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Polyacrylamide Ability for Protein Immobilization in One-phase Binary-solvent Systems

Kamahldin Haghbeen[1]*, Roshanak Agharafeie[2], and Saeed Maghsoudi[2]

(1) National Institute for Genetic Engineering and Biotechnology, P.O.Box:14155-6343 Tehran, I.R. Iran
(2) Biotechnology Department, Chemical Engineering Faculty, Iran University of Science and Technology, P.O. Box: 13145-1384, Tehran, I.R.Iran

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ABSTRACT

The protein immobilization ability of cross-linked polyacrylamide (CLP) was studied in one-phase binary-solvent systems. Four miscible solvents in water; 2 protic (methanol, 2-propanol) and 2 aprotic (THF, acetonitrile), and bovine serum albumin (BSA) as target protein were chosen. According to the results of a study on the sieve structure of CLP, the polymer which was made from 1.69 M acrylamide and 2.03×10^{-2} M bis-acryl amide was selected for the research. Results showed that CLP immobilization efficiency increased as the co-solvent share in the binary-solvents increased. However, it was not necessary to omit water. The immobilization efficiency of CLP was satisfying in the binary systems containing about 50% 2-propanol or acetonitrile, or 70% Methanol or THF. The CLP immobilization performance was very high when the organic solvent share was increased above 90% in all the studied binary media. The outcome suggests that the tolerable level of water and the role of co-solvent in the applied system are mainly affected by the amphiphilic nature of CLP. Assumingly, the amphiphilic structure helps CLP to be applied in one-phase binary systems containing co-solvent from various groups of protic or aprotic with different log P.

Key Words:
protein immobilization;
cross-linked polyacrylamide;
amphiphilic structure;
binary-solvent systems.

INTRODUCTION

Medium engineering has emerged as a hybrid branch of science and technology to address the increasing demand for biocatalysts. Enhancing both activity and stability of biocatalysts for in vitro applications are the major objectives in this field. Hence, it concerns not only studying the conventional and non-conventional media, but also the adaptation of the microenvironment of biocatalysts via immobilization or introduction of additives for stabilization purposes [1].

In fact, immobilizing biocata-

(*) To whom correspondence should be addressed.
E-mail: Kamahl@nrcgeb.ac.ir

lysts in the optimized media is one of the economically promising current researches in medium engineering. This is the reason why there are a considerable number of reports on the medium selection [2], immobilization materials, and techniques [3]. Nonetheless, examining the final combination of these components and the biocatalyst has proved to be vital [4-5]. Therefore by considering this trend, the results from investigations on the non-conventional media [2,6], and the polyacrylamide (PAA) physical and chemical properties [3,7], this research was devoted to a study on the cross-linked PAA (CLP) efficiency for entrapping macromolecules like enzymes in one-phase binary media.

Two properties of CLP, i.e., hydrophilicity and molecular-sieve like structure have made it applicable in some important processes such as electrophoresis of proteins [8], soil quality amendment [9], and entrapping cells and enzymes. However, because of its high hydrophilicity, CLP is usually preferred to be used for cells rather than macromolecules immobilization [3] in conventional media. Its application in non-conventional media has rarely come to attention.

In this study, it was intended to focus on the CLP interactions with the protein and the solvent system. So, instead of an enzyme, bovine serum albumin (BSA) was selected as a protein designate to avoid the possible interferences of other subjects like the effect of the total system on the transition state, rate of the enzymatic reaction, substrate solubility, and so on.

EXPERIMENTAL

Materials

Ammonium persulfate (APS), tetramethyl ethylene diamine (TEMED), BSA, acrylamide (Ac), bis-acrylamide (bAc), and solvents with extra pure grade were purchased from Merck. All the other chemicals used in this work were taken from the authentic samples.

Preparation of Different CLP

Two different groups of CLP polymers were made by changing the ratio of Ac/bAc and bAc/Ac as shown in Table 1 using the conventional method [10]. The CLP polymers of both groups were synthesized in the presence of constant amounts of TEMED (6.6 mM) and APS (2.2 mM) in 10 mL phosphate buffer (0.01 M) at pH 6.8 and room temperature.

