Article

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Property and Application of BACy-Based Functional Hydrogels

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Conventional polyacrylamide hydrogels prepared from the free radical polymerization between acrylamide and *N*,*N*′-methylenebisacrylamide (NMBA) have been frequently used in the biochemical technique like the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to resolve protein mixtures. In this study, we have prepared an alternative polyacrylamide hydrogel from the cross-linking of acrylamide and *N*,*N*′-bisacrylylcystamine (BACy). In addition, we have compared the BACy-based hydrogel with the NMBA-based polyacrylamide hydrogel for their physical properties such as swelling ratio, shear modulus, crosslink density and morphology. Moreover, we further determined whether BACy-based polyacrylamide hydrogel could be applied to SDS-PAGE and proteomics research. The results showed that this type of hydrogel is capable of separating proteins and facilitates further in-gel protein digestion and the following protein identifications by mass spectrometry. In summary, our study provides a basis for the putative application of BACy-based hydrogels.

Keywords: *N,N*′-Bisacrylylcystamine (BACy); *N,N*′-Methylenebisacrylamide (NMBA); Disulfide linkage; Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

SDS-PAGE has been frequently used method in biochemical analysis and provides the capability to resolve complex protein mixtures based on the difference of their molecular weights.[1] In addition, three-dimensional structure of polyacrylamide hydrogel is essential for SDS-PAGE.[2] The polyacrylamide hydrogel can be prepared from free radical polymerization of acrylamide in the presence of diverse two-functional cross-linkers such as the *N,N*′-methylenebisacrylamide (NMBA), 1,2-diacrylamide ethyleneglycol (DEG), ethyleneureabisacrylamide (EUB), *N,N*′-propylenebisacrylamide (PBA), *N,N*′-diallyltartardiamide (DATD), diacrylamide dimethylether, and ethylene diacrylate (EDA).[3,4] The resulting polyacrylamide hydrogels exhibit diverse pore size within the three-dimensional structures and provide distinct applications in molecular separation and drug loading/release. Currently, bioanalytical methodology like proteomics frequently uses polyacrylamide hydrogels to separate proteins in the one- or two-dimensional gel electrophoresis.

In addition, the cleavable polyacrylamide hydrogels have also been developed by incorporating a cleavable cross-linker into polymer structure. These cleavable cross-linkers include the oxidation-sensitive *N,N*′-1,2-dihydroxyethylene bisacrylamide (DHEA),[5,6] the alkaline-sensitive ethylene glycol diacrylate (EGDA)[7] and the reduction-sensitive *N,N*′-bisacrylylcystamine (BACy).[8] Expansion of pore size within the gel structure is anticipated when such labile crosslinkers were put under conditions for cleavage. Proteomics represents a large-scale investigation on structures and functions of total proteins in cells or tissues.[9-12] A typical proteomics approach includes the extraction of peptides from in-gel protein digestion by proteases, and the subsequent amino acid sequencing by mass spectrometry. The pore expansion resulted from the cleavable polyacrylamide hydrogel upon cleavage conditions may increase the amount of extracted peptides or proteins for the subsequent analysis. Indeed, the base-labile EDGA was previously used to prepare the reversible gel which facilitates collecting the sufficient amount of radio-isotope-labelled proteins for scintillation counting.[7,13]

We herein investigate the characteristics of the reduction-cleavable polyacrylamide hydrogel as well as its application potential in proteomics study. Since condition of oxidation or base treatment causes damage and modification to protein structure, the reducible BACy crosslinker

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was used to prepare the cleavable polyacrylamide hydrogel for the following study. The disulfide linkage of BACy can be effectively and efficiently cleaved into two thiol groups upon treatment of reducing agents like 1,4-dithiothreitol (DTT) or 2-mercaptoethanol (β-ME).[14] Therefore, such reductive cleavage within the three-dimensional structure of BACy-based polyacrylamide hydrogel provides a controllable manner to expand the gel pore size. In the present study, the swelling ratios and pore sizes of the BACy polyacrylamide hydrogel were measured and compared to those of the conventional NMBA polyacrylamide hydrogel. In addition to the protein separation efficiency of these hydrogels, the drug release experiment of caffeine and bovine serum albumin (BSA) from different hydrogels under the treatment with or without reducing agents were carried out to demonstrate the feasibility of reducible polyacrylamide hydrogel.

