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Preparation and stability tests for polyacrylamide-coated capillaries for capillary electrophoresis

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Abstract

The stability of polyacrylamide coatings at high pH values has been tested. It could be shown that overall stability is not limited by the coating of the fused-silica surface with organo silanes or Grignard reagents. The instability of polyacrylamide capillaries could be traced back to the hydrolysis of the amide bond at pH 10. The Si–O–Si surface bonds are not affected at this pH by hydrolysis.

The application of the sol–gel process to the preparation of stable homogeneous sublayers and a high density of olefinic groups within the capillary is described. The advantage of this method is the simple coating reaction and its independence of fused-silica surface properties.

1. Introduction

In the separation of biopolymers many efforts have been made to diminish the solute–capillary wall interactions. One approach was to use capillaries with a chemically modified surface. Usually two steps are applied to achieve the bonding [1–6]. In the first step olefinic groups are introduced on the surface of the fused-silica capillary and in the second step the final coating, in most cases acrylamide, is copolymerized with the olefinic sublayer. In capillary electrophoresis extreme pH values are desired, especially in the separation of proteins by capillary zone electrophoresis or isoelectric focussing. The stability of the surface coatings at high pH values is limited. This has been attributed to the instability of the bonds between the sublayer and the silica surface [2].

The methods described in the literature for the preparation of surface coatings differ mainly in the way the olefinic sublayer is bonded to the silica surface. Hjertén [1] condensed γ-methacryloxypropyltrimethoxysilane (MEMO) to the surface in an aqueous solution (pH 3.5 with acetic acid) at room temperature. This method (MEMO technique) has been widely applied for the preparation of olefinic sublayers. Because of the quoted instability, other approaches already known from the preparation of reversed-phase stationary phases for HPLC have been transferred to capillary surface modification [2–5]. Cobb et al. [2] treated the silica surface with thionylchloride and the silicic acid chloride prepared this way reacted further with vinyl magnesium bromide. Others used a long-chain alkoxysilane with a terminal olefinic group [3] or active silane transfer agents like acetylacetone vinyldimethylsilylenolate [4] for the preparation of the olefinic sublayer prior to acrylamide polymerization. Olefinic polysiloxanes have also

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been reacted with the capillary surface [6] to minimize the protein–capillary wall interactions.

For the second step – the polymerization of acrylamide – similar procedures have been used in all cases. Hjertén [1] applied ammonium persulfate as starter and TEMED (N,N,N′,N′-tetramethylethylenediamine) as catalyst in aqueous solutions for the polymerization. Then the formed gel was flushed out of the capillary, with the surface coating remaining. In organic solvents AIBN (azodiisobutyronitrile) has been used as starter [3].

Characterization of the coatings is extremely difficult due to the very small surface area [7]. The most convenient way is to measure the dependence of the electroosmotic flow (EOF) on the pH of the buffer. The EOF is correlated with the silanol concentration on the surface. For uncoated surfaces the silanol concentration has been calculated from the EOF, and a good correlation with surface silanol concentrations discussed in literature has been found [7]. The reduction of the EOF achieved by the coating procedure, and its constancy during capillary use are the only means to describe the efficacy and stability of the bonding procedure. In this paper the stability of different coatings at high pH has been tested. Special attention has been paid to the stability of the different coating steps, because some indications have been found in literature that coating instability may also be traced back to acrylamide hydrolysis [8]. A new coating procedure using the sol–gel process for the preparation of stable olefinic sublayers will also be described.

