Monolithic cryogels made of agarose–chitosan composite and loaded with agarose beads for purification of immunoglobulin G

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\textbf{A B S T R A C T}

In order to obtain a novel absorber with high adsorption capacity for the purification of immunoglobulin G (IgG), continuous supermacroporous agarose beads embedded agarose–chitosan composite monolithic cryogels (agarose–chitosan cryogels) were prepared by cryo-copolymerization of agarose–chitosan blend solutions with glutaraldehyde as the crosslinker in the presence of agarose beads. After coupling 2-mercaptopyridine onto divinylsulfone-activated matrix, the obtained cryogels were used for the purification of IgG. The microstructure morphologies of the cryogels were analyzed by scanning electron microscopy. The results showed that the obtained cryogels possess interconnected pores of 10–100 \textmu m size. The specific surface area was 350 m\textsuperscript{2}/g with maximum adsorption capacity of IgG 71.4 mg/g. The cryogels showed workable stability, and can be reused at least 15 times without significant loss in adsorption capacity. IgG purity after one-step purification from human plasma was monitored by electrophoresis and the average recovery was estimated to be 90%.

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1. Introduction

Immunoglobulin G (IgG), is a major serum immunoglobulin [1], and widely used in clinic for the treatment of diseases related to immunodeficiency [2]. For its purification, the most commonly used methods are the high specificity protein A or protein G based methods [3]. However, these methods also have some drawbacks, such as the high cost and leak of ligands, difficult to immobilize in the proper orientation, and susceptible to degradation by microorganisms, which severely limit their applicability [4,5]. Compared with those methods, the low cost 2-mercaptopyridine (2-MP) ligand based methods showed selective binding of IgG not only in high salt concentrations but also in salt-independent way. Its adsorption property in salt-independent way makes the tedious sample preparations could be avoided.

Cryogels, as a new generation of monolithic stationary phase, have several preferable attributes such as large pores, short diffusion path, low pressure drop, and very short residence time for both adsorption and elution. Since the pioneer work of the separation of microbial cells [6], cryogels have been evidenced to be very good alternative materials for direct purification of biomolecules from fermentation mixture [7,8] or crude cell homogenates [9,10]. Although the interconnected supermacropores within the matrix are necessary to capture the target biomolecules, they also lead to deteriorating the adsorption capacity [11–14]. To solve this problem, Savina et al. [15] and Yao et al. [16] prepared ion-exchange supermacroporous cryogels by in situ graft-polymerization, which could produce more binding sites for biomolecules on the graft-chains. Other groups prepared Fe\textsubscript{3}O\textsubscript{4} [17] or SiO\textsubscript{2} [18] nanoparticles, polymer beads [12] and bioparticles (Cu\textsuperscript{ii+}-attached sporepollenin particles) [19] embedded cryogels, respectively, which result the increase of surface areas within cryogels.

In our previous study, we found agarose beads embedded cryogels could also greatly improve the adsorption capacities for targeted biomolecules (published elsewhere [20]). As the most frequently used matrix, agarose beads embedded cryogels show the fascinating characteristics of high stability, biological inertness, and facile activation. Encouraging by these results, in this study, we prepared continuous supermacroporous agarose beads embedded agarose–chitosan composite monolithic cryogels (agarose–chitosan cryogels) by cryo-copolymerization.

2. Experimental

2.1. Materials

Agarose (Type V), DVS, IgG, bovine serum albumin (BSA) and lysozyme (Lys) were purchased from Aldrich (Milwaukee,
USA). Chitosan (MW: 590,000; degree of deacetylation, DDA: 80.0–95.0%), acetic acid, glutaraldehyde (GA, 50%), NaH2PO4, Na2CO3, NaOH, 2-MP and Coomassie Brilliant blue (R250) were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ethanolamine was obtained from Tianjin Chemical Reagent Plant (Tianjin, China). Na2HPO4 was purchased from Xi’an Chemical Reagent Plant (Xi’an, China).

