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Anion-exchange supermacroporous monolithic matrices with grafted polymer brushes of *N*,*N*-dimethylaminoethyl-methacrylate

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Abstract

Graft polymerization using potassium diperiodatocuprate as initiator was found to be an effective and convenient method for grafting functional polymer of *N*,*N*-dimethylaminoethyl methacrylate (DMAEMA) onto superporous polyacrylamide gels, so-called cryogels (pAAm cryogels). It was possible to achieve grafting degrees up to 110% (w/w). The two-step graft polymerization i.e. first activation of the matrix followed by displacement of initiator solution with the monomer solution, decreased pronouncedly the soluble homopolymer formation. The efficiency of graft polymerization using a two-step technique increased up to 50% (w/w) at a monomer conversion of 10%, compared to 10% graft efficiency with 60–70% monomer conversion for one-step direct graft polymerization. The pAAm cryogels grafted in one-step and two-step procedures, respectively, behaved similarly when binding low-molecular weight ligand but showed very different behavior for sorption of a high-molecular-weight ligand, bovine serum albumin (BSA). The differences in behavior were rationalized assuming different structure of the graft polymer layers and tentacle-type BSA binding to the grafted polymer.

Keywords: Supermacroporous polyacrylamide; *N*,*N*-dimethylaminoethylmethacrylate; Graft polymerization; Protein binding

1. Introduction

Monoliths or continuous bed chromatographic columns represent a new class of chromatographic materials introduced as an alternative to traditional packed bed columns [1–2]. The structure of interconnected pores of the monolith, convective solute flow through the pores and low mass transfer limitations [3–4] make monoliths an attractive stationary phase for chromatography of protein, plasmids and viruses [5–7].

The pore size plays a crucial role in chromatographic performance of monolithic columns. The monoliths with sufficiently large pores have a low flow resistance allowing rapid separation processes. Moreover, large pores allow using monolithic columns for the separation of nano- and macroparticles. However, one of the drawbacks of monolithic materials with large pores is a limited surface area of pore walls and as the result a limited amount of functional groups available on the pore wall surface.

The introduction of functional groups onto the surface of porous chromatographic support via graft polymerization of vinyl monomers originally containing functional groups is a versatile approach for the preparation of materials with controlled incorporation of functional groups [8–11]. Polymer chains grafted onto a substrate are designated as polymer brushes or tentacles. Chromatographic supports with polymer brushes have several advantages. The capacity of the tentacle-type chromatographic supports depends no longer exclusively on the surface area of the pore walls but could be enhanced by increasing the length of the polymer chain grafted. The dynamic binding capacity of tentacle-based chromatographic supports is sufficiently high [12–14]. During the process of protein binding the tentacles behave to some extent like polymers in solution and the binding of the target molecule is

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favored by a number of cooperative effects coming into play [15–16].

The grafted monoliths could be prepared by initiating monomer polymerization from the surface by forming active sites on the backbone of the polymer. During the polymerization reaction, the polymer chains "grow" from the surface of the monolith (*grafting* from Section 2.2). Thus, graft polymerization at the pore surface of capillary monoliths was carried out by UV initiated photografting [17–19]. Both positively and negatively charged polymer brushes were prepared in this way. The length of the grafts and thus the overall extent of grafting were controlled by the reaction time and the monomer concentration [20]. Another approach used for graft polymerization at the pore wall surface of monoliths is using cerium(IV) as a catalyst for grafting on the substrates containing hydroxyl groups. For example, cation-exchanging sulfonic groups were introduced by cerium(IV)-initiated grafting onto poly(glycidyl methacrylate-co-ethylene dimethacrylate) monoliths [21].

Recently, a new type of macroporous, monolithic, polyacrylamide cryogel (pAAm cryogel) has been developed [22]. These cryogels (from the Greek κριοσ (kryos) meaning frost or ice) are produced by radical copolymerization of acrylamide (AAm) and cross-linker *N*,*N*′-methylene-bis-acrylamide (MBAA) in partially frozen media, the dissolved monomers and initiator are concentrated in small fractions of non-frozen fluid. In these non-frozen fractions the polymerization proceeds despite that the whole system looks like a frozen ice block. The ice crystals formed after partial freezing perform as porogen. Each crystal continues to grow until contacting another crystal, thereby forming a network of connecting ice crystals.