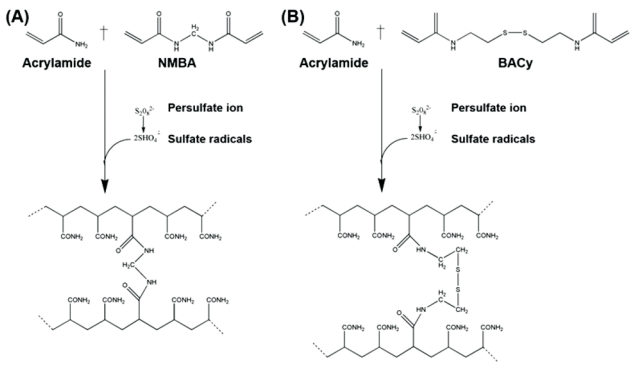
RESULTS AND DISCUSSION

Characterization of the Polyacrylamide Hydrogels

BACy was synthesized at good quality according to the modified procedure from our recent report (Figure 1).[14] The corresponding polyacrylamide hydrogels were prepared by mixing acrylamide with either NMBA or BACy as crosslinker (as shown in Scheme 1) and the resulting hydrogels were respectively named as NP, BP_a and BP_b. NP hydrogel was prepared following the conventional recipe of acrylamide (0.967 g, 13.6 mmole) and NMBA ratio (0.033 g, 0.216 mmole) for SDS-PAGE. In addition, BP_a hydrogel was prepared using the same weight ratio of acrylamide (0.967 g, 13.6 mmole) and crosslinker (BACy, 0.033 g, 0.128 mmole) as in the NP hydrogel whereas BP_b hydrogel used the same molar ratio of acrylamide (13.6 mmole) and crosslinker (BACy, 0.056 g, 0.216 mmole) as in the NP hydrogel. The feed compositions as well as the obtained physical and mechanical properties for all three polyacrylamide hydrogels were summarized in Table 1. In addition, the appearance of two BP hydrogels resembles the NP hydrogel in the aspect of transparency, softness, protein separation efficacy and effect of gel staining, as shown in Figure 2. We further measured the swelling ratio for each three hydrogels and the results shown in Table 1 indicated that the BP_b hydrogel has the highest swelling ratio (13.91 g/g) whereas NP and BP_a hydrogels have similar values, 9.78 g/g and 11.89 g/g respectively. This is because the BP_b hydrogel has the BACy crosslinker effect than NP hydrogels, which led to a larger structure in BP_b hydrogel.

Swelling kinetics profile of all three polyacrylamide hydrogels in deionized water at 25 °C is shown in Figure 3. The measured values of SReqs for polyacrylamide hydrogels indicated, i.e. BP_b (13.91 g/g) > BP_a (11.89 g/g) > NP (9.78 g/g). As shown in Table 1, the G and ρ_x values of the

Scheme 1 Polymerization of the polyacrylamide hydrogels using acrylamide with NMBA (A) and BACy (B) respectively