Electrophoretic Mobility Shift Assays

BSA samples (10 µl containing 0.17 µg) were loaded onto the CLP polymers and electrophoresed under the non-denaturing conditions [10] using phosphate buffer solution at constant field (200 V). Then, the fixed gels were stained by Coomassie blue G-250 solution. After destaining, the BSA shift on each CLP was precisely measured. The results are reported in Table 1.

Immobilization of BSA in CLP

A slightly modified procedure described by Skryabin and Koshcheenko [11] was used for entrapment of BSA in CLP. Hence; Ac (1.69 M), bAc (2.03×10^{-2} M), TEMED (6.6 mM), and BSA (188 mg) were mixed in 20 mL phosphate buffer (0.01 M, pH = 6.8) at room temperature. The mixture was degassed and APS (2.2 mM) was added. The reaction mixture was transferred into a conventional electrophoresis frame to solidify. The resulting gel was then cut into disks with the average weight of 1.265 g.

CLP Immobilization Efficiency

17 freshly prepared discs of CLP containing the entrapped BSA (9.4 mg per gram of gel) were precisely weighed. 17 solvent systems were also prepared; one phosphate buffer solution and 16 one-phase binary sys-

Table 1. Different synthesized CLP with the corresponding BSA mobility shift. Ten different PAA hydrogels were synthesized by changing the amounts of Ac and bAc in the presence of a constant amount of the initiator.

Concentration	A	B	C	D	E	F	G	H	I	J
(Ac) M	1.69	1.69	1.69	1.69	1.69	1.12	1.4	1.69	1.97	2.53
(bAc) mM	25.3	20.3	16.9	14.5	11.3	16.9	16.9	16.9	16.9	16.9
Ratio of (Ac)/(bAc)	66.8	83.2	100	116.5	149.5	66.3	82.4	100	116.5	149.7
BSA Shift (cm)	1.5	1.8	2	2.2	2.6	3.4	2.5	2	1.4	1.2

tems. The latter systems, each, contained phosphate buffer and either of 25, 50, 75, and 100% of four different co-solvents. The selected co-solvents were methanol, 2-propanol, tetrahydrofuran (THF), and acetonitrile. Then, each CLP disc was transferred into one of the 17 solvent systems (10 mL). Considering the amount of the released water from the disks (Figure 2), the final share of the organic solvents in the above mentioned binary media were 23.04, 47.17, 71.77, and 90.90%, respectively.

The amount of the released BSA from the CLP matrix into the solution was monitored spectrophotometrically at 280 nm using the same solvent system as the blank [12]. All the 17 systems were checked for any precipitate prior to absorbance reading of the corresponding supernatants. In none of the examined systems any precipitate was not observed even after one week. The absorbance of dissolved BSA (2.82 mg) in phosphate buffer solution (3 mL) was considered as 100% release. All the results introduced in this paper are the average of triplicate, measurements.

RESULTS AND DISCUSSION

CLP Preparation

It has been shown that the length of polyacrylamide strands and the number of the cross-linkings directly affect the quality of the sieve structure of CLP [13]. Since the mechanical quality of CLP gel can be controlled via adjusting the ratio of Ac to bAc in the presence of a constant amount of the initiator at room temperature, some different CLP polymers were first synthesized by changing the ratio of the starting materials [14]. The BSA mobility shift on the obtained polymers in a constant electrophoretic field was considered as an index for a tentative comparison between their pores. The conditions and the results of these experiments are summarized in Table 1.

The data in Table 1 clearly indicates the mobility shift of BSA on CLP polymers of A to E increases as the ratio of [Ac]/[bAc] increases, while opposite trend is seen on the polymers of F to J. It means that the results of BSA shift on two CLP(s) of A and F are opposite despite alike [Ac]/[bAc] ratio. These data suggest that the quality of the sieve structure of CLP can not be simply predicted from the [Ac]/[bAc] ratio, and the detail has to be considered. For instance in these experiments, the number of bAC molecules in CLP A is bigger than that in CLP F. As a result, the possible number of the cross-linking in the resulting polymer structure is higher, which brings about a smaller mobility shift of BSA on CLP A.