2. Experimental

2.1. Reagent and materials

Fused-silica capillaries were purchased from Polymicro Technologies (German distributor: Laser 2000, Munich). Reagents for surface modification were purchased from different suppliers: γ-methacryloxypropyltrimethoxysilane (ABCR, Karlsruhe, Germany), acrylamide (BioRad, Munich, Germany), N,N-dimethylacrylamide, AIBN and acrylic acid (Fluka, Neu-Ulm, Germany), ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine (Electran, UK) and vinyl magnesium bromide (Aldrich, Steinheim, Germany). The proteins were obtained from different suppliers such as cytochrome c (Sigma, Deisenhofen, Germany), chymotrypsinogen (Serva, Heidelberg, Germany) and lysozyme and ribonuclease A (Fluka, Neu-Ulm, Germany). Vinyltrichlorosilane, thionylchloride as well as all buffer substances were obtained from Fluka.

2.2. Coating procedures

Capillary pretreatment and modification with trichlorovinylsilane were performed according to a procedure described previously [5]. For MEMO modifications the capillary was filled with a solution of silane–methanol (50:50, v/v) and left for reaction over night at room temperature. Vinyl magnesium bromide was bonded as described in Ref. [2]. The procedure of Hjertén was used for linear polyacrylamide as well as for poly-N,N-dimethylacrylamide coatings [1]. In the same way capillaries were modified with polyacrylic acid.

2.3. Sol–gel procedure

The starting sol was obtained by adding 0.1 *M* hydrochloric acid in molar ratio to MEMO (MEMO–H_2O, 1:1.5 or 1:3) and stirring at room temperature for 2 h. Then, the capillary was dynamically coated with this solution and heated to 130°C for 1 h in a GC oven under a slight nitrogen stream. After washing with dichloromethane, methanol and water the capillary was ready for use.

2.4. Apparatus

For all measurements a Beckman P/ACE System 2050 was used. Data acquisition was accomplished with Beckman Gold Software (V 7.12) and an IBM PS/2 personal computer. For coating procedures at higher temperatures the oven of a Carlo Erba GC 6000 Vega Series was used.

EOF/pH measurements always started at pH 3 (hysteresis). Between the individual runs the capillary was rinsed for several minutes with methanol, water and the corresponding running buffer. For stability measurements an aqueous solution of benzyl alcohol was injected and an electrical field was applied for 2 h at pH 10. Between the different runs the capillary was rinsed with fresh buffer. When after 2 h no peak appeared, an EOF of $6 \cdot 10^{-6}$ cm^2/Vs was assumed which corresponds to that migration time.

3. Results and discussion

3.1. Characterization and stability of the olefinic sublayer

The EOF is the most important parameter in CZE separations. Its stability is important for the reproducibility of migration times. Absence of EOF is required for isoelectric focussing and capillary gel electrophoresis. The dependence of the EOF on pH shows a typical sigmoidal curve, caused by the dissociation of the surface silanol groups. In Fig. 1 the EOF measured for different preparation techniques of the olefinic sublayer is compared to the EOF obtained with the uncoated capillary of the same batch and identical surface pretreatment. It is important that the capillaries are taken from the same batch,

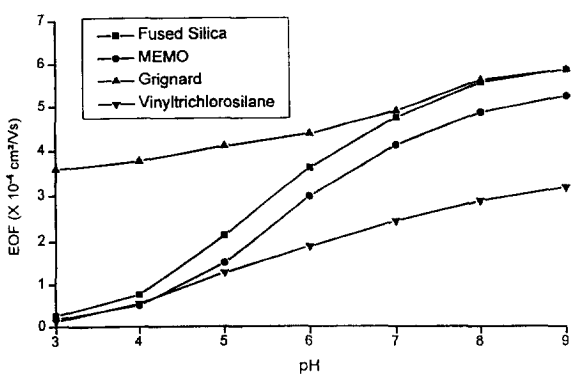

Fig. 1. EOF vs. pH curves for different vinyl-modified capillaries. Conditions: $l = 30/37$ cm; buffer, 10 mM phosphate; neutral marker, benzyl alcohol; field, 432 V/cm.

because the EOF characteristics are also a function of the storage age of the capillary. A capillary stored for one year in the laboratory showed only half of the initial EOF measured immediately after delivery and opening.