The pore size depends on the freezing conditions and concentration of monomers and cross-linker used [22]. The cryogels have large interconnected pores of 5–100 μm size with a mean pore size of 35 μm [23]. The pores are separated by walls of dense polymer gel formed in the non-frozen fluids of concentrated reaction mixture. The gels prepared by this way have a sponge-like morphology. The large pore size in cryogel in combination with highly interconnected pore morphology allows substances of different molecular weights, bovine serum albumin (69 kDa), Blue Dextran (2000 kDa) or even microbial cells (3–10 m) to pass freely through the cryogel matrix without retention [22]. The large pore size in combination with convective flow of aqueous media through the gel matrix have attracted a considerable attention for using cryogels as chromatographic matrices for separation of nano- and macroparticles [24–26]. However, pAAm cryogel monoliths with large pores have low protein binding capacity [27]. We have therefore attempted to implement the controlled grafting of chains of functional polymers onto the gel surface within the large pores of polymer monoliths to produce pAAm cryogel monoliths with a high amount of functional groups, per unit of the monolith volume.

Free-radical graft polymerization of *N*,*N*-dimethylaminoethyl methacrylate (DMAEMA) was initiated on pAAm-cryogel using potassium diperiodatocuprate, $K_5[Cu(HIO_6)_2]$. The sorption of a low molecular weight (Orange G) and a high molecular weight (bovine serum albumin, BSA) substances was studied on polyDMAEMA-grafted monolithic columns.

2. Materials and methods

2.1. Materials

N,*N*-dimethylaminoethyl methacrylate (DMAEMA, 98% purity), acrylamide (AAm, more than 99.9% purity, electrophoresis reagent), *N*,*N*′-methylene-bis-acrylamide (MBAA), *N*,*N*,*N*′,*N*′-tetra-methyl-ethylenediamine (TEMED) and ammonium persulfate (APS) were from Aldrich (Aldrich, Steinheim, FRG). Orange G and bovine serum albumin were from Sigma (St. Louis, USA). The buffer salts were of the best quality available.

2.2. Methods

2.2.1. Cryogenic co-polymerization of AAm with MBAA

The supermacroporous cryogels were prepared in glass tubes (total weight of monomers AAm + MBAA = 6%, AAm/MBAA = 8/1, the amount of TEMED as well as APS = 1.2% of that of the total AAm+MBAA weight). The solution in the tubes was frozen at −12 °C and kept at this temperature for 20 h. After thawing and washing with water (200 ml) the gel matrix was dried at 60 °C and stored in dry state.

2.2.2. Preparation of potassium diperiodatocuprate (Cu(III)) solution

The Cu(III) solution was prepared according to [28] as follows: $CuSO_4 \cdot 5H_2O$ (3.54 g), KIO_4 (6.82 g), $K_2S_2O_8$ (2.20 g) and KOH (9.00 g) were added to 200 ml of deionised water. The mixture was boiled for 40 min. After cooling to room temperature, the mixture was filtered and the filtrate was diluted to 250 ml with deionised water. The final concentration of Cu(III) was 0.0562 M.

2.2.3. Graft polymerization of DMAEMA onto AAm-cryogel monolith

Graft polymerization of DMAEMA onto the AAm-cryogel was carried out by using two approaches. A dried pAAm cryogel (0.15 ± 0.03 g) was directly submerged in the 10 ml of the reaction solution of monomer and initiator (Cu(III) 0.008 M). The reaction mixture was flashed with nitrogen for 10 min before Cu(III) solution was added. Polymerization was carried out for 2 h at 40 °C.

Graft polymerization was carried out also by using two steps technique. Alternatively, a dried pAAm cryogel was placed in a glass tube and saturated with 5 ml of 0.033 M Cu(III) solution in 1 M NaOH. The dry cryogels re-hydrated

within less then a minute after contact with aqueous solution filling up the glass tubes so that the liquid was passing through the interconnected porous system of the monolith. The samples saturated with Cu(III) were incubated at 40 °C for 30 min, that was found optimal for the maximal efficiency of graft polymerization. Then the initiator system was displaced from the cryogel with 8 ml of degassed monomer solution that was passed through the cryogel matrix at a flow rate of 4 ml/min. The flow was stopped with a cork. The graft polymerization proceeded at 40 °C for 1 h.

