Towards rational design of porous nanostructured biopolymeric microparticles for biomacromolecules separation: A case study of intraparticle diffusion facilitation and BSA adsorption on agarose microspheres

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\textbf{ABSTRACT}

Synthesis and employing advanced materials for emerging applications is of great challenge for the scientific community. Recombinant proteins production and purification is one of the fastest growing fields in the global economy. In this regard, it is essential to fabricate biocompatible low-cost materials with high specificity to enhance purification efficiency. This requires the regulation of mass transfer based on the protein molecular size and interactions with the matrix interface; thus, needs synthesizing novel materials with tuned porosity.

In this study, we proposed rational alteration in porous structure of biopolymeric microspheres using appropriate-sized porogen to facilitate intraparticle molecular diffusion. The tailored porous nanostructures, which were generated by phase separation in the polymer blend of agarose and polyethylene glycol, were analyzed with optical and scanning electron microscopy, Fourier transform infrared spectroscopy, water diffusion, and albumin adsorption. The well-tuned beads possessed highly porous structures with dominant mesopores owing to PEG phase migration out of the network. The high speed homogenizer caused an uncommon dense morphology with interwoven two-type porosity. Optimally tuned mesoporous beads with considerably high specific surface area exhibited dramatically fast and enhanced intraparticle diffusion of both water and protein molecules. Thus, the introduced porosity modification is a promising design for enhancing mass transfer in the bioseparation process. Finally, useful insights for developing future smart hydrogel microparticles with tuned porous network for biomolecules purification are provided by the conducted experiments.

1. Introduction

A broad range of applications for hydrogel biopolymers is introduced and explored in the literature [1]. Practically, the most important issue for improving the efficiency of these materials is tuning their porous nanostructure more nearly commensurate with the geometry of the specimens (films, macro/microparticles), and the application in which they will be used [2,3]. Their porous structures, especially surface area and pore size (macro- (> 50 nm), meso- (50–2 nm), and microporous (< 2 nm)) are distinctive features which determine their application that vary from molecular recognition to affinity chromatography [4].

A well-known hydrogel biopolymer used in affinity chromatography for biomolecules separation is agarose [5]. This linear polymer is made up of helices and their aggregates (long fibers) consisting repeating units of agarobiose. The long fibers join together and form a three-dimensional (3D) network [6,7] Agarose microparticles’ 3D structure and chemical stability have made them qualified matrices as packing in chromatography columns during the last 70 years [8]. These beads are synthesized by several methods including microfluidic assay, membrane emulsification technique, spray gelation, or stirring-induced emulsification of agarose solution in oil phase [9,10].

The common agarose beads’ drawbacks such as low porosity and mechanical strength have urged the researchers to study new methods of fabrication through which improved 3D network characteristics are obtained [9,11]. Moreover, since the intraparticle proteins diffusion controls the protein binding capacity of agarose beads [12], modifying the nanostructure of the hydrogel, especially pore size distribution and surface area, as a tool for regulating molecular diffusion is required for efficient proteins separation [12–14]. Previous studies have suggested...
variety of methods for increasing the hydrogel network porosity. Parameters such as stirring speed, cross-link density, polymer content, aqueous phase ionic strength, quenching temperature beside the amount and type of porogen (microbubbles, salts, polymers, etc.) play prominent roles in porosity alteration [9,11,15–21]. Among all, utilizing porogenic reagents has been proven to be the most influencing parameter on porosity modification [13,18,21].

An optimal porous structure is achieved for an agarose bead when the porosity and the specific surface area are tuned commensurate with the penetrant biomacromolecules’ size [22,23]. For this purpose, two criteria should be considered: (i) enhancing intraparticle molecular diffusion which requires enlarging the pore widths, and (ii) increasing the surface area available for protein adsorption which is provided by small pores [9]. However, these criteria are in conflict with each other. Therefore, an optimally increased pore size with the least decrease in the surface area is needed. Hence, in a compromise between large surface area and pore size, mesopores are the appropriate choice [9,13,24]. Additionally, since most of the proteins have diameters smaller than 20 nm [25], mesopores can be large enough to allow proteins diffusion. Previously, we reported maximum protein separation by utilizing mesoporous magnetic agarose beads which possessed 2–20 nm diameter pores in dry state [27].

However, many of the introduced pore-forming agents in the literature have resulted to micro and/or macro pores formation [9,11,16,18,26]. Thus, in order to obtain mesoporous structure, it is essential to use proper pore forming agent. Among porogens, polyethylene glycol (PEG), as a quite biocompatible polymer, is successfully used for uniform round shape pore formation in hydrogel matrix [28,29]. Contrary to long chained PEGs that migrate difficulty out of the particles [30], low molecular weight PEGs can effectively leave the polymer blend and form micro- and mesopores [13,29]. Thus, employing such polymers with proper length can serve the purpose of making mesoporous agarose particles.

In addition to a well-tuned porosity, small particle sizes are favorable for common chromatography applications [4]. Increasing the stirring speed in water in oil (W/O) emulsion method is a common approach for making small sized agarose beads [11]. However, changing the mixing regime can lead to porosity alterations [20].