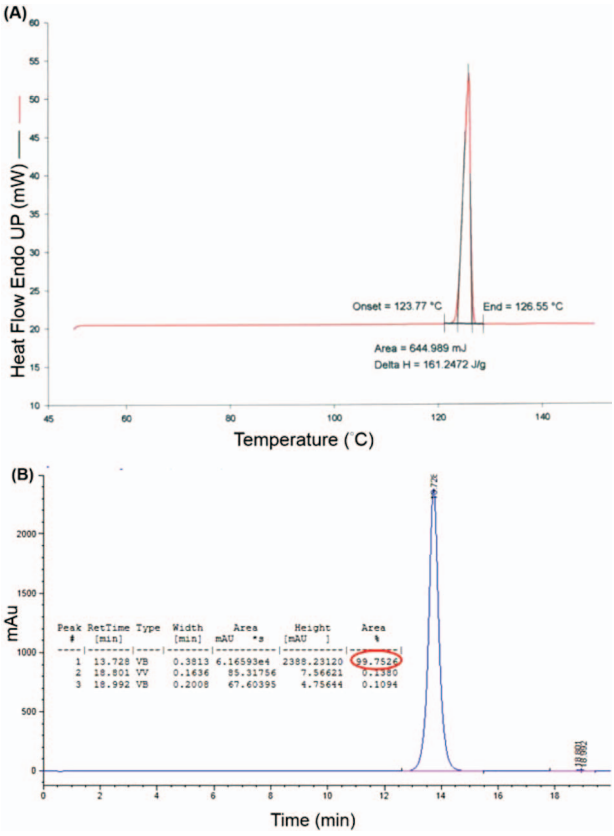


Fig. 1. The BACy has m.p. of 126-127 °C (A) and purity more than 99% based on result of High Pressure Liquid Chromatography (B).

Table 1. Feed compositions and physical properties of the copolymeric hydrogels

Gel code	AAm[a] (mmole)	NMBA[b] (mmole)	BACy[b] (mmole)	SReq[c] (g/g)	G × 10^4[d] (N/m^2)	wet gel[e] $\rho_x \times 10^{-2}$ ($mmole/cm^3$)
NP	13.6	0.216	-	9.78 ± 0.10	2.26 ± 0.30	2.10 ± 0.30
BP_a	13.6	-	0.128	11.89 ± 0.12	2.07 ± 0.20	2.05 ± 0.20
BP_b	13.6	-	0.216	13.91 ± 0.04	1.50 ± 0.11	1.58 ± 0.12

[a] AAm: Acrylamide. [b] NP: hydrogel with 0.216 mmole of NMBA; BP_a: hydrogel with 0.128 mmole of BACy; BP_b: hydrogel with 0.216 mmole of BACy. [c] SR_{eq}: equilibrium swelling ratio. [d] G: shear modulus. [e] ρ_x. effective cross-linking density.

polyacrylamide hydrogels are 2.26×10^4 N/m^2, 2.10×10^{-2} $mmol/cm^3$ for NP gel, 2.07×10^4 N/m^2, 2.05×10^{-2} $mmol/cm^3$ for BP_a gel and 1.50×10^4 N/m^2, 1.58×10^{-2} $mmol/cm^3$ for BP_b gel. These values indicated the higher the gel strength (G) and the higher effective crosslinking density (ρ_x), the lower the swelling ratio of the hydrogels, i.e. NP > BP_a > BP_b. In addition, the low effective crosslinking density (ρ_x) of BP_b indicated the less degree of crosslinking in the hydrogel structure, which allowed more water absorption.

Scanning electron microscopy (SEM) was used to investigate the internal morphologies of the polyacrylamide hydrogels and the resulting micrographs of SEM clearly showed the pore sizes in these gels (Figure 4). Whereas BP_a and BP_b gels have the larger pore size, which is explained the difference of swelling ratios among these gels. i.e. BP_b > BP_a > NP.

Effects of BACy on Protein Separation Efficacy

All three polyacrylamide hydrogels were examined for their ability to resolve protein mixtures. As shown in Figure 5A, the BSA (M.W. 66 kDa), PhaZ (M.W. 34 kDa) and mixture of standard protein weight markers (ranged from 17 kDa to 170 kDa) were loaded into NP, BP_a and BP_b hydrogels respectively for the SDS-PAGE analysis. Our data showed that both BP hydrogels are capable to resolve proteins and the separation efficacy is comparable to NP hydrogel. Notably, all proteins exhibit slower mobility in BP_b hydrogel than in other two hydrogels. This phenome-

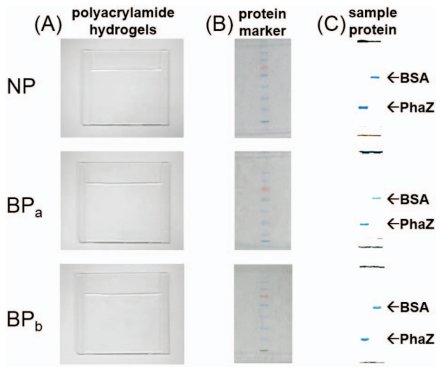


Fig. 2. Appearance of NP, BP_a and BP_b SDS-PAGE gels. (A) Transparency of polyacrylamide hydrogels (B) Separation of pre-stained protein markers without gel staining (C) Coomassie blue staining of gels loaded with sample proteins of PhaZ (lane 1) and BSA (lane 2).