After completion of the reaction, the cryogels were washed with 30 ml 0.1 M HCl followed by washing with an excess of deionized water. The washings containing homopolymer were collected and any remaining monomer was removed by dialyzing against water for 30 h. The water was changed in the meantime four times. The final homopolymer was then freeze-dried to the constant weight under vacuum.

The grafting degree (G), grafting efficiency (EG) and monomer conversion (C) of the graft polymerization were defined and calculated as follows:

$$G\,(\%) = \left[\frac{(W_1 - W_0)}{W_0}\right] \times 100\%,$$

$$\mathrm{EG}\,(\%) = \frac{W_1 - W_0}{(W_1 - W_0) + W_2} \times 100\%,$$

$$C\,(\%) = \frac{(W_1 - W_0) + W_2}{W_3} \times 100\%,$$

where W_0 and W_1 are the weights (g) of original and grafted samples; W_2 and W_3 are the weights (g) of soluble homopolymer and monomer used, respectively.

2.2.4. Sorption experiments

Adsorption of low molecular anions like Orange G by pAAm cryogels grafted with DMAEMA (polyDMAEMA-grafted pAAm cryogel) was measured as follows. Dry samples of grafted and non-grafted pAAm cryogel were cut into small pieces and incubated with 40 ml of Orange G solution (5 mM, pH 5.0) for 40 h at room temperature and constant shaking. The amount of adsorbed Orange G was quantified spectrophotometrically as decrease in the concentration of Orange G in the solution before and after incubation with the cryogel. The control experiment with non-grafted pAAm cryogel showed no adsorption of the dye.

Chromatography of BSA was monitored using a LKB UVI-cord with a 276 nm filter. A monolith of grafted cryogel was put into a glass column (inner diameter 10 mm, 3 ml volume) equipped with upper and lower adapters. BSA solution (1 mg/ml in running buffer, 20 mM Tris–HCl buffer, pH 7.0) was applied to the column followed by washing with running buffer until the absorbance of the eluate at 276 nm was down to baseline. Elution was performed with 1.5 M NaCl in running buffer. Fractions of 5 ml were collected and optical density at 280 nm was measured. BSA content was calculated using a calibration curve for BSA (0.1–1 mg/ml) established at 280 nm.

3. Results and discussion

A sponge like, macroporous cryogel matrix was produced by radical copolymerization of AAm with MBAA in partially frozen reaction media, followed by defrosting and intensive washing. Cryogels produced in such a way have large continuous interconnected pores filled with water [22]. Dry polymer constitutes only 3–4% of the total weight of completely swollen cryogel. Cryogels produced from polyacrylamide can be dried at 60 °C and re-swollen when submerged into water within less than a minute without deterioration of their supermacroporous structure. Thus, the submerging of the dried cryogel matrix into reaction mixture allows reducing the time of contact of the polymer with reaction mixture used since the reactants will be sucked into the gel upon swelling. The superporous structure of the cryogels promotes grafting by providing an ample surface of the gel for grafting, ensuring good mass transfer inside the gel sample and allows convenient and efficient washing away of both homopolymer and insoluble by-products formed via oxidation of Cu(III) during the polymerization reaction.

The potassium diperiodatocuprate ($K_5[Cu(HIO_6)_2]$) is capable via redox reaction with amide groups of the acrylamide cryogel backbone, to form amidyl radicals on the cryogel surface, thus initiating the polymerization of vinyl monomers [29]. The mechanism of initiating graft polymerization onto pAAm backbone could be presented as follows (Fig. 1).

Potassium diperiodatocuprate-initiated graft polymerization was found to be an efficient and convenient method for grafting of DMAEMA onto superporous polyacrylamide cryogels. It was possible to achieve up to 110% (w/w) DMAEMA grafting on pAAm cryogel. The graft density of polyDMAEMA-pAAm cryogels increased with increasing monomer concentration (Fig. 2).