The current study explores rational tailoring of the porous nanostucture of agarose microspheres for the purpose of facilitating intraparticle molecular diffusion while keeping the specific surface area high. Agarose-based beads are synthesized in a W/O emulsion process followed by a phase separation stage. Tailored porous nanostructures are achieved as a consequence of the low molecular weight PEG removal from the hydrogel blend. In order to obtain uniform particle size distribution, a stirrer and a high speed homogenizer are employed. After characterizing the beads, we study in detail the effects of PEG removal and stirring speed on beads and nanostructures. Furthermore, since the intraparticle water penetration is a reliable criterion for investigating the diffusion permeation of hydrogel beads [14,31], water molecular diffusion kinetics and mechanism are evaluated and discussed. Finally, adsorption kinetics of Bovine Serum Albumin (BSA), as the model protein, on the fabricated beads was studied to understand the applicability of our introduced porosity modification.

The resulting agarose microparticles are low-cost tunable nanostructured and optimally pore sized materials that permit rapid mass transfer of biomacromolecules.

2. Experiments

2.1. Materials

Agarose powder (biotechnology grade) was purchased from Medicago (Sweden). Analytical PEG (molecular weight of 200 g/gmol, PEG200), and calcium chloride (CaCl2) were purchased from Merck (Germany), and ethanol was brought from Mojallali reagent chemicals Co. (Iran). Corn oil (food grade) was purchased from a local market, and used as received. Distilled water (DW) was used throughout the work.

2.2. Bead fabrication

Agarose (6 wt%) beads were prepared similar to our previous work [27]. For polymer blends (Agars-PEG), 1.8 g of each polymer was dissolved in 30 ml of 200 mM CaCl2 solution under stirring at 150 rpm and 85°C. The mixture was added to 150 ml hot oil phase (95°C) under two distinct continuous stirring regimes utilizing an agitator (500 rpm), and a rotor-stator homogenizer (5000 rpm) (SilentCrusher M, Heidelberg, Germany). Well dispersed aqueous droplets were cooled to form microparticles. Afterward, for quick removal of oil, beads were washed with 10 volumes of DW. Beads were filtered using 20 μm stainless steel mesh filters. Finally, beads were washed with 20% ethanol. Agarose-PEG beads were mildly stirred for 24 h in 20% ethanol for PEG removal. Porogen molecules migrated out of the agarose matrix since PEG200 is a water soluble liquid polymer which does not chemically react with agarose chains [32].

In brief, the characteristics of four bead types are discussed here, including: Agars-S (6%-agarose at low stirring speed), Agars-H (6%-agarose at high stirring speed), Agars-PEG-S (6%-agarose-PEG200 at low stirring speed), and Agars-PEG-H (6%-agarose-PEG200 at high stirring speed).

2.3. Physicochemical characterization

2.3.1. Morphology, particle and pore size distributions

The microparticles shape was observed under an inverse stereo microscope. After determining the shape factors of the microparticles by analyzing the microscopic images [33], particle size distribution (PSD) of the wet samples was measured using a particle size analyzer (HELOS/RF, SYMPATEC©) as explained in [33]. Then, a fraction of 150–400 μm diameter of each bead sample was meshed, freeze-dried, and used for further investigations. Bound and unbound liquid (water and ethanol) in hydrogel network was evaporated during freeze drying process following the same procedure as reported in [11]. Fourier transform infrared (FTIR) spectrophotometer (PerkinElmer Spectrum Gx, Perkin-Elmer©) was used to study the chemical structure of the freeze-dried beads. Morphological surface evaluation of the freeze-dried beads was conducted using a Mira 3 TESCAN XMU field emission scanning electron microscope (FE-SEM) at low voltages (4–15 kV).

Furthermore, porosimetry analysis (pore size distribution, pore volume, specific surface area) of the freeze-dried samples was performed using an accelerated surface area and porosimetry (ASAP) 2020-M micropore physisorption analyzer (Micromeritics©). Nitrogen adsorption-desorption isotherms were measured at 77 K. Pore size distributions of the microparticles were calculated from the adsorption branches of the isotherms using Barrett–Joyner–Halanda (BJH) model. Also, the specific surface areas and pore volumes were calculated by Brunauer–Emmett–Teller (BET) (total pore volume measurement at a relative pressure P/P0 of 0.95) and BJH (for pore width between 1.7 and 300 nm) methods.

2.3.2. Intraparticle molecular diffusion

To investigate the intraparticle molecular diffusion, a plate containing distilled water was placed under an inverse stereo microscope. Afterwards, a small amount of freeze-dried microparticles was immersed in water by placement in the plate using a stainless steel needle. Imaging was started immediately after particles placement in water. At regular time intervals, microscopic images of the swollen samples were taken. Each experiment was performed in triplicates or more at room temperature. Average initial diameters of the dried particles (average of all of the replicates) were 132.8, 75.5, 65.9, and 57.8 μm, respectively.
for Agars-S, Agars-PEG-S, Agars-H, and Agars-PEG-H. At the end, images were processed using ImageJ software to measure the radius of spherical beads. Image analysis was continued until average radii of swollen beads reached to a constant. The swelling capacity (water absorption) was defined as [34]:

\[
\text{Swelling capacity (\%)} = \left( \frac{V_t - V_0}{V_0} \right) \times 100\% 
\]

(1)

where \(V_t (\mu m^3)\) is the average volume of the swollen beads at time \(t\), and \(V_0 (\mu m^3)\) is the initial average volume of the dry beads.