2.2. Preparation of 2-MP covalent agarose–chitosan cryogels

2.2.1. Preparation of agarose beads

Agarose beads were prepared by an emulsion procedure [21]. Briefly, agarose was dissolved in boiling water to give a concentration of 6% and used as the water phase. A mixture of hexamethylenetetramine and carbon tetrachloride (v/v: 4:1) containing 0.3–4.0 wt.% of Span-85 or Tween emulsifier was used as the oil phase. The water phase was sprayed into ice cold oil phase under 0.5 MPa pressure of nitrogen gas at 60–65 °C by a stainless steel container with microporous nozzle form uniform W/O emulsion. The emulsion was kept cooled on stirring for 10 min. After settling for 1 h, the organic phase was discarded, and the water phase was collected and completely washed with water, 50% ethanol, and water, respectively. Then 10 μm uniformly sized agarose beads were screened.

2.2.2. Preparation of agarose–chitosan cryogels

Supermacroporous agarose–chitosan cryogels were prepared as follows [22,23]: agarose and chitosan (the weight of agarose to chitosan was 3:1) were dissolved in 30 mL 5% acetic acid to give a 4% (w/v) agarose/chitosan complex solution by stirring in the boiling water. After cooling the complex solution to 60 °C, 1 mL of 6% agarose beads were added into the solution with vigorous stirring. Then the crosslinker GA (final concentration, 0.07%, w/v) [24,25] was added when the mixed solution was cooled to 50 °C, the reaction mixture was stirred for 30 s. Then the solution was quickly poured into glass column (150 mm × 13 mm I.D.) with closed outlets, and frozen under variation of freezing-temperature as the reported [17]. Firstly, the mixture solution was frozen linearly from 50 °C to −20 °C in 2 h, then the freezing temperature was increased linearly from −20 to +5 °C in 1 h and maintained for 0.5 h. Consequently, the freezing temperature was re-decreased linearly from +5 to −20 °C in 1 h and maintained for 18 h. After that the frozen monolith within columns was defrosted without seal at 4 °C water bath, then the column was equipped with adaptors and connected to a pump and washed with water until the solution was neutralized. Supermacroporous non-beads embedded agarose–chitosan cryogels were prepared with the same procedure but without adding agarose beads.

2.2.3. Divinyl sulfone activated the agarose–chitosan cryogels

Functional vinyl-groups were coupled to the surface of agarose–chitosan cryogels by passing divinyl sulfone (DVS) (10% (v/v), 4 mL 1.0 M Na2CO3) solution for 4 h at room temperature, at a flow rate of 2 mL/min in recycling mode. Finally the activated cryogels were washed with deionized water until neutral. The number of vinyl groups in the DVS-activated cryogels were determined using the revised method of Pepper [26]. The DVS-activated cryogels were dried in an oven at 60 °C to a constant mass and cut into small pieces, then 1 g cryogel pieces were mixed with 5 mL 1.0 M sodium thiosulfate solution, and rotated for 24 h at room temperature. Subsequently, the supernatant was titrated with 0.1 M hydrochloric acid until pH 7.0 using phenolphthalein as an indicator. The amount of hydrochloric acid consumed was used to calculate the number of vinyl groups immobilized on the agarose–chitosan cryogels.

2.2.4. Covalent attachment of 2-MP for the divinyl sulfone activated agarose–chitosan cryogels

The DVS-activated agarose–chitosan cryogels were washed with 150 mL 0.5 M sodium carbonate solution, then the mixed solution of 4 mL 50 mM 2-MP (dissolved in 0.5 M NaOH) and 1 mL glycerol was passed though the composite cryogels in a recycling mode for 12 h at room temperature, at a flow rate of 2 mL/min. Finally the 2-MP covalent agarose–chitosan were washed with 100 mL deionized water. Quantification of immobilized 2-MP ligand was carried out according to the method of elemental analysis of sulfur using an infrared carbon–sulfur analyzer (Beijing, China).