The direct graft polymerization of DMAEMA onto pAAm cryogel by submerging of dry pAAm cryogel directly in the reaction mixture containing initiator and monomer entailed the formation of large amount of soluble homopolymer (Fig. 3). The amount of soluble homopolymer increased with increasing the monomer concentration. Potassium diperiodatocuprate initiated also the homopolymerization of DMAEMA as there was an intensive homopolymer formation in free solution when the potassium diperiodatocuprate was added to the monomer solution (Fig. 3). Thus, during the graft polymerization by submerging of dry pAAm cryogel in solution of monomer and initiator the generation of radicals proceeded both onto pAAm backbone and in solution. That resulted in an intensive homopolymer formation in free solution during graft polymerization thereby decreasing the efficiency of graft polymerization. The efficiency of graft polymerization with respect to the total polymer forma-

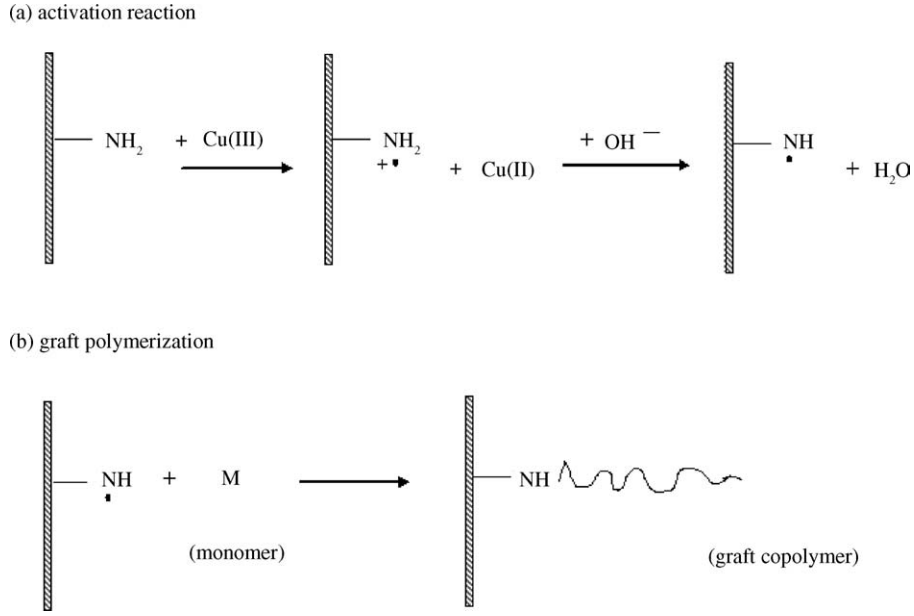

Fig. 1. The mechanism of initiation of graft polymerization onto pAAm cryogel using potassium diperiodatocuprate.

tion was only 10% at 60–70% monomer conversion (Fig. 4). It was mostly the soluble homopolymer which was formed during the direct graft polymerization by submerging of dry cryogel in the monomer containing reaction mixture.

The two-step graft polymerization via activating the polymer matrix first and then via saturation with the monomer solution, allowed to avoid the intensive homopolymer formation in free solution during the graft polymerization (Fig. 3). The radicals are generated only on the pAAm cryogel surface. The polymerization of DMAEMA was initiated from the active center onto gel surface restricting the formation of homopolymer in solution and increasing the efficiency of graft polymerization up to 50% (Fig. 4a). However, the utilization of monomer for polymerization decreased. The monomer conversion was only 10–15% for two-step procedure as compared to 60–70% for the one-step direct graft polymerization (Fig. 4b).

The activation conditions in two-step procedure have been optimized for the maximal efficiency of radical generation. However, even under optimal conditions, the grafting percentage was lower as compared to direct grafting (Fig. 2). The decrease of graft density for two-step graft polymerization is presumably due to the contact of monomer solution with less radical sites on the pAAm backbone as the initiator has been already removed when cryogel came into contact with the monomer solution and the possibility

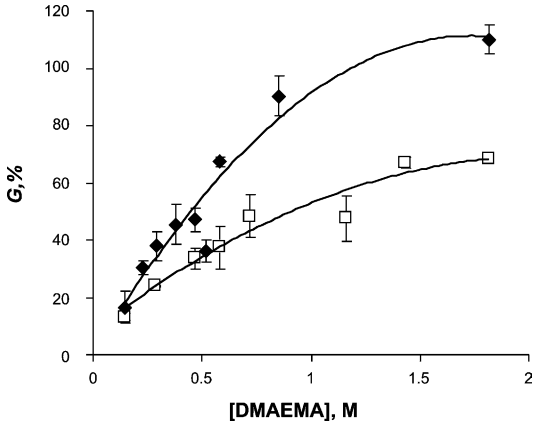

Fig. 2. Effect of monomer concentration on grafting degree of polyDMAEMA-grafted pAAm cryogel. Direct one-step grafting when dry pAAm cryogel was submerged directly in the reaction solution containing the monomer and initiator (closed rhombus). Two-step grafting when the cryogel was activated before the saturation with the monomer solution (open squares). The bars present the standard deviation calculated from four individual experiments.