The equilibrium water absorption \(S_{eq} \) was measured by [35]:

\[
S_{eq} (\%) = \left( \frac{V_{eq} - V_0}{V_0} \right) \times 100\%
\]

(2)

where \(V_{eq} (\mu m^3)\) is the average volume of the swollen beads at equilibrium.

2.3.3. Protein adsorption

For further investigation of the applicability of our introduced porosity modification, adsorption kinetics of Bovine Serum Albumin (BSA), as the model protein, was determined by adsorption experiments. For this purpose, fabricated beads were functionalized with an affinity dye ligand, Cibacron Blue F3GA (CB), using the same procedure in our previous study [27].

Protein adsorption experiments were conducted in a 96-well Terasaki plate, each well containing 16 mg of drained beads. To equilibrate the beads, 200 μl of equilibration buffer (0.1 M phosphate buffer pH = 5.6) was added to each of the wells. Beads were allowed to settle by interchange of the droplets breakup and coalescence [36]. Based on the statistical analysis, presence of PEG200 in aqueous phase had no significant effect on PSD of the beads, while homogenizer had an almost significant effect (p-value = 0.09).

The FTIR spectra of the fabricated beads and PEG200 are shown in Fig. 2. In the bead's spectra (a-d), the wide peaks at \(~3450 \text{ cm}^{-1}\) belong to the stretching vibration of O–H groups, and the peaks at almost 2950 cm\(^{-1}\) and 2850 cm\(^{-1}\) are assigned to the stretching vibration of C–H groups. Also, the peaks around 1065 cm\(^{-1}\) reveal the C–O stretching vibration from the primary alcohol in agarose [38,39]. Generally, carbohydrates show their characteristic bands and the C–O and C–C groups vibration in the wavenumber range of 1145 cm\(^{-1}\) to 554 cm\(^{-1}\) [40].

In addition, the PEG200 spectrum (Fig. 2(e)) showed bands at 2874.2 cm\(^{-1}\) and 1103.9 cm\(^{-1}\) which are assigned to the C–H and C–O–C groups, respectively [41]. This characteristic peak is not seen in the spectra of polymer blended beads (Agars-PEG-S and Agars-PEG-H) (Fig. 2(b) and (d)). Since no obvious shift of the bands or new peaks is observed, it can be concluded that the native chemical structure of the agarose beads is almost maintained and the remained PEG200 in the polymer blend is negligible.

To evaluate the surface morphology of the beads, FE-SEM images were analyzed. Fig. 3 shows the presence of different pore sizes on the surface of the beaded samples. Based on Fig. 3(a, b), it is seen that simple agarose beads (Agars-S) are almost nonporous. According to Fig. 3(d), image large pores corroborate the migration of PEG200 out of the polymer blend of Agars-PEG-S beads. Mesoporous structure and the large porogen-formed pores are clearly seen. As expected, the remaining PEG200 upon polymerization can dissolve in water and diffuse out of the agarose network due to its small size and linear chains.

Based on Fig. 3(e–h), homogenizer-synthesized beads have relatively rough surfaces with a multitude number of peaks and valleys. This distinct morphology is observed after PEG removal too; however, with deeper valleys. Apparently, homogenizer alters the nanostructure of the beads as speculated. This nano-structural change can be attributed to two reasons. First, the high shear mixing regime which is applied upon homogenization forms an O/W/O microemulsion. Very tiny oil droplets are almost homogeneously encapsulated within the aqueous dispersed microdroplets containing dissolved polymer. The oil micro-droplets leave the polymer beads, playing the role of nonsolvent porogen [4]. Consequently, rough interlinked porous network is formed as a result of aggregated polymer chains. In the final bead network macro pores are predominant [4]. Some of the macro pores are visible in the optical microscopic images of homogenizer-synthesized beads (Fig. 1(c,d)). Corstens et al. reported precise data from X-ray tomography of alginate beads’ 3D image, showing the macro pores generation in O/W/O microemulsion by a rotor-stator homogenizer [42].

As explained previously, agarose gelation occurs in a two-step process: 1 - double-helices formation from agarose chains, 2 - self-assembly of the double-helices and formation of physical aggregates known as microcrystalline junctions. These cross-links which are held together by hydrogen bonds form the agarose 3D structure. Thus, the population and distribution of these junctions affect the hydrogel physicochemical properties and nanostructure [43]. The more the number of junction zones, the less the network mesh size, \(\xi\), (average distance between the cross-links) [1]. On the other hand, applying high shear mixing methods leads to generation of higher cross-linked
hydrogel specimens [44]. For this reason, as the second cause of the nano-structural change, homogenizer-synthesized beads possess more junction zones in comparison with stirrer-synthesized samples. Consequently, it is hypothesized that the former has a denser structure, and therefore rough and dense facial morphology is observed.