Although the extent by which the number of cross-linkings affects the pore size in the CLP structure has to be worked out in the structural and rheological studies. It is effective on the solvation radius of an entrapped macromolecule [15], which directly influences the microenvironment of the immobilized macromolecule. This is why Hicks and Updick could achieve to the highest activity of lactate dehydrogenase only in the 10% CLP gel [16]. Munjel and Sawheney had also reported different activities for the entrapped mush-

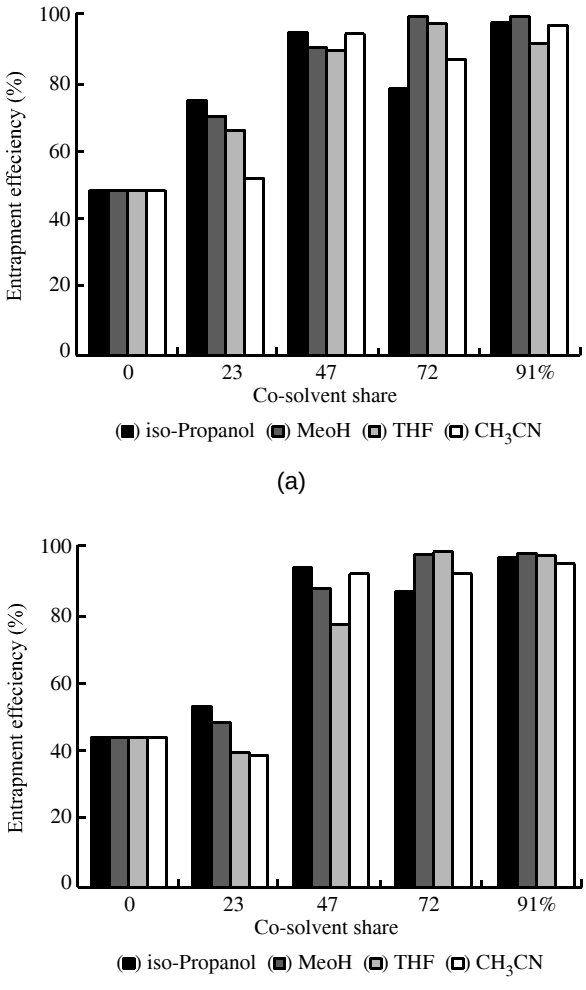


Figure 1. The amount (%) of the entrapped BSA in CLP in the studied one-phase binary-solvent systems after (a) 20 h and (b) 144 h.

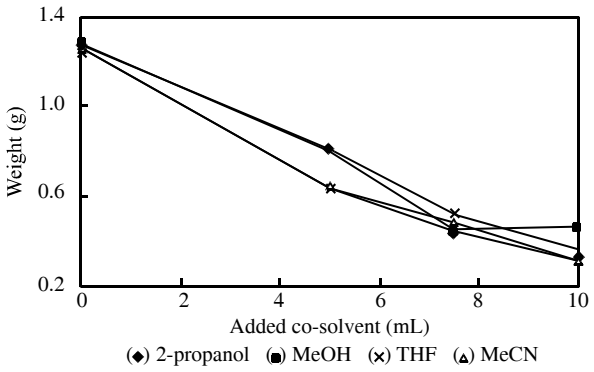


Figure 2. The weight loss of CLP disc in the studied one-phase binary-systems. CLP Disc (1.248 g) gained weight (1.793 g) after incubation in phosphate buffer 0.01 M and pH 6.8.

room tyrosinase in different CLP polymers [17].

Co-solvent Selection

Four different solvents, two protic and two aprotic, were selected for these experiments. The most prominent property of these solvents was their miscibility in water so that they could easily form one-phase binary systems with water. In such systems, the solubility of organic substrates is increased and the molecules of the solvent could reach to the solvation shell of the entrapped protein and contribute to its stabilization [18].

Regarding the organic solvent selection, most of the previous researches were not directly related to this type of work but could be enlightening it. For instance, Khmelnitsky et al. had tried to introduce a parameter for choosing the right co-solvent for running enzymatic reactions in non-aqueous media [19]. Although they had not used immobilization technique and the applicability of their whole idea is still a matter of controversy [20]; it was preferred to consider the invaluable results of those experiments. Hence, the selected organic solvents for this research were not belonging to the so called sub-group "bad solvents". Moreover, the log P and denaturation capacity (DC) indexes, Table 2, of the selected solvents were different enough to cover almost the whole range of the introduced data for various solvents [19].