As can be seen in Fig. 1, the capillary coated by the MEMO technique showed only a slight decrease of the EOF at high pH (approximately 10% reduction compared to that of the untreated one). When the surface concentration of dissociated silanols is the only cause for the EOF, one can assume that in this case only a small amount of these groups have been reacted by this treatment. The adsorption of the reagent onto the surface–a prerequisite for the condensation reaction [9]–is low in the polar reaction medium. Alkoxysilanes are also relatively stable at pH values between 3 and 5 in aqueous solution and the polycondensation reaction is slow [10]. On the other hand, chlorosilanes are very reactive with silanol groups. For capillaries treated with pure vinyltrichlorosilane (VTCS) and without catalyst the reduction of the EOF is about 50% of its initial value. Of course, in this case only part of the silanol groups are reacted and new ones are introduced by the trifunctional silanization reagent. When treating such a capillary with an active silanization reagent like bis-trimethylsilylacetamide an additional EOF reduction of 20% has been observed. However, further studies showed that this procedure is not important for the preparation of good acrylamide-coated capillaries, and therefore it has been omitted.

The situation is different with regard to the capillaries prepared by the Grignard reaction. The treatment of fused-silica capillaries with thionyl chloride resulted in an increase of the EOF (after flushing the capillary with water). This can be attributed to an increase of the surface silanol concentration due to acid treatment by the formed hydrochloric acid [11]. After reaction with vinyl magnesium bromide the EOF is also at low pH values significantly higher than in the untreated capillary. At high pH values the EOF of the Grignard capillary is similar to that of the initial fused-silica capillary. The decrease of the EOF from the thionylchloride-treated

capillary to that reacted with vinyl magnesium bromide was around 10%.

Because of the small differences in EOF between the untreated capillary and those coated by the MEMO and Grignard technique, long-term stability measurements were not very reliable. However, it can be assumed that their behaviour is similar to that of the VTCS treated capillary, which is shown in Fig. 2. The capillary was continuously swept with the pH 10 buffer by the EOF. After every 2 h the buffer was changed and the EOF remeasured. Over a 80-h period of continuous treatment with buffer of pH 10 no increase in EOF could be noticed. This indicates that the sublayer is stable. One reason for this stability might be that the hydrophobic surface layer prevents the hydroxyl ions attacking the Si–O–Si bonds of the vinyl groups to the surface. The achievable surface coverage with VTCS is very high. With porous silica and identical reaction conditions surface concentrations around 5 μmol/m^2 have been achieved [12].

3.2. Characterization of the polyacrylamide layer

The same test procedure was applied to the different capillaries after the polymerization of acrylamide onto the surface. The results are summarized in Fig. 3. The importance of the

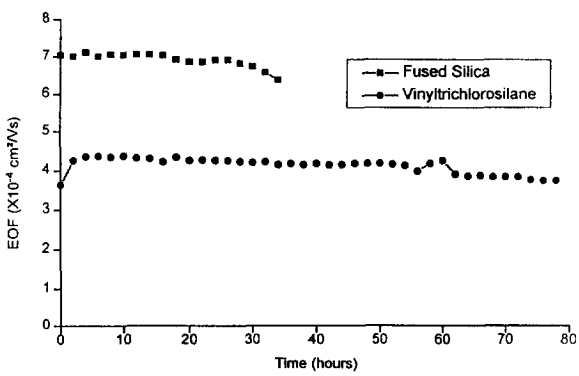

Fig. 2. Stability at pH 10 of a capillary modified with vinyltrichlorosilane. Conditions: l = 20/27 cm; buffer, 10 mM phosphate pH 10; neutral marker, benzyl alcohol; field, 444 V/cm; measurement by procedure described in the text.