In the case of using nonsolvent porogens, phase separation occurs before the gelation point. On the other hand, PEG200 molecules leave the particles after gelation i.e. after the gel network skeleton is formed with its dense structure and more junction zones [4]. Thus, considering these two phenomena for Agars-PEG-H beads, it is expected that PEG200 molecules cannot alter the agarose chains aggregation, contrary to their effect on changing chain placement, and/or distances on their way outward the agarose particles. Hence, in SEM images, Agars-PEG-H beads show similar surface morphology with Agars-H beads, except for deeper valleys and sharper peaks which are caused by PEG200 removal.

In order to have a quantitative insight of porosities and pore size distributions of the prepared beads, ASAP test (as a quite reliable and most straightforward porosimetry method [4]) was performed. Figs. 4–6 represent the obtained porosimetry data. The nitrogen adsorption-desorption isotherms of the four beaded agarose samples are shown in Fig. 4. The very low adsorption capacity of the unmodified beads (Agars-S) shows these beads are nonporous or slightly microporous. The hysteresis loop for Agars-S at higher P/P₀ values indicates some mesoporosity caused by interparticle adsorption.

The sorption isotherms of homogenizer-synthesized beads seem closest to a combination of two IUPAC classification types II and IV isotherms with an H3 hysteresis loop which is a characteristic of a macroporous material with plate-like aggregates in their porous structure [45]. The hysteresis loop for Agars-PEG-H beads is closed in 0.5 < P/P₀ < 0.6 which shows some mesoporosity as a result of PEG200 porogen removal [46]. Agars-PEG-S sorption-desorption plot exhibits noticeable mesoporosity as demonstrated by its hybrid type I and type IV isotherms and type H4 hysteresis loops, indicating a mesoporous structure [45]. Based on the literature, carbohydrate hydrogels such as agarose particles consist of interconnected cylindrical pores [9,47]. Also, when appropriate PEGs are used as porogens, carbohydrate hydrogels forms large cylindrical meso- or macropores [28]. Considering the SEM images and N₂ isotherm data of Agars-PEG-S beads besides the literature, it is inferred that these beads possess interconnected cylindrical micro- and mesoporous networks.

Furthermore, Fig. 5 represents the pore size distributions of the beads, from adsorption branches by BJH model. As seen in Figs. 4 and 5, pore sizes of modified agarose beads are increased dramatically. Unmodified beads possess a very small number of inherent micropores...
Therefore, they are counted as nonporous. On the other hand, homogenizer-synthesized samples have uniformly distributed meso- and macro-sized pores (Fig. 5(c, d)) due to the microemulsion formation. Moreover, after PEG removal the number of mesopores in homogenizer-synthesized beads of Agars-PEG-H is increased (Fig. 5(d)). Also, mesopores are the dominant pores in Agars-PEG-S beads due to PEG200 removal.

The BET porosimetry data for total porosities and total surface areas are presented in Table 1. The total pore volume of unmodified agarose beads is too small (< 5mm³/g) that can be neglected. However, utilizing PEG200 has an effective impact on increasing the pore volume of Agars-PEG-S beads (25.6 ± 1.0mm³/g) to 8 times that of Agars-S beads. In addition, the total pore volume of homogenizer-synthesized beads is increased to 26.0 ± 2.0 and 29.5 ± 2.0mm³/g in Agars-H and Agars-PEG-H samples, respectively. Results of comparing the mean values of the total porosities are represented in Table 1 by letters A to C. Consequently, the total pore volumes of the three modified beads significantly differ from that of Agars-S beads (p-value < 0.05).

In addition to the total pore volumes, Fig. 6 shows the pore volumes and the specific surface areas by pore types of each beaded sample. Though the total pore volumes of the homogenizer-synthesized and Agars-PEG-S beads are close in amount (Table 1), the macropore volume of the former is more than the latter (Fig. 6(a)). In other words, migration of PEG200 molecules out of the agarose network results in mesopores generation.

Fig. 5(c, d) shows the pore volumes and the specific surface areas by pore types of each beaded sample. Though the total pore volumes of the homogenizer-synthesized and Agars-PEG-S beads are close in amount (Table 1), the macropore volume of the former is more than the latter (Fig. 5(a)). In other words, migration of PEG200 molecules out of the agarose network results in mesopores generation.

As seen in Fig. 5(b), the simple agarose beads’ BJH surface area is quite negligible. Contrary, tailoring the nanostructure of the agarose beads resulted in raising the BJH surface areas of Agars-PEG-S (24.68 ± 1.23m²/g), Agars-H (10.61 ± 0.66m²/g), and Agars-PEG-H (19.37 ± 0.96m²/g) beads. Fig. 5(b) shows that the mesopores provide
Fig. 4. N₂ at 77 K physisorption isotherms of the synthesized Agars-S, Agars-PEG-S, Agars-H, and Agars-PEG-H beads.

