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Effects of pore morphology and size on antimicrobial activity of chitosan/poly(ethylene glycol) diacrylate macromer semi-IPN hydrogels

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ABSTRACT: The objective of this study was to obtain antibacterial active chitosan/poly(ethylene glycol) diacrylate macromere (CS/PEGM) semi-IPN hydrogels near a neutral pH level by changing their pore size and morphology. These hydrogels were prepared from CS and PEGM with different molecular weights in the presence of pore-forming agents, poly (ethylene glycol) (PEG) or sodium bicarbonate ($NaHCO_3$), by using two different initiator system, namely chemical or UV. A combination of CS with PEG or $NaHCO_3$ in the presence of PEGM could be able to create desired pore formation in both initiator systems. The antibacterial activity of hydrogels changed with the molecular weight (g/mol) of PEGM in the order 2000>400>8000. A chemical initiation system was found more suitable than the UV initiation system for antibacterial activity. Hydrogels showing the highest antibacterial activity on *Staphylococcus aureus* and *Escherichia coli* have medium or distributed pore size and interconnected pores. Hydrogels prepared with PEGM (M_n: 2000 g/mol) were proposed for antibacterial wound dressing and soft tissue regeneration applications owing to their antibacterial activity and elastic modulus. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42707.

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INTRODUCTION

Synthetic and natural forms of polymeric hydrogels have been used to repair and assist in the regeneration of a variety of tissues.[1–4] Enhancing the effect of macroporosity on the growing of stem or blood cells and vascular structures was presented in studies using biocompatible PEGM[5] based synthetic hydrogel with an average pore size of 200 μm by using $NaHCO_3$ as pore former. Macroporous hydrogels could also be obtained with higher isotactic polymeric structures instead of pore formers.[6,7] The effect of scaffold microporosity on the chondrogenesis of human mesenchymal stem cells was studied using PEGM macromonomer and PEG pore-forming agent containing photopolymerizable semi-interpenetrating networks by changing the PEGM/PEG ratio and the molecular weight of PEGM.[8] PEG carrying different functional groups were also used as macromonomers to obtain porous and hyperbranched hydrogels and macromolecules.[9–13] Because of their low interfacial free energy and capability to bind metals, PEG containing polymers have a potential for antibacterial applications.[14,15]

Semi-IPN hydrogels of hydrolytically degradable PEGM with collagen, gelatin and hyaluronic acid were also investigated as an approach for accelerating cellular remodeling.[16] Because of its biocompatibility and antibacterial activity, the copolymers and composites[17–25] of CS is popular for many applications. Because of their superior properties, a combination of CS and PEGM in the same hydrogel structure has attracted the attention of some researchers. In research, IPN hydrogels composed of CS and PEGM were synthesized by using UV irradiation in a mild aqueous medium.[26] In another study, semi-IPN hydrogels composed of CS and PEGM that were synthesized with UV irradiation in the presence of a photoinitiator has potential biomedical applications.[27] Although the crystallinity, thermal and mechanical properties of CS/PEGM semi-IPN structures at different ratios were investigated in this work, the effect of the preparation method and molecular weight of PEGM on the interior morphology and antibacterial characteristics of the polymer has not been researched in any paper, until now.

In our study, porous CS/PEGM semi-IPN hydrogels were prepared with different molecular weight of the PEGM in the presence of pore-forming agents $NaHCO_3$ or PEG by using UV irradiation or a chemical initiator pair (APS/TEMED). The use of CS and pore-forming agents $NaHCO_3$ or PEG together caused the creation of pores at different morphologies inside