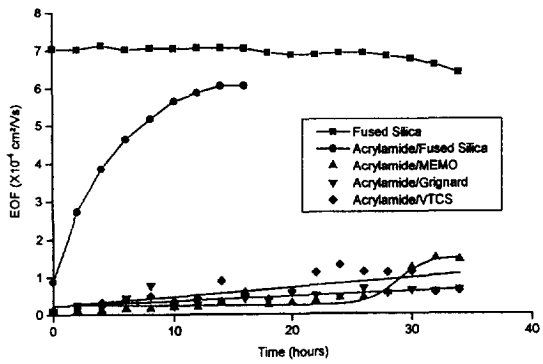

Fig. 3. Influence of the olefinic sublayer on the stability of polyacrylamide capillaries at pH 10. Conditions: see Fig. 2.

sublayer in the copolymerization of acrylamide with the olefinic groups bonded to the surface can be deduced from the behaviour of the capillary on which only acrylamide has been polymerized. In this capillary, where no covalent attachment of the polymer to the surface is possible, no constant EOF could be observed and after 10–15 h the EOF of the uncoated capillary was approached. Thus modification of a capillary surface only with physically adsorbed polyacrylamide does not give a stable coating.

The behaviour of the three other capillaries, where the subcoating was achieved by different methods, is very similar. The measurements at pH 10 exhibit a much lower EOF ($<10^{-5}$ cm^2/Vs) than achieved with capillaries coated solely with the olefinic sublayer. The reason for this is the high viscosity of the polymeric acrylamide layer in the region of the electric double layer [1]. The identical behaviour of the three different capillaries supports the statement given above that the three different subcoatings are of identical stability, despite the different degrees of shielding of the surface silanols. An additional reason for this stability may be the extremely low diffusion coefficients of catalytic hydroxyl ions through the thick polymeric layer.

The increase of the EOF can only be attributed to the slow decomposition of the acrylamide groups. The instability of polyacrylamide is a well known fact from classical flat-bed gel electrophoresis [8,14]. The formed carboxylic groups are fully dissociated at this pH and contribute to

the EOF. Our results are partly in contradiction with those of others [13], where a different behaviour of the Grignard and MEMO sublayer was found. The experimental set-up differs, however, because only pH 8 has been used (here pH 10). At this pH hydrolysis of the amide group is less important. Moreover, the capillaries were stored in buffer, whereas in our more stringent experiments the buffer was continuously swept through the capillary by the EOF, and the electrical field was applied continuously. It has also been demonstrated with another coating than acrylamide that the MEMO sublayer is stable at pH 12 [15].

To prove the hypothesis that the obvious instability of polyacrylamide capillaries stems from the hydrolysis of the amide bond, acrylic acid itself has been polymerized either as pure acrylic acid or in a 1:1 mixture with acrylamide. As can be seen in Fig. 4, the EOF is lower than in the untreated capillary and remains constant over the whole test period. Surprisingly, the EOF of the polymeric layer obtained by co-polymerization of acrylic acid and acrylamide also remains constant. One possible explanation for this stability might be the hindered attack of hydroxyl ions to the negatively charged polymeric layer. In the same figure the stability test of the acrylamide capillary from the previous figure is included for comparison. The behaviour of a capillary coated with N,N-dimethylacrylamide (DMA) is also shown. It has

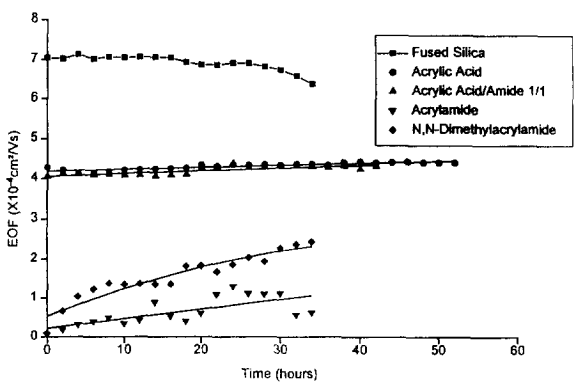

Fig. 4. Influence of the polymerized monomer on the stability at pH 10. Olefinic sublayer: vinyltrichlorosilane. Conditions: see Fig. 2.