Fig. 5. Pore size distributions from adsorption branches by BJH for the synthesized (a) Agars-S, (b) Agars-PEG-S, (c) Agars-H, and (d) Agars-PEG-H beads.
higher specific surface areas. Thus, porogen-tuned mesoporous structure of Agars-PEG-S and Agars-PEG-H beads record the highest specific surface areas. However, since increasing in the number of macropores leads to decreasing the specific surface area [48], the specific surface area of Agars-PEG-H beads is less than Agars-PEG-S ones.

Additionally, Table 1 shows the BET surface areas of the samples. Statistical analysis showed significant difference between the BET surface areas of the four samples (p-value < 0.05). Comparing the main effects, it is confirmed that PEG200 and homogenizer respectively have positive and negative effects on BET surface areas. It is worthy to note that while both homogenizer and PEG200 have significant effects on beads porosity, the effect of former (more agarose chains aggregation) on the nanostructure of the homogenizer-synthesized beads is larger than the latter's as described previously.

Results of comparing the mean values of the BET surface areas are represented in Table 1 by letters A to D. The BET surface area of Agars-PEG-S sample is significantly more than the other three samples. Accordingly, represented data confirmed the success of utilizing PEG200 as porogen for designing mesoporous agarose beads.

### 3.2. Intraparticle water diffusion

#### 3.2.1. Water absorption capacity

In order to evaluate the effectiveness of tailored mesoporous nanostructure in enhancing intraparticle molecular diffusion, the water absorption capacity (swelling capacity) of the beads was studied.

The swelling capacity of agarose beads through time is represented in Fig. 7. Generally, the hydrogel beads swell by absorbing water till reaching the equilibrium water absorption ($S_{eq}$). As seen in Fig. 7, the equilibrium water absorption of unmodified beads is too small and does not exceed 120 ± 12 (%), whereas Agars-PEG-S beads show a much higher water absorption capacity ($S_{eq}$ = 655 ± 11 (%)). Also, Agars-H and Agars-PEG-H beads equilibrium water absorptions are 282 ± 21 (%) and 330 % ± 25 (%), respectively. Statistical analysis shows significant difference between the equilibrium water absorption of Agars-PEG-S beads and the other three bead types (p-value < 0.05). Also, the equilibrium water absorptions of homogenizer-synthesized beads significantly differ from the other two samples.

Considering porosimetry results, it can be concluded that increasing the pore volumes of the beads has led to rise in water absorption capacity of the three modified samples. Low porosity of the beads delays intraparticle molecular diffusion [49]. Also, Gharekhani et al. [50] reported same results on increase in equilibrium water absorption due to higher porosity. However, higher pore volume is not the only influential parameter on the beads swelling capacity. Indeed, the specific surface area can be more effective in enhancing water absorption [50]. Therefore, Agars-PEG-S beads showed the highest equilibrium water absorption since these mesoporous beads possess the highest specific surface area (Fig. 6(b)).

Moreover, homogenizer-synthesized beads show remarkably smaller water absorption capacities than Agars-PEG-S beads. Considering these beads' morphology (Fig. 3), it can be deduced that homogenizer causes dense nanostructures with meso- and macro-porosity and relatively lower specific surface area. The lower specific surface area hinders the hydrogel network to absorb water and swell freely. Maleki et al. also reported a declined equilibrium swelling for a hydrogel structure with dense interconnected network [51].

In our previous study we found that the maximum protein adsorption capacity of mesoporous agarose beads (packed in columns) with pore width < 20 nm was strikingly higher than the beads possessing larger mesopores for Bovine Serum Albumin (BSA) [27]. Interestingly, in the current study utilizing PEG200 as porogen in agarose beads led to the maximum swelling capacity due to optimal mesoporosity (2–20 nm). It is believed that pores are opened in the swollen state. Thus, pores with 20 nm diameter reach to appropriate sizes which allow the proteins to diffuse within the mesoporous network, while keeping the specific surface area high. The applied design successfully provided high specific surface area and water absorption capacity.

### Table 1

Brunauer–Emmett–Teller (BET) porosimetry results. The same letters (A–D) in column show that their corresponding data have no significant difference (p-value > 0.05).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total pore volume (mm$^3$/g)</th>
<th>BET surface area (m$^2$/g)</th>
<th>Average pore diameter (nm)</th>
</tr>
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<tr>
<td>Agars-S</td>
<td>&lt; 5.0$^a$</td>
<td>0.20 ± 0.20$^a$</td>
<td>3.0 ± 0.1$^a$</td>
</tr>
<tr>
<td>Agars-PEG-S</td>
<td>25.6 ± 1.0$^a$</td>
<td>19.16 ± 0.33$^b$</td>
<td>4.8 ± 0.1$^b$</td>
</tr>
<tr>
<td>Agars-H</td>
<td>26.0 ± 2.0$^a$</td>
<td>4.14 ± 0.30$^c$</td>
<td>15.2 ± 1.3$^c$</td>
</tr>
<tr>
<td>Agars-PEG-H</td>
<td>29.5 ± 2.0$^a$</td>
<td>7.81 ± 2.03$^b$</td>
<td>14.5 ± 0.2$^c$</td>
</tr>
</tbody>
</table>