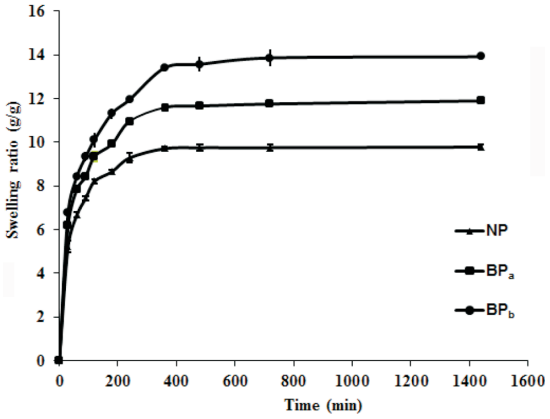


Fig. 3. Measurement of swelling kinetics of NP, BP_a and BP_b hydrogels.

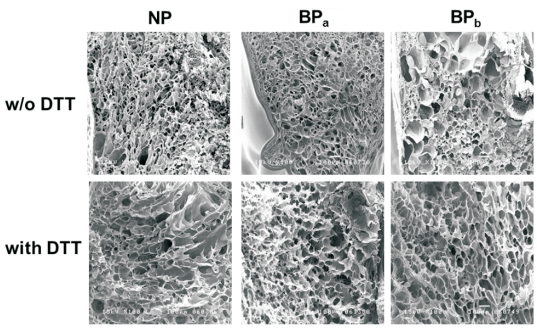


Fig. 4. Morphology of NP, BP_a and BP_b hydrogels with or without DTT treatment using SEM photography at 100x.

non is likely due to the fact that BP_b gel has a higher content of crosslinker in the composition of polyacrylamide, which results in denser structure and smaller space for protein penetration. In addition, the retention factor (RF) which reflects the relative mobility of proteins in hydrogels during electrophoresis was calculated and listed in Table 2. Furthermore, plot of RF against molecular weight (M.W.) of protein weight markers was shown in Figure 5B. In summary, proteins with higher molecular weight travel fast in BP_a hydrogel than in NP hydrogel whereas all proteins move relatively slowly in BP_b hydrogel. However, both BP hydrogels exhibit good protein-separating ability comparing to NP hydrogel.

Effects of BACy on Protein Identification Efficacy

In a typical proteomics study involving SDS-PAGE, the in-gel trypsin digestion of selected protein band (or spot) into peptides and the subsequent mass spectrometric (MS) analysis are carried out to identify proteins within the polyacrylamide hydrogel. Although the disulfide linkage of BACy in polyacrylamide hydrogels may possibly react with the free thiol group in any protein, whether the presence of the disulfide bond interferes with the trypsin digestion of proteins or the MS analysis of tryptic peptides remain unknown. We next investigated effects of BACy on the protein identification efficacy from NP, BP_a and BP_b hydrogels by a MALDI-TOF mass spectrometry (MS) (Figure 6). The BSA protein was chosen for the following in-gel digestion and MS identification. BSA has 35 cysteines, but in its native state, 34 are in disulfide linkage. It has a limited ability to potentially react with disulfide linkage of BACy in hydrogels. As shown in Table 3, the identification of BSA was achieved from all three hydrogels with similar identification score, sequence coverage and number of the identified peptides. These results indicated that the BACy content or free cystein on BSA does not interfere with the identification process of BSA proteins, including the in-gel trypsin digestion procedure to generate peptides and the MS-based analysis of peptide masses. In summary, the BACy-based polyacrylamide hydrogels showed no interference with the typical protein identification and provide a good alternative for NP hydrogels.

