatible with protein identification by MS and immunoblot analysis with specific antibodies. It is also more efficient in HMW protein separation of red cells exposed to oxidative stress than standard PA matrix, suggesting its possible use in studying pathological red cells or other cell types characterized by oxidative damage. In conclusion, allyl-PVA gels represent an excellent tool for the characterization and identification of HMW proteins from complex mixture of proteins such as those extracted from cell system(s).

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The authors have declared no conflict of interest.

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