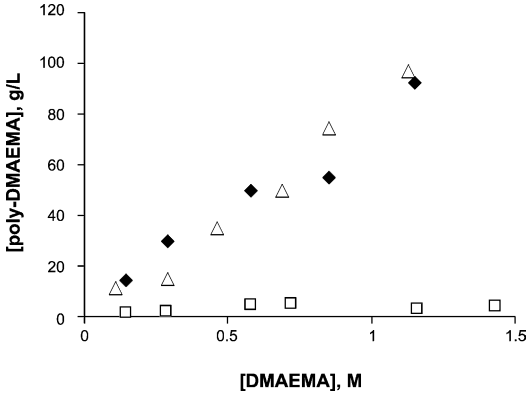

Fig. 3. Homopolymer formation in free solution during polymerization of DMAEMA initiated by potassium diperiodatocuprate. Direct one-step grafting when dry pAAm cryogel was submerged directly in the reaction solution containing the monomer and initiator (closed rhombus). Two-step grafting when the cryogel was activated before the saturation with the monomer solution (open squares). Homopolymerization in the monomer solution (open triangles).

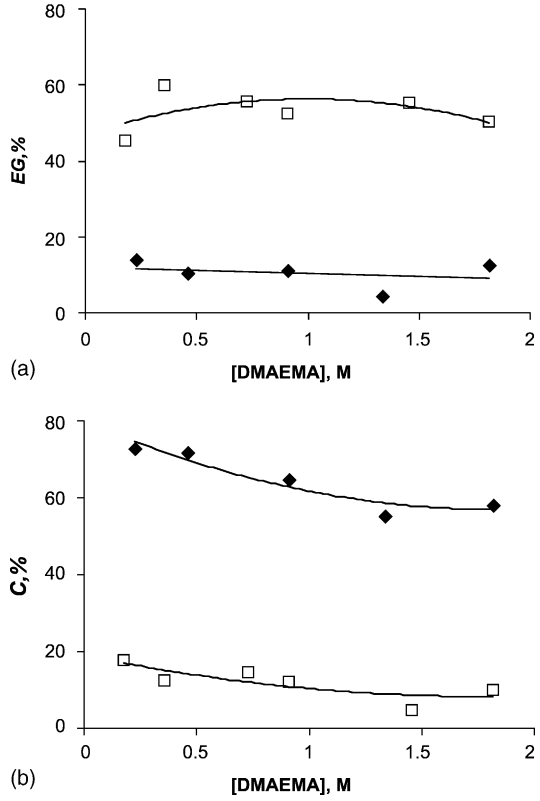

Fig. 4. Efficiency of graft polymerization (a) and conversion of monomer to polymer (b) for graft polymerization of DMAEMA onto pAAm cryogel implemented in different modes. Direct one-step grafting when dry pAAm cryogel was submerged directly in the reaction solution containing the monomer and initiator (closed rhombus). Two-step grafting when the cryogel was activated before the saturation with the monomer solution (open squares).

for free radicals to get quenched by impurities and oxygen entered with monomer solution.

The pAAm cryogels grafted with polyDMAEMA using both grafting procedures behaved similarly with respect to binding low-molecular-weight ligand, dye Orange G which is negatively charged due to two $-SO_3^-$ groups in its structure (Fig. 5). The binding of the dye increased linearly with increasing grafting density independently of the grafting method (Fig. 6). So, the positively charged tertiary amino groups on the polymer chains grafted using different methods are equally accessible for the dye.

On the contrary, the binding of high-molecular-weight ligand, bovine serum albumin depended pronouncedly on the

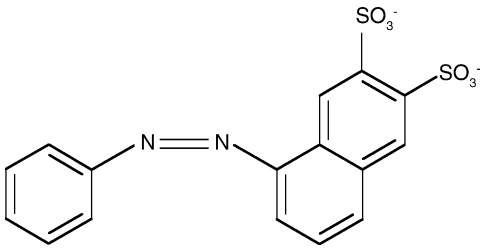

Fig. 5. Structure of Orange G.