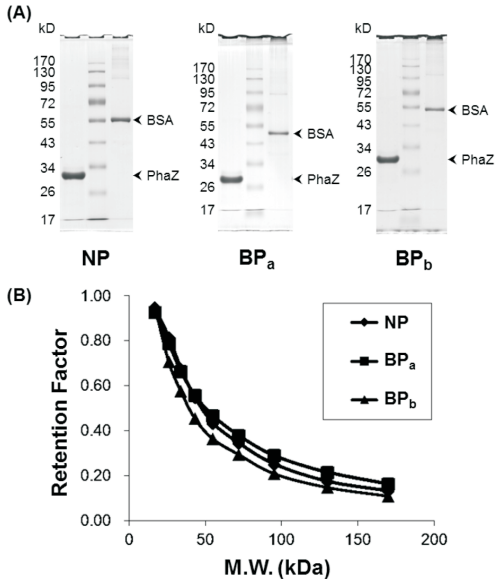


Fig. 5. SDS-PAGE with NMBA or BACy as crosslinker. (A) Protein separation results on NP, BP_a and BP_b gels; lane 1, PhaZ (34 kDa); lane 2, commercial MW markers; lane 3, BSA (66 kDa). (B) Retention factors (RF) of lane 2 MW markers on NP, BP_a and BP_b gels were measured.

Table 2. Relative mobility based on the RF value of BSA, PhaZ and protein weight markers on NP, BP_a and BP_b hydrogels

Proteins	Retention factor (RF)		
	NP hydrogel	BP_a hydrogel	BP_b hydrogel
BSA (66 kDa)	0.43	0.50	0.38
PhaZ (34 kDa)	0.73	0.76	0.64
170 kDa marker	0.13	0.16	0.11
130 kDa marker	0.17	0.22	0.15
95 kDa marker	0.25	0.29	0.21
72 kDa marker	0.34	0.38	0.29
55 kDa marker	0.43	0.47	0.36
43kDa marker	0.55	0.56	0.45
34 kDa marker	0.68	0.66	0.57
26 kDa marker	0.81	0.79	0.70
17 kDa marker	0.95	0.93	0.95

Effects of BACy on Caffeine and BSA Release

As indicated in Table 3, release of caffeine from caffeine-incubated hydrogels was slightly increased upon the elevation of BACy contents from BP_a (3.20 mg/g) to BP_b (3.49 mg/g). Under DTT treatment, the released caffeine in hydrogels was also slightly increased with an elevation of BACy content in the hydrogels from BP_a (3.27 mg/g) to BP_b (4.08 mg/g). These results indicate that higher swelling ratio of BACy in hydrogels did not significantly affect the release amount of caffeine.

On the other hand, all three hydrogels exhibited the clear increase of BSA released from hydrogels upon DTT

Table 3. Effects of BACy on the protein identification and the swelling ratio as well as Caffeine and BSA release upon DTT reduction

	BSA Protein Identification by MS			Swelling Ratios (g/g)		Substance Release (mg/g)			
						Caffeine		Bovine Serum Albumin (BSA)	
Gel code	ID Score	ID Coverage	Peptide number	w/o DTT	w/ DTT	w/o DTT (HPLC)	w/ DTT (HPLC)	w/o DTT (WAKO)	w/ DTT (HPLC)
NP	1537.07	41.97%	28	9.78 ± 0.10	9.89 ± 0.16	2.61	2.75	1.10	13.90
BP_a	1456.28	42.77%	27	11.89 ± 0.12	29.56 ± 0.78	3.20	3.27	2.05	21.25
BP_b	1584.78	42.77%	28	13.91 ± 0.04	34.62 ± 0.99	3.49	4.08	3.05	32.64

treatment. As shown in Table 3, released amount of BSA were increased from 2.05 mg/g to 21.25 mg/g for BP_a gel and from 3.05 mg/g to 32.64 mg/g for BP_b gel. In addition, more BACy content resulted in more increase of released BSA upon DTT treatment, as BP_b contains more BACy than BP_a (0.216 *vs.* 0.128 mmol). Although NP gel doesn't not contain disulfide bond for cleavage by DTT, it is anticipated that disulfide linkage of BSA proteins within NP hydrogel was also reduced by DTT to adapt more linear conformation, which allows BSA to be easily released from gel network (increased from 1.10 to 13.90 mg/g in NP gel). Notably, the BP_b hydrogel which has more molar ratio of