Fig. 6. (a) Pore volumes, and (b) specific surface areas of the synthesized beads by pore types (micro-, meso-, and macro pores). Unmodified agarose beads (Agars-S) are nonporous. Homogenizer-synthesized beads (Agars-H & Agars-PEG-H) have porosities with meso- and macropores. PEG200 removal leads to mesopore formation and specific surface area elevation (Agars-PEG-H & Agars-PEG-S). The dominant mesopores of Agars-PEG-S microbeads provide the highest specific surface area. Though the total pore volume (sum of the bars of panel a for each sample) of the modified samples are almost similar, much mesoporous samples (Agars-PEG-H & Agars-PEG-S) have higher specific surface areas.
3.2.2. Swelling kinetics

In order to assess the structural impact of agarose beads on water molecule diffusion, swelling kinetics of the four bead types were evaluated by regression analysis of swelling data. It was found that pseudo-second order kinetic model had the best compliance with the experimental data. The model is described as:

\[ \frac{t}{S_t} = A + Bt \]  

where \( S_t (\mu m^3 \cdot \mu m^{-3}) \) is the average water absorption of the swollen beads at time \( t \) (min), \( A \) parameter (\( \mu m^3 \cdot \min \cdot \mu m^{-3} \)) is regarded to the reciprocal hydrogel initial swelling rate \( \left( \frac{dS}{dt} \right)_{t=0} \), and \( B \) parameter (\( \mu m^3 \cdot \mu m^{-3} \)) attributes to the inverse of theoretical equilibrium water absorption capacity \( \left( \frac{1}{S_{eq,\text{cal}}} \right) \) [44].

Table 2 shows the regression data for Eq (1). Higher amounts of parameter \( A \) represents lower swelling rate of the beads. As reported in Table 2, Agars-H beads showed the highest parameter \( A \) (0.1 \( \times 10^{-4} \mu m^3 \cdot \min \cdot \mu m^{-3} \)). Thus, it is inferred that Agars-H sample had the slowest swelling rate. This can be explained by discussing the mesh size of the networks \( \xi \). As explained previously, high shear mixing regime upon homogenization results in formation of more self-assembled agarose chain aggregates and junction zones. This phenomenon reduces \( \xi \) [44]. Consequently, homogenizer-synthesized beads possess lower mesh sizes besides dense structures. This characteristic reduced the swelling rate of Agars-H.

It is observed that Agars-H and Agars-PEG-H beads showed similar equilibrium water absorption. However, the latter showed relatively facilitated intraparticle molecular diffusion mechanism (higher swelling rate). This behavior can be attributed to two reasons. First, Agars-PEG-H is slightly much mesoporous with higher specific surface area. Similar result for facilitating molecular diffusion rate as a function of increasing specific surface area is reported by Chen et al. [52]. Second, moving PEG200 chains towards the outer space of the beads can force the agarose chains to displace and widen the mesh size of the network. Consequently, the swelling rate of PEG200-tuned nanostructure of Agars-PEG-H beads was not declined significantly.

3.2.3. Intraparticle diffusion mechanism

In order to find out the intraparticle water diffusion mechanism, the swelling power number \( (n) \) was calculated based on fitting Eq. (4) on the first 60% of the equilibrium swelling data.

\[ \frac{V_t}{V_{eq}} = k\cdot t^n \]  

where \( V_t (\mu m^3) \) is the average volume of the swollen beads at time \( t \) (min), \( V_{eq} (\mu m^3) \) is the average volume of the swollen beads at equilibrium, \( k \) is a constant, and \( n \) is the diffusional exponent characteristic of the swelling mechanism [53]. The values of \( n \) and \( k \) were calculated from the slope and intercept of \( \ln(V_t/V_{eq}) \) versus \( \ln(t) \) plot. Results are shown in Table 2.

The diffusional coefficient \( n \) gives us valuable information about the physical mechanism controlling water uptake of the beads. For