2.3. Characterization of agarose–chitosan cryogels

2.3.1. The porosity test of the cryogels

The porosity (ϕ) value was estimated as the reported [17,19]. Briefly, a piece of cryogel sample was saturated with deionized water, and then immersed in water to yield volume V1. After that, the total volume of cylinder was measured as volume V2. Water-saturated cryogel volume V0 was calculated by the volume difference, i.e., V0 = V2 − V1. The mass of water-saturated cryogel, mw, was weighted. After squeezing the free water, the mass of the cryogel sample without free water, ms, was weighted. The porosity was calculated by the following formula:

\[
\phi = \frac{m_w - m_s}{\rho_w V_0}
\]

where ρw is the density of deionized water. Then the cryogel sample was dried in the oven at 60 °C to a constant mass (mD), and the total water fraction (TWF) was calculated by the following formula:

\[
TWF = \frac{m_w - m_d}{\rho_w V_0}
\]

Each measurement was carried out in triplicate.

2.3.2. The surface morphology of the cryogels

The surface morphology of agarose–chitosan cryogels was examined using scanning electron microscopy (SEM). The sample for SEM was prepared as the reported [27]. The sample was fixed in 2.5% glutaraldehyde for overnight. Then the sample was dehydrated at −50 °C in Christ Alpha 1-2 LD plus Lyophilizer (Osterode, Germany). Finally, it was coated with gold–palladium (40:60) and examined using a JEOL JSM 5600 scanning electron microscope (Tokyo, Japan).

The specific surface area (S, m²/mg) was estimated according to the adsorption of methylene blue based on the theory of single-layer molecular adsorption of MB on solid surface [28]. The cryogels were dried in an oven at 60 °C to a constant mass and cut into small pieces, and then 100 mg cryogel pieces were immersed into 50 mL MB standard solution with known concentration for 24 h at room temperature. Subsequently, the MB uptake onto the cryogels was calculated from the difference between the MB concentration before and after adsorption onto the cryogels. Five replications were carried out for the cryogels.

\[
S = \frac{(C_0 - C_{eq}) V_{MB}}{m_{cryogel} 2.45}
\]

where \(V_{MB}\) is the volume of added methylene blue solution (mL), \(m_{cryogel}\) is the weight of the sample cryogels (mL), \(C_0\) and \(C_{eq}\) are the initial and equilibrium concentrations of MB (mg/mL), respectively. The constant 2.45 (m²/mg of methylene blue) indicated that 1 mg of methylene blue could cover an area of 2.45 m².
2.4. Chromatography of IgG on 2-MP covalent agarose–chitosan cryogels

2.4.1. IgG separation from aqueous solution

The chromatographic processes were performed on an ÄKTA Prime Plus Chromatography System from GE Healthcare (Uppsala, Sweden). IgG (dissolved in running buffer, 25 mM pH 7.5 PBS) was used to elucidate the adsorption and elution behavior. Elution was performed with 20 mM NaOH. The eluted IgG contents were analyzed by the Bradford method using BSA as the standard protein. The composite cryogels were washed with 30 mL of water and then equilibrated with pH 7.5 25 mM PBS. Then, the prepared IgG solution was pumped through the column. The maximum adsorption capacity was estimated according to its adsorption isotherm. Effects of pH (6–10), flow rate (1.0–7.0 mL/min), and the concentration of IgG (0–2.5 mg/mL) on the adsorption capacity were studied.