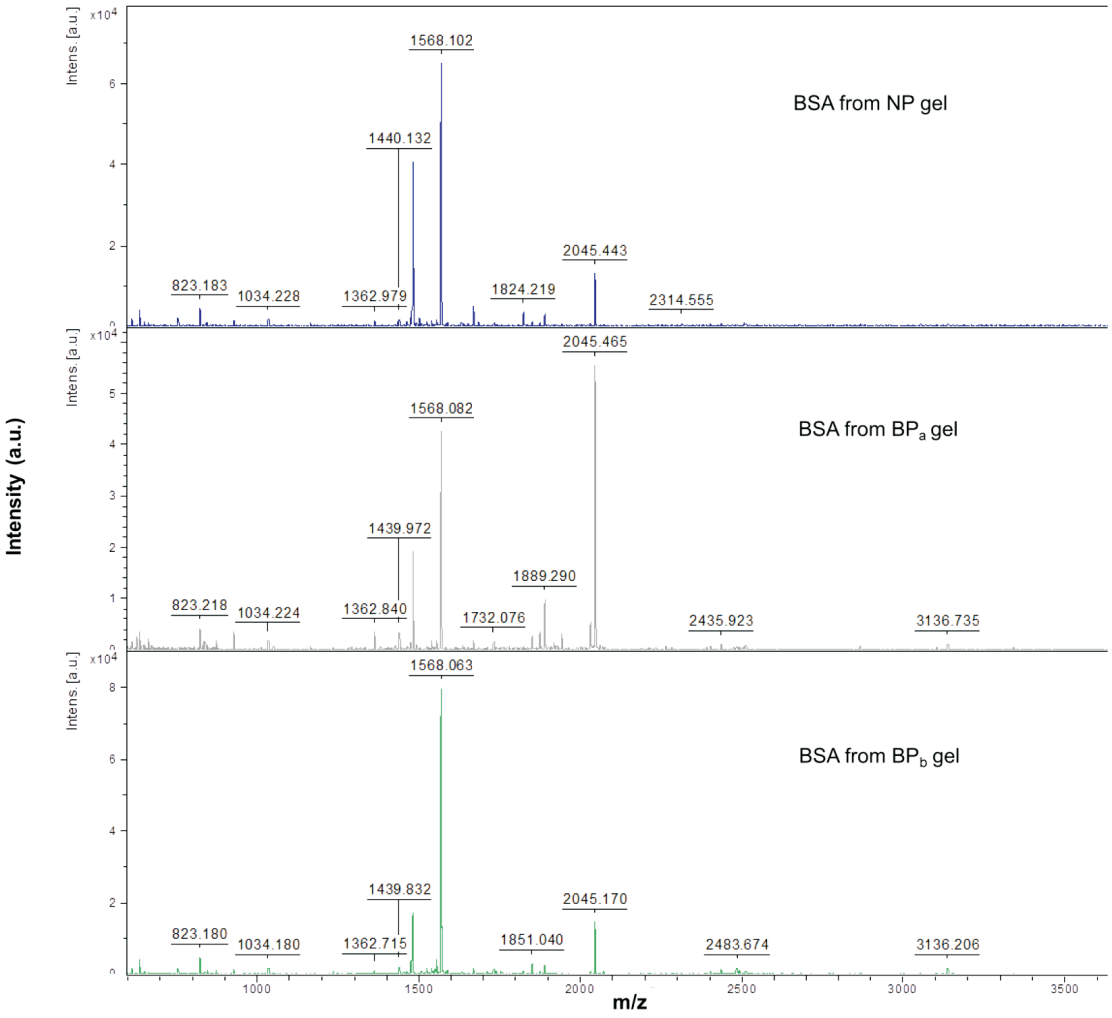


Fig. 6. Mass spectra of the identified BSA from NP, BP_a and BP_b gels using a MALDI-TOF MS.