been described [8] that disubstituted amides are substantially more resistant to alkaline hydrolysis than the unsubstituted species. Surprisingly, we found that the stability of the DMA-coated capillary seems to be lower than that of the plain acrylamide coating. One reason may be the higher hydrophobicity of the monomer which can result in a lower degree of polymerization (thin coating) due to a diminished incorporation of the monomer into the polymeric layer [8]. Polymerization at elevated temperatures should diminish these problems [8]. However, such an experiment has not been included in our studies.

To sum up, the instability of polyacrylamide-coated capillaries seems mainly to originate from hydrolysis of the amide group at the applied pH of 10. So far, no difference could be found between the different procedures for the preparation of the olefinic sublayer. The application of hydrolysis-stable monomers, however, is limited by their diminished accessibility to polymerization. Cross-linking with formaldehyde has also been mentioned to improve acrylamide stability [6]. Polymerization of other polar monomers with non-hydrolysable functional groups [14–17] might result in superior stable polar capillaries for the CZE of biopolymers.

Another way to characterize coatings is the analysis of proteins, where high plate numbers should be achieved. Fig. 5 shows the separation of basic standard proteins with a VTCS/polyacrylamide capillary at four different pHs. Even at pH 6 a highly efficient separation is achieved. As can be seen in Table 1, the plate numbers vary between 300 000 and 800 000.

3.3. Sublayers by the sol–gel process

Concerning the previously applied techniques, the efficiency and reproducibility of the sublayer preparation depends on the concentration and reactivity of the surface silanol groups on the fused-silica capillaries. As described and determined by EOF measurements [7], this concentration depends on the supplier and the history of the capillary. It is, on the other hand, desirable to have a constant homogenous and high-density surface concentration of polymerizable

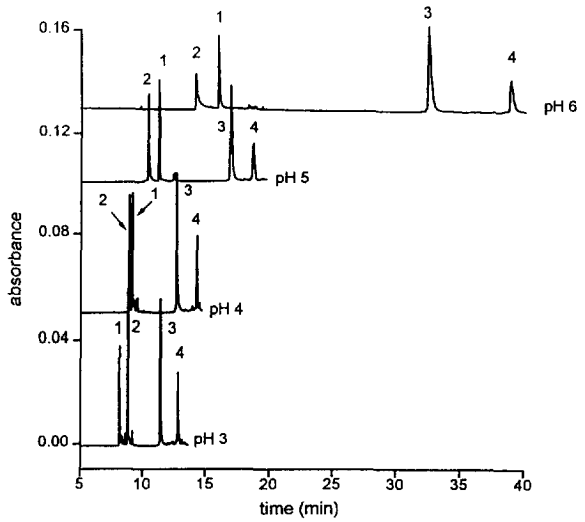

Fig. 5. Influence of pH on the separation of standard basic proteins with a polyacrylamide–vinyltrichlorosilane capillary. Peaks: 1 = cytochrome c, 2 = lysozyme, 3 = ribonuclease A, 4 = chymotrypsinogen. Conditions: l = 30/37 cm; buffer, 50 mM phosphate; field, 270 V/cm; injection, pressure (0.5 p.s.i.), 1 s.

functional groups immobilized on the fused-silica surface. The only way to achieve this is by immobilizing a polysiloxane with easily polymerizable olefinic groups [6].