The adsorption isotherm can be described by the Langmuir (4) and Freundlich isotherms models (5).

\[
q_{eq} = \frac{q_{max} m C_{eq}}{1 + m C_{eq}} \quad (4)
\]

\[
q_{eq} = K_f C_{eq}^{1/n} \quad (5)
\]

where \( q_{eq} \) is the equilibrium IgG concentration in the composite cryogels (mg IgG/g cryogel), \( C_{eq} \) is the equilibrium IgG concentration in solution (mg/mL), \( q_{max} \) and \( m \) are the maximum adsorption capacity (mg IgG/g cryogel) and the Langmuir adsorption equilibrium constant (mL/mg IgG), respectively, and \( K_f \) and \( n \) are the Freundlich adsorption constant (mg IgG/g cryogel) and the Freundlich exponent. According to the linearized form of equations (Eqs. (6) and (7)) and least-squares algorithms, the data about these models can be obtained.

\[
\frac{1}{q_{eq}} = \frac{1}{q_{max}} \frac{1}{m C_{eq}} + \frac{1}{q_{max}} \quad (6)
\]

\[
\ln q_{eq} = \ln K_f + \frac{1}{n} \ln C_{eq} \quad (7)
\]

The selectivity of 2-MP covalent agarose–chitosan cryogels were evaluated by using proteins, bovine serum albumin (BSA), lysozyme (Lys), ribonuclease (bovine pancreas) (RNase), albumin, trypsin, and papain, as model samples.

2.4.2. IgG separation from human plasma

Freshly frozen normal human plasma donated by healthy volunteers was a kind gift from the Shaanxi Maternal and Child Care Service Centre. 1 mL human plasma was diluted 10-fold with loading buffer solution (25 mM PBS, pH 7.5) and filtered through a 0.45 μm filter.

2-MP covalent coupled agarose–chitosan cryogels were equilibrated with pH 7.5 25 mM PBS. 2.0 mL of the human plasma sample solution was loaded onto the monolith, and then the monolith was washed with 25 mM pH 7.5 PBS until the protein absorption at 280 nm in the effluent reached the base line. Elution was performed with 20 mM NaOH.

2.4.3. Protein analysis

The protein contents were determined according to the method of Bradford [29] using BSA as the standard protein. Fractions eluted from the 2-MP covalent agarose–chitosan cryogels were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 12% acrylamide separating gel and 5% stacking gel containing 0.1% SDS. Protein samples were resolved at 120 V for 20 min followed by Coomassie Brilliant blue R250 staining.

3. Results and discussion

3.1. Properties of the agarose–chitosan cryogels

The SEM images of the internal structure of the agarose–chitosan cryogels were shown in Fig. 1. As showed in Fig. 1a, the composite cryogels had large continuous inter-connected pores (10–100 μm in diameter) with thin walls that provide channels for the mobile phase to flow through. The porosity, \( \psi \), and the total water content (TWC), of the agarose–chitosan cryogels were measured as 59.5% and 87.8% (v/v), respectively, which were lower than the non-beads embedded agarose–chitosan cryogels (\( \psi \), 65.2% and TWC, 88.4%). Resultantly, ca. 28.3% of the total water was bound by the small pores of the agarose–chitosan cryogels compared with only 23.2% in non-beads embedded agarose–chitosan
cryogels. The specific surface area for agarose–chitosan cryogels was found to be 350 m²/g cryogel, which was about three times as large as that of the non agarose–chitosan cryogels. This result indicated specific surface area of agarose–chitosan cryogels could be greatly increased because of the embedding of agarose beads.

The titration results demonstrated that the amount of available vinyl groups in the activated cryogels was approximately 54.1 μmol/mL cryogel. Moreover, infrared carbon–sulfur analyzer results shown that the 2-MP coupled on agarose–chitosan cryogels were approximately 40.1 μmol/mL cryogel, which suggested that more than 74% epoxide groups could be quantitatively converted into mercaptopropyidine groups in the cryogels matrix.

At the highest flow rate of 16 mL/min, the agarose–chitosan cryogels gave a backpressure of 0.2 MPa, compared with non-beads embedded agarose–chitosan cryogels with a backpressure of 0.2 MPa at their highest flow rate of 12 mL/min. This suggested that agarose–chitosan cryogels had higher mechanical strength compared with non-beads embedded agarose–chitosan cryogels.