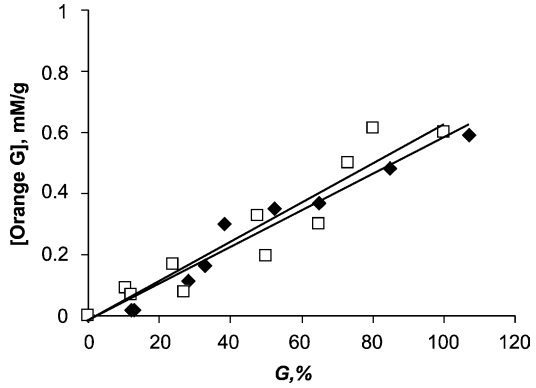

Fig. 6. Orange G binding by polyDMAEMA-grafted pAAm cryogel prepared by one-step procedure (closed rhombus) and two-step grafting (opened square), respectively. The pieces of about 2 mm size of polyDMAEMA-grafted pAAm cryogel were incubated with 5 mM Orange G solution in water, pH 5.0 for 40 h at room temperature and constant shaking. The amount of Orange G adsorbed was estimated spectrophotometrically as decreasing of the concentration of Orange G in the solution before and after incubation with the cryogel.

graft polymerization method used. BSA is, at neutral conditions, a negatively charged protein with molecular weight 69,000 Da that could be bound by positively charged polyDMAEMA grafted chains. One could expect that the capacity for BSA binding would increase monotonously as the number of ion-exchange groups increased with increasing grafting density. However, the BSA binding capacity increased slightly with increasing graft density for grafted cryogels prepared by direct graft polymerization and even at high grafting density (70%), the BSA binding capacity did not exceed 7 mg/g of dry polymer (Fig. 7). In a completely different manner, the BSA binding capacity of polyDMAEMA-grafted pAAm cryogel prepared using two-step procedure, increased significantly with increasing graft density up to 27 mg/g at grafting density of 70%.

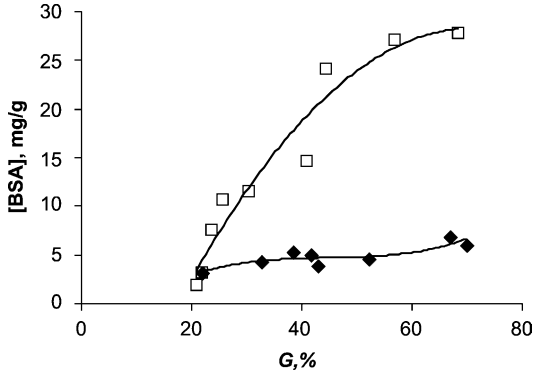

Fig. 7. BSA binding by polyDMAEMA-grafted pAAm cryogel. The pAAm cryogel was submerged directly in the reaction mixture containing the monomer and the initiator (closed rhombus) and the pAAm cryogel was activated with the initiator before exposure to the monomer solution (opened square). The monolith of polyDMAEMA-grafted pAAm cryogel were saturated with BSA solution (1 mg/ml) dissolved in 20 mM Tris–HCl buffer, pH 7.0, unbound BSA was washed off with the same buffer and bound BSA was eluted with 1.5 M NaCl dissolved in the same buffer.