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Swelling kinetics</th>
<th>Swelling kinetics Parameters</th>
<th>Diffusion Mechanism Parameters ( (V_t/V_{eq} \leq 0.6) )</th>
<th>Diffusion Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A (( \mu m^3 \cdot \min \cdot \mu m^{-3} ))</td>
<td>B (( \mu m^3 \cdot \mu m^{-3} ))</td>
<td>R²</td>
</tr>
<tr>
<td>Agars-S</td>
<td>Pseudo-second</td>
<td>0.2 ( \times 10^{-5} )</td>
<td>0.6 ( \times 10^{-6} )</td>
<td>0.98</td>
</tr>
<tr>
<td>Agars-PEG-S</td>
<td>Pseudo-second</td>
<td>0.2 ( \times 10^{-5} )</td>
<td>0.6 ( \times 10^{-6} )</td>
<td>0.98</td>
</tr>
<tr>
<td>Agars-H</td>
<td>Pseudo-second</td>
<td>0.1 ( \times 10^{-4} )</td>
<td>0.6 ( \times 10^{-5} )</td>
<td>0.997</td>
</tr>
<tr>
<td>Agars-PEG-H</td>
<td>Pseudo-second</td>
<td>0.1 ( \times 10^{-5} )</td>
<td>0.6 ( \times 10^{-6} )</td>
<td>0.98</td>
</tr>
</tbody>
</table>
spherical particles, n = 0.43 indicates Fickian diffusion, 0.43 < n < 0.85 shows anomalous transport, and n ≥ 0.85 implies case II (relaxation-controlled) transport [54]. When water molecules can penetrate and transport freely within the hydrogel network, without network limitations affecting the transport, Fickian diffusion mechanism controls the mass transfer. In contrast, case II transport occurs when the polymer chains restrict the molecular transport within the hydrogel network. In these hydrogels cross-links, make the pores as a function of the network mesh size (ξ). Therefore, these materials lack penetrating pores, and thus polymer chains must move to allow penetrants diffuse within the network. As a result, slow intraparticle diffusion rate controlled by polymer chain relaxation is observed. In case of anomalous transport, both the molecular diffusion and polymer relaxation rates control water uptake of the hydrogel [55].

Based on the results (Table 2), it is seen that the diffusional coefficients of Agars-S and Agars-H beads are larger than 1.0. In other words, the hydrogel network of these two bead types restrict the water molecules transport into the network. Since both Agars-S and Agars-H beads suffer low mesh sizes (ξ), slow polymer chain relaxation hindered intraparticle water diffusion.

Contrary, the diffusional coefficients of Agars-PEG-S and Agars-PEG-H beads are measured within anomalous range (0.72 and 0.44, respectively). This observation indicates that the applied modification on hydrogel structure for raising the polymer chain relaxation rate by increasing the specific surface area and network mesh size is quite successful. Hence, the water molecule diffusion through mesoporous agarose beads is facilitated by tailoring the structural characteristics of the hydrogel matrix.

### 3.3. Protein adsorption kinetics

For further evaluation of the effectiveness of tailored mesoporous nanostructure in enhancing intraparticle molecular diffusion, BSA adsorption kinetics on CB-functionalized agarose beads was studied. Albumin adsorption through time is shown in Fig. 8. Interestingly, the protein adsorption data is in general agreement with water diffusion results. Similar to water absorption capacities, equilibrium protein adsorption capacities of the two homogenizer-synthesized bead types have no significant difference due to insignificant total pore volumes (Table 1). However, higher amounts of BSA are adsorbed at equilibrium \( q_e \) on modified beads with larger specific surface areas. Specifically, rationally tuned Agars-PEG-S beads showed the greatest equilibrium protein adsorption (3.2 (mg · g\(^{-1}\))).

The adsorption data was fitted into scattered plots to determine the initial adsorption rates of BSA based on pseudo-second order kinetic model. The pseudo-second order equation is [56]:

\[
\frac{dq}{dt} = k_2 (q_e - q_t)^2
\]

In Table 3, it shows that the adsorption kinetics parameters (equilibrium BSA adsorbed \( q_e \), initial adsorption rate \( h \), pseudo-second-order rate constant \( k_2 \), and half-adsorption time \( t_{1/2} \)) of BSA adsorption on CB-functionalized agarose beads are as follows:

<table>
<thead>
<tr>
<th>Sample</th>
<th>( q_e ) (exp) (mg · g(^{-1}))</th>
<th>( q_e ) (cal) (mg · g(^{-1}))</th>
<th>( h ) (mg · g(^{-1}) · min(^{-1}))</th>
<th>( k_2 ) (g · mg(^{-1}) · min(^{-1}))</th>
<th>( t_{1/2} ) (min)</th>
<th>( R^2 )</th>
<th>RMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agars-S</td>
<td>1.0</td>
<td>1.1</td>
<td>0.04</td>
<td>0.035</td>
<td>26</td>
<td>0.938</td>
<td>0.36</td>
</tr>
<tr>
<td>Agars-PEG-S</td>
<td>3.2</td>
<td>3.3</td>
<td>0.47</td>
<td>0.042</td>
<td>7</td>
<td>0.997</td>
<td>0.03</td>
</tr>
<tr>
<td>Agars-H</td>
<td>1.9</td>
<td>2.2</td>
<td>0.11</td>
<td>0.024</td>
<td>19</td>
<td>0.906</td>
<td>0.79</td>
</tr>
<tr>
<td>Agars-PEG-H</td>
<td>1.7</td>
<td>1.8</td>
<td>0.23</td>
<td>0.071</td>
<td>8</td>
<td>0.995</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Fig. 8. BSA adsorption on Cibacron Blue-coupled beads through time (error bars represent the standard deviation for three replicates of the experiments). Microporous agarose beads (Agars-S) showed the least equilibrium BSA adsorption. Homogenizer-synthesized beads (Agars-H & Agars-PEG-H) showed almost the same diffusion capacities with no significant difference. Modified mesoporous Agars-PEG-S beads recorded the highest water equilibrium protein adsorption.
where $q_e$ and $q_t$ (mg·g$^{-1}$) are respectively the amount of BSA adsorbed at equilibrium and at time $t$; and $k_2$ is the pseudo-second-order rate constant (g·mg$^{-1}$·min$^{-1}$). Integrating Eq. (5) and considering the boundary conditions (at $t = 0$, $q_i = 0$, and at $t = t_1/2$, $q_e = q_i$) gives:

$$\frac{t}{q_i} = \frac{1}{k_2q_e^2} + \frac{t}{q_e}$$

(6)