Besides, in case of the immobilized enzyme, it is highly probable that the co-solvent molecules compete with water or substrate molecules for the active site of the enzyme in one-phase binary media [21]. Therefore, to extend the scope of this research for such works, the aprotic solvents with different functionalities were selected beside the protic solvents with different steric factors.

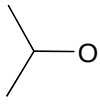
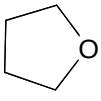
Immobilization Efficiency Studies

CLP Is usually considered as a poor support for the macromolecule entrapment due to its high water permeability. Although covering the solid-gel phase with permeable membrane avoids the macromolecules' escape [22]; it was the major objective of this research to study the CLP immobilization ability in the absence of any permeable membrane in the aforementioned media. Therefore; CLP B, with a milder mobility index mentioned in Table 1 was selected for this purpose and the escape of the entrapped BSA from the CLP B in each introduced solvent system was measured.

Figures 1a and 1b show the amount of the entrapped BSA in the CLP after 20 and 144 h, respectively. These figures do not seem very different at first glance. It means that the immobilization of BSA in CLP reaches a sustainable equilibrium in the studied systems. This stability is particularly more evident in the binary systems containing more than 70% organic solvent. Nevertheless, in a more precise examine, it seems that the immobilization ability of CLP has been increased after 144 h in a few cases. One explanation for this observation could be the re-adsorption of the released BSA on the CLP surface.

Figure 1 indicates that the BSA immobilization ability of CLP does not depend on the protic or aprotic nature of the co-solvent. Besides, omitting water or using a binary system with very low water content is

Table 2. Log P and DC indexes [18] and structures of the applied organic solvents in this study.

Solvent	Log P	DC	Structure
Methanol	-0.74	30.5	H_3C-OH
2-Propanol	0.14	70.2	
Acetonitrile	-0.34	64.3	$H_3C-C\equiv N$
THF	0.46	100.0	

not a necessity. For instance, a proper immobilization of BSA in CLP has achieved in the system containing about 50% 2-propanol or acetonitrile. However, this phenomenon has happened for the binary systems of methanol and THF at 70% of the co-solvent share.

Finally, the immobilization efficiency is almost identical for all the studied solvents above 90% of the co-solvent share. In fact this result can be extended to the indexes of log P and DC of the applied solvents. Since methanol and THF which have very different DC and log P indexes (Table 2) have produced similar results.

For understanding the results, chemical structure of CLP has to be fully considered. Although CLP enjoys a vast number of amide functional groups in its chemical structure; the main skeleton of CLP is made of carbon. Likewise, if a protein dissolves in water; it does not mean that it will not respond to the hydrophobic interactions. In fact, detailed research has proved that there is a threshold for the organic solvent share in the binary systems under which proteins maintain their native structures [6,23]. Hence, what is seen in Figure 1 is presumably the outcome of the overall interactions between the amphiphilic structure of CLP, BSA, and the solvent systems.

There is another determinative factor which has to be watched, the water content of the immobilization system. Low or excess amount of water, both could be disadvantageous. It has been shown that the amount of water in the solvation shell of a "dissolved protein" in a one-phase binary-solvent system depends on the nature of the co-solvent. In this case, protic organic solvents can attract water molecules to them [21], as a result, reduce the accessible water for the protein. But when the protein is entrapped in CLP, the amphiphilic structure of CLP affects the final behaviour of the solvent components. Figure 2 shows weights of the identical CLP disks which had been incubated in the introduced media. The data indicates that the weight of each disk has been decreased after increasing the co-solvent share in the corresponding system, and the inclination trend has been alike for the different, protic or aprotic, solvents. It means that CLP has almost balanced the ability of different solvents for removing water molecules from the gel. Therefore, CLP is assumingly able to influence the amount and the nature of the exerted solvent force on the entrapped macromolecule body which, in turn, affects its long-term stability.

CONCLUSION

In this study, it was shown that the lack of efficiency of CLP for entrapping macromolecules in conventional media can be cured using one phase binary solvent systems. Besides, the amphiphilic structure of CLP helps the immobilized system to adopt different co-solvents with various polarities. CLP Also lets the system maintains a considerable amount of water while it holds the macromolecules efficiently.

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