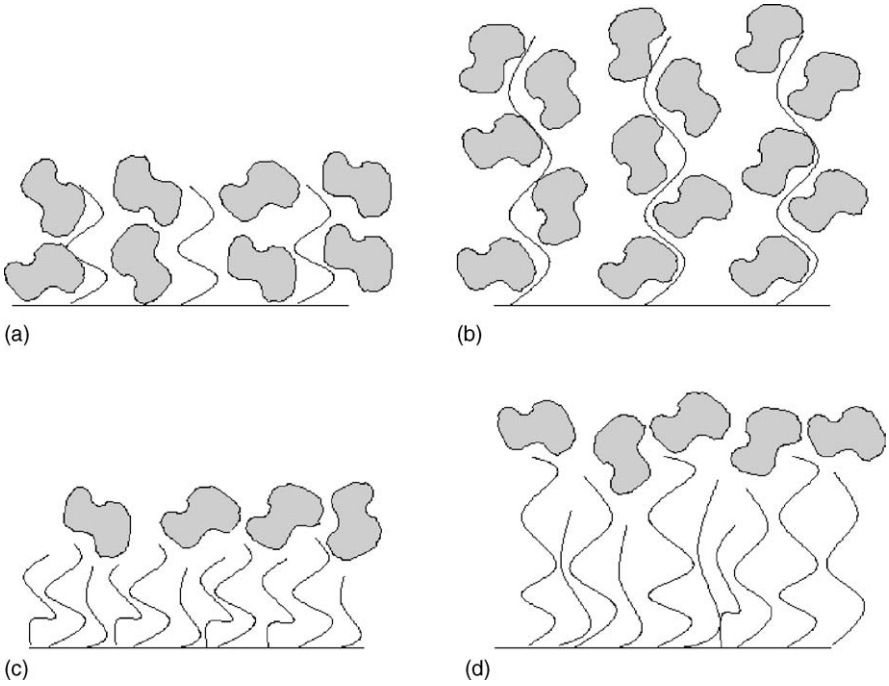

Fig. 8. Schematic presentation of BSA sorption on the surface of pAAm cryogel grafted by using different techniques: (a) two-step technique, low grafting degree; (b) two-step technique, high grafting degree; (c) one-step technique, low grafting degree; and (d) one-step technique, high grafting degree.

The significant differences in the BSA binding capacity could presumably be due to the different architecture of the grafted polymer layer. The degree of grafting shows only the amount of the polymer grafted saying nothing about how dense the grafting is and how long the grafted chains are. The same degree of grafting could be achieved either by grafting a large number of short polymer chains or by grafting a small number of long polymer chains.

The structure of the grafted layer as well as accessibility of functional groups for binding is very important for the adsorption of large multifunctional ligands as BSA is [14].

One could presume that the dense layer of short polymer chains was formed during the direct graft polymerization as the polymerization proceeded in a medium containing initiator capable of producing new radicals on pAAm backbone during the entire reaction time. The presence of numerous sites for starting graft polymerization resulted in the dense population of grafted chains, however, the simultaneous polymerization proceeding in solution could favor the termination of grafted chains due to recombination.

For the two-step procedure, grafting proceeded under the conditions when no new radicals are formed on pAAm backbone hence resulting in a less efficient conversion of monomers but in a highly restricted formation of homopolymer in solution. These conditions could favor the growth of long grafted chains at the initial radical sites on the pAAm backbone. One could expect, hence the formation of sparsely populated but long polymer chains.

Sparse long grafted polymer chains are preferable for binding large protein molecules like BSA. They are more flexible and could protrude in solution performing as “tentacles” capable of multipoint interactions with negatively charged BSA molecules. The flexibility of these tentacles allowed them to change their conformation in order to adapt for the most efficient BSA binding and thus to increase the efficiency of utilization of positive charges (Fig. 8a). The higher the grafting degree (which means probably that the grafted chains are longer), the higher is the BSA binding capacity (Fig. 8b).

On the contrary, the short and dense grafted chains provide poor possibilities for protein binding. One could expect significant shielding of short chains by bound protein molecules, hence decreasing significantly the BSA binding capacity as compared to long grafted chains (Fig. 8c). The small increase in binding capacity with grafting degree for polyDMAEMA-grafted pAAm cryogel prepared by direct grafting corroborates the proposed scheme of protein binding as new short chains of grafted polymer hardly improve the BSA binding capacity due to the screening effect (Fig. 8d).

4. Conclusion

DMAEMA was efficiently and conveniently grafted onto pAAm cryogel using potassium diperiodatocuprate as an initiator. It was possible to achieve 110% (w/w) grafting degree by increasing the monomer concentration. The BSA binding capacity of polyDMAEMA-grafted pAAm cryogels with the same grafting degree, depended significantly on the grafting procedure, whether one-step direct grafting or two-step procedure was used. It could be speculated that much higher BSA binding capacity for polyDMAEMA-grafted pAAm cryogels

prepared using two-step procedure is attributed to the different architecture of grafted polymer. Smaller number of longer chains of grafted polymer could provide better BSA binding capacity as compared to a higher number of shorter grafted chains, as the long chains could perform as tentacles when binding protein molecules.

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