From Eq. (6), $h = k_2q_e^2$ (mg·g$^{-1}$·min$^{-1}$) can be expressed as the initial adsorption rate. Considering Eq. (4), a plot of $h$ versus $t$ gives a linear relationship from which $h$, $k_2$, and $q_e$ can be calculated from the intercept and the slope, respectively.

Also, the time required for the functionalized beads to take up half of the equilibrium BSA adsorption is defined as half-adsorption time ($t_{1/2}$). This time is expressed as Eq. (7), and can be used as a criterion for adsorption rates comparison.

$$t_{1/2} = \frac{1}{k_2q_e}$$

(7)

The regression data for Eq. (6) is represented in Table 3. As reported in Table 3, initial adsorption rates ($h$) have a remarkable increase when BSA is adsorbed on modified agarose beads. Therefore, the highest adsorption rate (0.47 (mg·g$^{-1}$·min$^{-1}$)) belongs to mesoporous Agars-PEG-S beads due to higher porosity and semi-optimal pore sizes.

Furthermore, the initial adsorption rate of BSA on homogenizer-synthesized beads is lower than Agars-PEG-S since these beads possess lower mesh sizes in their polymeric network. However, Agars-PEG-H showed relatively facilitated intraparticle protein diffusion (higher initial adsorption rate) than Agars-H. As explained in the previous section, Agars-PEG-H is slightly much mesoporous.

Amazingly, the half-adsorption time is dramatically decreased for BSA adsorption on modified beads. Contrary to nonporous Agars-S beads with $t_{1/2} = 25$ min, fast protein adsorption is achieved for Agars-H and Agars-PEG-H beads (respectively 19 and 8 min). However, due to lower specific surface area and tight porous network (small $ξ$), protein adsorption enhancement on homogenizer-synthesized beads is less than Agars-PEG-S. Apparently, increasing the number of ~20 nm width mesopores resulted in raising the protein’s chance to approach the affinity ligand. In our previous study we observed almost same results for mesoporous agarose beads which possessed mesopores of 2–20 nm [27].

Based on the results, it can be concluded that the introduced porosity modification successfully resulted in protein intraparticle mass transfer enhancement and reduction of time taken to reach the static adsorption capacity.

4. Conclusion

Rationally tuned mesoporous microbeads were prepared by blending agarose and PEG200 as porogen. Two mixing regimes were employed to achieve the desired macro and microscopic characteristics of the beads as a potential material for biomacromolecules separation.

Porosimetry results showed a very negligible porosity (~nonporous) for unmodified beads, mostly caused by micropores. However, porogen removal after phase separation effectively increased the total porosities of the beads up to 8 folds. Successful migration of porogen molecules out of the beads and utilizing homogenizer led to formation of meso- and macro-pores, respectively. Statistical analysis of the results revealed that the effects of each of these two independent variables on increasing the porosity and changing pore types of the beads were significant.

Our results indicate that high shear mixing provides fine-sized particles and easy manipulation. However, homogenization leads to formation of meso- and macro- porosity, and increases the number of junction zones. These features slow down intraparticle molecular diffusion.

On the other hand, using mesopore-forming porogen (PEG200) increased the specific surface area of the modified beads (Agars-PEG-S and Agars-PEG-H). The equilibrium swelling capacity and swelling kinetics beside protein adsorption kinetics of the tuned mesoporous beads confirmed the hypothesized structural effects on molecular diffusion. The more mesoporous beads showed higher equilibrium water absorption and protein adsorption. Indeed, the higher specific surface areas facilitated molecular diffusion; thus, anomalous diffusion mechanism occurred. It was concluded that, by increasing the specific surface area of the beads, besides designing a relatively sparse porous structure, molecular diffusion through hydrogel matrix can be facilitated.

In conclusion, utilizing a low molecular weight PEG, as porogen, is a quite successful method for tailoring the nanostructure of other carbohydrate biopolymer beads with the purpose of facilitating protein diffusion through mesoporous structure.

The following is the supplementary data related to this article.

**Figure S1**

Cumulative Volume Frequency-Q3 (%) - Particle Size (µm)


47. M.G. Orkoulas, P.G. Koutsoukos, Dissolution effects on specific surface area, particle size, and porosity of pectinic marble, J. Colloid Interface Sci. 239 (2001) 483–488.