### 3.2. IgG adsorption from aqueous solutions

As shown in Fig. 2, the pH value of the binding buffer had a great effect on the adsorption of IgG. In the examined pH range, the poorest adsorption was at pH 4.0, excellent adsorption capacities were observed from pH 5.0 to 7.5. Above pH 7.5, the adsorption capacities sharply decreased. Moreover, around pH 7.0, the IgG had the strongest biological activity and could be safely preserved without inactivation [29]. In this work, pH 7.5 PBS (25 mM) was used as the binding buffer.

The adsorption capacities of IgG on the 2-MP covalent agarose–chitosan cryogels at different flow rates were studied. As shown in Fig. 3, the adsorption capacity of IgG on the obtained cryogels was strongly affected by flow rate. The IgG adsorption capacity decreased significantly from 28.5 to 4.0 mg/g with the increase of the flow rate from 1.0 to 7.0 mL/min. This may due to the decrease in contact time between IgG and the matrix at higher flow rates, these results were in good agreement with those reported [27,30]. Moreover, the column pressure drop only increased slightly during the whole experiment, indicating the obtained cryogels could tolerate high flow rates as the existence supermacroporous within the composite cryogels.

In order to observe the effect of equilibrium IgG concentration on adsorption capacity, the equilibrium IgG concentration in the range of 0–2.5 mg/mL was investigated. The adsorption isotherm for IgG was shown in Fig. 4. As shown in Fig. 4, the equilibrium adsorption of IgG increased with increasing IgG concentration in buffer solution.

The Langmuir and Freundlich models were used for describing the adsorption actions between IgG and the obtained cryogels. The related data about Langmuir and Freundlich isotherms models were shown in Table 1. The data demonstrated that the experimental data tended to better fitted with Langmuir \((R^2 = 0.992)\) rather than Freundlich \((R^2 = 0.937)\) isotherm model, suggesting that the adsorption of IgG onto 2-MP covalent agarose–chitosan cryogels is a monolayer adsorption. The calculated maximum adsorption capacity was 71.4 mg/g.

The obtained maximum adsorption capacity for 2-MP covalent agarose–chitosan cryogels was 24.3 mg/g. This result could further demonstrated that the improvement of the adsorption capacity for 2-MP covalent agarose–chitosan cryogels was due to the embedded

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**Table 1**
The constant of Langmuir and Freundlich model.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Langmuir model</th>
<th>Freundlich model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(q_{\text{max}}) (mg/g)</td>
<td>(m) (mL/mg)</td>
</tr>
<tr>
<td>Composite cryogel</td>
<td>71.4</td>
<td>2.33</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Effect of pH on IgG adsorption capacity. Experimental conditions: binding buffer: 25 mM phosphate buffer; elution buffer: 20 mM NaOH; T: 25 °C; IgG concentration: 0.5 mg/mL; flow rate: 1 mL/min.

**Fig. 3.** Effect of flow rate on IgG adsorption capacity. Experimental conditions: binding buffer: 20 mM phosphate buffer; elution buffer: 20 mM NaOH; T: 25 °C; IgG concentration: 0.5 mg/mL; pH: 7.5.

**Fig. 4.** Effect of IgG equilibrium concentration on IgG adsorption capacity. Experimental conditions: binding buffer: 25 mM phosphate buffer; elution buffer: 20 mM NaOH; T: 25 °C; pH: 7.5; flow rate: 1 mL/min.
of agarose beads within cryogels. Excitingly, the obtained adsorption capacity was higher compared to other reported cryogels, in which the maximum capacity was only 25.6 mg/g [31].

### 3.3. Selectivity of the obtained cryogels

To study the selectivity of the interaction of 2-MP covalent agarose–chitosan cryogels with human IgG, some proteins such as BSA, Lys, Ribonuclease (bovine pancreas) (RNase), albumin, trypsin and papain, were injected into 2-MP covalent agarose–chitosan cryogels and eluted under the elution conditions studied (the data were not showed). The results showed that these proteins were not retained on the cryogels and the obtained materials exhibited no any non-specific bindings for these proteins with their concentrations less than 0.5 mg/mL.