BACy than BP_a hydrogel released significant amount of BSA proteins than other hydrogels upon DTT reduction, which indicates the advantage of using BACy as cross-linking reagents in polyacrylamide hydrogel. Currently, the typical method to extract proteins from polyacrylamide hydrogel needs technique like electroelution with limited yield. Therefore, the BP hydrogels could provide an alternative choice for researchers that intend to extract proteins of interest from protein-separating gel electrophoresis. Further investigation on conditions of protein extraction using BACy-based polyacrylamide hydrogel is suggested.

EXPERIMENTAL

Materials: Acrylamide (AAm, Bio-RAD, 99.9%) was used as monomer to prepare polymeric gels. *N,N′*-methylenebisacrylamide (NMBA) (Sigma) and *N,N′*-bisacrylylcystamine (BACy) were used as cross-linking agents with AAm in the preparation of three-dimensional polyacrylamide hydrogels. BACy was synthesized by the coupling reaction of cystamine dihydrochloride (Cy·2HCl) (ACROS, 97%) and acryloyl chloride (AC) (Alfa Aesar, 96%), as described in our previous report. Ammonium persulfate (APS) (Sigma, 98%) as initiator and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) (Sigma, 99.5%) as an accelerator were used as received. 1,4-Dithiothreitol (DTT) (USB, 99.5%) is used as reducing reagent to convert disulfide bond to thiols. The PhaZ protein is purified from *E. coli* as described previously.[15] The pre-stained protein molecular weight marker was purchased from Thermo Scientific. The coomassie brilliant blue (G250) was used for protein staining and purchased from BIO-RAD. Protein Assay Rapid Kit (WAKO) is the high sensitive reagent to determine protein concentration at room temperature. Sinapinic acid (SA) and peptide mass standard kit were purchased from Bruker Daltonics for mass spectrometry. Tris(hydroxymethyl)aminomethane, sodium dodecyl sulfate (SDS), trifluoroacetic acid (TFA), glycine, imidazole, ethylenediamine tetraacetic acid (EDTA) were purchased from ACROS. Bovine serum albumin (BSA) as a model protein and caffeine were used in drug release experiment.

Instrumentation: For protein identification, the MALDI-TOF mass spectrometer (Ultraflex, Bruker Daltonics) was used. High performance liquid chromatography (HPLC, Agilent 1100 dissolution analysis system G1312A binary pump, Minnesota, USA) and DU 800 UV/VIS Spectrophotometer were used to determine protein and caffeine concentration assay. Gel strength measured by uniaxial compression experiment with a Lloyd LRX universal tester (JJ Lloyd, Poole, UK). Morphology of dry gel was determined using a sputter coater with gold (JEOL, JFC-1200) and a scanning electron microscopy (SEM) (JSM-5300, JEOL).

Synthesis of BACy: BACy was prepared according to our previous protocol.[14] Briefly, cystamine dihydrochloride (1 equiv.) and TEA (4.2 equiv.) were first mixed in 80 mL of THF and then added with acryloyl chloride (2.2 equiv.) dissolved in 20 mL of THF dropwise at a rate of 1 mL/min. The resulting solution was allowed to stir at 40 °C under nitrogen atmosphere for 24 h. After evaporation of solvent, reaction product was purified by column chromatography with hexane/ethyl acetate (Hex/EA) as mobile phase and analyzed for purity by thin layer chromatography. The obtained BACy was white crystals in 76% yield; Mp: 126-127 °C.

Preparation of Polyacrylamide Hydrogels: Polyacrylamide gels were prepared using acrylamide (0.967 g, 13.6 mmole) with varied amount of NMBA or BACy (0.216 mmole or 0.128 mmole, 0.216 mmole) as cross-linker, as indicated in Table 1. Briefly, acrylamide and the selected cross-linker were dissolved in 6 mL of deionized water at 40 °C and added with tris-glycine and 0.1 mL of sodium dodecyl sulfate (SDS). The following polymerization was initiated when the 0.1 mL of 10% ammonium persulfate (APS) and 0.004 mL of tetramethylethylenediamine (TEMED) in deionized water were added to make a final volume of 10 mL. The resulting solution was loaded into a 7 × 7 cm^2 gel cast with 0.5 cm spacer and allowed to reaction at room temperature for 2 h.