The sol–gel process [18,19] is a well-known chemical synthesis method for the preparation of glasses and ceramics. Starting from alkoxysilanes, a growth process is initiated leading to oligomeric molecules or colloids, which form a three-dimensional network by condensation reactions. These intermediates can be stabilized in solution (sols), and can be used for coating techniques. In general, the alkoxysilanes are reacted with a stoichiometric amount of water in

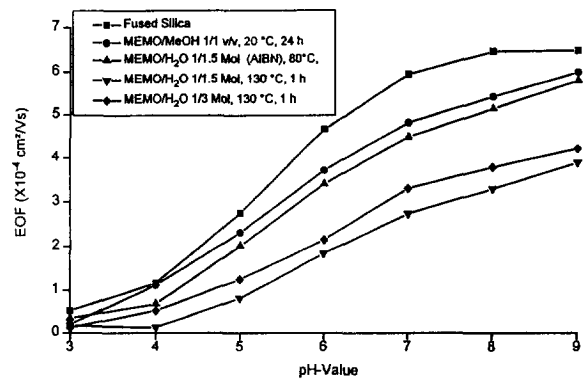

Fig. 6. EOF vs. pH curves for different MEMO modified capillaries using sol–gel process. Conditions: see Fig. 1.

the presence of a catalyst (acid or base). By applying organoalkoxysilanes with reactive functional groups, a mixed organic–inorganic network is generated under very smooth conditions at room temperature. By this process extremely stable layers with interesting mechanical properties can be obtained on different materials ranging from plastics to glass and metals.

Here, the sol–gel process has been used to prepare stable layers of MEMO within the capillary. The influence of the sol preparation and its bonding to the surface is demonstrated via EOF measurements in Fig. 6. For comparison the classical MEMO coating discussed in Fig. 1 has been included. As discussed, in this case the EOF was reduced only by about 10%. For the preparation of the sol, MEMO has been reacted at room temperature for 2 h with molar amounts of 0.1 M hydrochloric acid. Under these conditions mainly linear polycondensation products are formed, which still contain sufficient unreacted alkoxy groups (more than 5%

Table 1
Number of theoretical plates per meter for standard basic proteins at different pH

	Cytochrome c	Lysozyme	Ribonuclease A	Chymotrypsinogen
pH 3	765 000	573 000	565 000	737 000
pH 4	615 000	754 000	427 000	746 000
pH 5	448 000	382 000	232 000	332 000
pH 6	851 000	230 000	506 000	358 000

Conditions: see Fig. 5.

after 14 days) [20]. The sol has then been dynamically coated onto the surface of the capillary and the condensation reaction with the surface has been achieved through heating to 130°C. As can be seen in Fig. 6, the EOF was decreased to about half its initial value. A similar reduction has been observed with the VTCS technique; however, with the sol–gel process the reproducibility was much higher. The ruggedness of the sol preparation and its immobilization has also been demonstrated. The amount of added aqueous acid (1.5 or 3.0 mol) had negligible influence on the EOF behaviour. Thus, a similar coating could be assumed.

In addition to the polycondensation process a cross-linking of the organic olefinic groups can be initiated in the sol–gel process by the addition of a radical starter such as AIBN (azodiisobutyronitrile). This procedure was also performed in a capillary with a precondensed MEMO. In this case the amount of sol immobilized on the surface is relatively small, as can be deduced from the still high EOF. The coating efficiency was similar to that of the initial MEMO procedure, at least a similar EOF/pH dependence has been achieved.

With these efficient coatings stability measurements have also been performed. It could be demonstrated again that the olefinic sublayer is stable over a long period of time with a constant low EOF at pH 10. It is no problem to react these fixed layers with monomers like acrylamide, which showed similar long term stability as the other polyacrylamide coatings. Only with the sol that had been additionally cross-linked in the organic part with AIBN no stable acrylamide layer could be formed due to the reduced concentration of remaining olefinic groups.

With these capillaries highly efficient protein separations have also been achieved. The advantage of the sol–gel process is the fact that, being independent on the initial surface properties of the fused-silica capillary, a homogenous sublayer with high reproducibility and high concentration of olefinic groups can be achieved. Of course, this process is not limited to MEMO. This sol–gel process can be applied to a great variety of organosilica derivatives for the preparation of surface-coated capillaries for CE.

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