### 3.4. Reusability and reproducibility

To investigate the reusability of the same 2-MP covalent agarose–chitosan cryogels monolith, the adsorption–desorption cycle was repeated 15 times using the same monolithic cryogel. The results (Fig. 5) showed that the 2-MP covalent agarose–chitosan cryogels could be used at least 15 times without significant decrease in the adsorption amount of IgG. The reproducibility of the supermacroporous 2-MP covalent agarose–chitosan was evaluated by measuring the RSDs of the maximum capacity of IgG for the five 2-MP covalent agarose–chitosan cryogels monoliths from the same batch and six different batches. As showed in Table 2, reasonable RSDs for column to column reproducibility (<7.0%) and batch-to-batch reproducibility (<9.3%) were obtained, which showed the preparation of 2-MP covalent agarose–chitosan cryogels were highly reproducible.

![Fig. 5. Reusability of the obtained cryogels. Experimental conditions: binding buffer: 25 mM phosphate buffer; elution buffer: 20 mM NaOH; T: 25 °C; pH: 7.5; flow rate: 1 mL/min; IgG concentration: 0.5 mg/mL.](
![Fig. 5. Reusability of the obtained cryogels. Experimental conditions: binding buffer: 25 mM phosphate buffer; elution buffer: 20 mM NaOH; T: 25 °C; pH: 7.5; flow rate: 1 mL/min; IgG concentration: 0.5 mg/mL.]

### 3.5. Purification of IgG from human plasma with 2-MP covalent agarose–chitosan cryogels

The purification of IgG from diluted human plasma was achieved with 2-MP covalent agarose–chitosan cryogels system (Fig. 6). The purity of IgG (160 kDa) eluted from the system was confirmed by SDS-PAGE under reducing conditions (Fig. 6 lane 4). The obtained IgG had high purity since only two bands corresponding to the heavy (55 kDa) and light (25 kDa) chains of IgG were present. Moreover, the average recovery of the purified IgG was 90% (Table 3). These results suggested that when coupled with 2-MP the agarose beads embedded cryogels would be efficient materials for the purification of IgG from human plasma.

### 4. Conclusion

In this study, agarose–chitosan cryogels were prepared by cryo-copolymerization under freezing temperature. After coupling 2-MP ligand on vinyl groups activated agarose–chitosan cryogels, the cryogels possess specific surface area as high as 350 m²/g and the maximum adsorption capacity of IgG was 71.4 mg/g. The cryogels could be reused many times without significant decrease in

### Table 2
The reproducibility of column-to-column and batch-to-batch.

<table>
<thead>
<tr>
<th></th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
<th>Batch 4</th>
<th>Batch 5</th>
<th>Batch 6</th>
<th>RSD (%)</th>
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<td>64.2</td>
<td>5.8</td>
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<td>69.9</td>
<td>67.4</td>
<td>63.5</td>
<td>71.9</td>
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</tr>
<tr>
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<td>63.2</td>
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<td>64.6</td>
<td>68.5</td>
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<tr>
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<td>4.7</td>
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<td>71.9</td>
<td>68.5</td>
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<td>61.0</td>
<td>74.8</td>
<td>9.3</td>
</tr>
<tr>
<td>RSD (%)</td>
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<td>6.0</td>
<td>–</td>
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</table>

### Table 3
IgG recovery.

<table>
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<tr>
<th>Loaded volume (mL)</th>
<th>Concentration (mg/mL)</th>
<th>Protein recovered (mg)</th>
<th>Average recovery yield (%)</th>
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<tr>
<td>2</td>
<td>0.5</td>
<td>0.9</td>
<td>90.0</td>
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<td>2</td>
<td>0.75</td>
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<td>1.0</td>
<td>1.82</td>
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</table>

* Values are the average of triplicate runs.
its adsorption capacity. Compared with the previous non-bead embedded cryogels, the obtained cryogels had higher mechanical strength and greater adsorption capacity.

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