Measurement of Equilibrium Swelling Ratios: Dry gels were equilibrated in 10 mL of deionized water at different temperatures on 25 °C and weighed to calculate their equilibrium swelling ratios (SR_{eq}).

$$SR_{eq} = (W\infty - Wd)/Wd \quad (1)$$

where W∞ and Wd were the weight of the wet gel at equilibrium and the weight of the dry gel, respectively.

Measurement of mechanical properties: Dry gels were equilibrated in 10 mL of deionized water at 25 °C and weighed. The gel strength (τ) was assessed with gel shear modulus (G) and measured by uniaxial compression experiment with a Lloyd LRX universal tester (JJ Lloyd, Poole, UK). The shear modulus (G) was calculated from equation (2):[16,17]

$$\tau = F/A = -G(\lambda - \lambda^{-2}) \quad (2)$$

where τ is the compression stress, F is the compression load, A is

the cross-sectional area of swollen gels, and λ is the compression strain ($\Delta L/L_o$) where ΔL is the difference of the thickness of deformed gel and initial swollen gel L_o. At low strains, a plot of shear stress versus ($\lambda-\lambda^{-2}$) would yield a straight line whose slope is shear modulus (G). The effective crosslink density (ρ_x) could be calculated from the shear modulus and polymer volume fraction (ν_2) as follows:

$$\rho_x = G/\nu_2^{1/3}RT \quad (3)$$

where R is the gas constant and T is the absolute temperature.

Gel Morphology and Pore Size: The dried polyacrylamide hydrogels were equilibrated in deionized water at 25 °C for 24 h, and the resulting swollen hydrogels were freeze-vacuum dried on –50 °C for 48 h. The hydrogels were then immersed in liquid nitrogen and fractured into pieces. The fractured specimens were coated with a gold layer for proper surface conduction, and determined for their morphological details using a scanning electron microscope (SEM, JEOL, JSM-5300, and Tokyo, Japan).

In Vitro BSA and Caffeine release experiment: Dry pieces of polyacrylamide hydrogel with fixed size were rehydrated in the aqueous solution of bovine serum albumin (BSA) (1 mg/mL) or caffeine (0.3 mg/mL) at 25 °C for 24 h. The resulting BSA or caffeine-containing hydrogels were transferred into a vial with DTT (1 mmole in 1 mL of deionized water), and controlled not DTT, then maintained at 37 °C in a water bath for 24 h. A 0.1 mL of solution was then collected for measuring the concentration of released BSA or caffeine using high performance liquid chromatography (HPLC, Agilent 1100 dissolution analysis system G1312A binary pump, Minnesota, USA) and DU 800 UV/VIS Spectrophotometer were used to determine protein and caffeine concentration assay.

Protein Identification from Polyacrylamide Gels Using In-gel Digestion and MS Analysis: The 1 μg of BSA, 1 μg of PhaZ, and 5 μg of protein molecular weight markers was separated respectively or together on the NMBA- or BACy-based polyacrylamide gels. After gel staining with Coomassie brilliant blue, protein bands were excised, cut into small pieces and digested with trypsin according to our previous protocol. The resulting peptides from trypsin digestion were analyzed using a MALDI-TOF mass spectrometer to determine the identity of protein.

CONCLUSIONS

In this study, we have demonstrated that BP hydrogels exhibited comparable protein separation efficacy to NP hydrogel in SDS-PAGE analysis. In addition, the contents of BACy did not interfere with protein separation or the subsequent protein identification for proteomics study. Measurement of swelling ratios of NP, BP_a and BP_b hydrogels showed that BP_b has the highest swelling ratio. In addition, the SEM analysis also verified that BP_b hydrogel has the largest pore size. Further treatment of DTT on these gels showed that the swelling ratios of the reduced BP hydrogels were increased but not for NP hydrogel. This phenomenon is supported by the significant increase of BSA release upon DTT treatment in BP hydrogels. The BP_b hydrogel exhibited the most degree of BSA release by DTT-mediated reduction of gel structure. Such protein release may be advantageous to researchers who use protein extraction to obtain proteins from gel electrophoresis. In summary, the current study provides a basis for the putative application of BACy-based polyacrylamide hydrogels in biochemical and proteomics study.

ACKNOWLEDGEMENTS

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