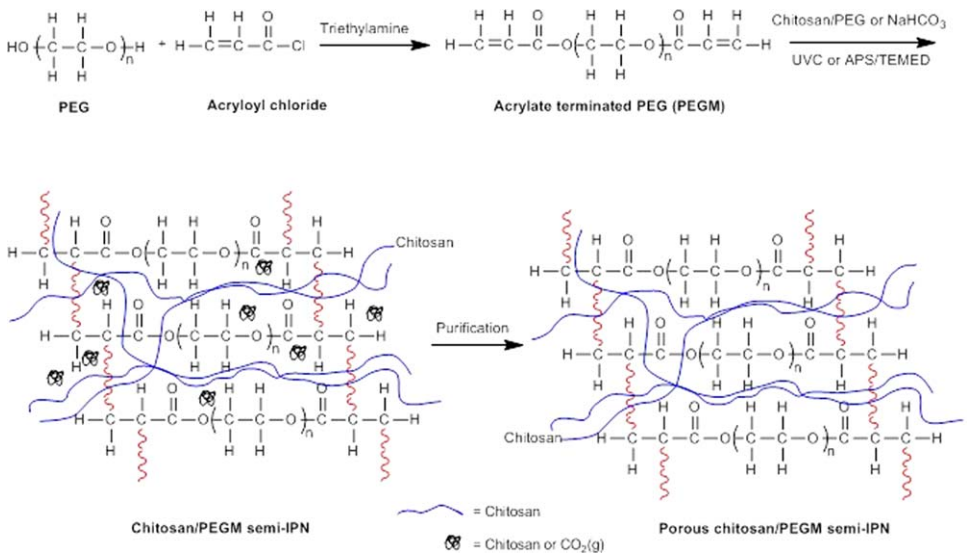

Figure 1. The method of porous chitosan/PEGM semi-IPN hydrogel synthesis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the hydrogels. The effect of a pore former, the molecular weight of PEGM and the type of initiation system on the pore structure, interior morphology and the antibacterial activity of hydrogels were presented.

EXPERIMENTAL

Chemicals

CS (76% deacetylated) with low viscosity was purchased from Sigma. Poly (ethylene glycol) (PEG) at different molecular weights (M_n: 400, 2000, 8000 g/mol), acryloyl chloride (≥97%), triethylamine (≥ 99%), anhydrous hexane (≥ 95%) were the products of Aldrich Chemical Inc. In order to remove the inhibitors in PEGs, they were washed using absolute ethanol and dried in a vacuum at 40°C before use. The photoinitiator, 2,2-dimethoxy-2-phenylacetophenone (DPAC, Aldrich), the chemical initiator pair N,N,N,N-tetramethylethylenediamine (TEMED, Selva)/ammonium persulfate (APS, Merck) and all other chemicals were reagent grade and they were used as they were received. CS solution 1% (w/v) in 2% (v/v) acetic acid aqueous solution was filtered from 2G1 glass filter (Schott) before use.

Synthesis of CS/PEGM Semi-IPN Hydrogels

First, PEGMs with different molecular weights were synthesized by using different molecular weights of PEG, acryloyl chloride and triethylamine.[26] These PEGMs, CS/PEGM semi-IPN hydrogels were prepared using two methods as presented schematically in Figure 1. In the chemical initiated method, first 0.18 g PEGM with a specified molecular weight was dissolved in 1.5 mL 1% (w/v) CS solution in 2% (v/v) acetic acid in a plastic tube. Then, 0.05 g PEG (M_n: 2000) (PEG2000) as a pore-former was added and mixed. The gelation of PEGM was initiated with the addition of 0.1 mL from each of aqueous solution of APS and TEMED under a nitrogen gas atmosphere, respectively. The reaction mixture was maintained for eight hours at room temperature for the completion of gelation.

In the UV initiated method, 0.18 g PEGM was dissolved in 1.5 mL 1% (w/v) CS solution in 2% (v/v) acetic acid in a plastic tube. A 0.002 g photoinitiator (DPAC) was dissolved in the PEGM containing CS solution. Then, 0.001 g $NaHCO_3$ was added as a pore former and the lid of the tube was immediately closed. The tube was lightly shaken by hand for five minutes and then it was exposed to UV irradiation by means of a 20 Watt ultraviolet (UVC) lamp (Ace Glass Co.) for four hours at room temperature. After completion of gelation, hydrogels produced from both methods were removed from the tubes and sliced into small thin discs (1mm in height and 9 mm in diameter). Hydrogel discs were immersed in distilled water for purification by extracting unreacted PEGM during the water phase. Distilled water was refreshed twice a day during the purification stage. Formulations for effectiveness of feed compositions on pore size and morphology of synthesized CS and/or PEGM based hydrogels are provided in Table I.

Purified CS/PEGM hydrogel discs were used in antibacterial tests in swollen or dry form. To use them in the swollen form, they were removed from the water and the excess water around them was removed with filter paper. The dry form of the hydrogel was obtained by drying them at room temperature under ventilation. The feed compositions of CS/PEGM semi-IPN hydrogels used in the antibacterial activity tests are provided in Table II.

SEM Characterization

For SEM characterization, both non-purified and purified samples remained at −80°C for two days were freeze dried at −50°C and 0.1mBar for 24 hours using a Christ Alpha 1–4 LSC freeze dryer (Martin Christ Freeze Dryers GmbH, Osterode an Harz). Then, the SEM pictures were taken by SEM, JSM-7600 F FEG after a gold coating.

Determination of Antibacterial Activity

According to the preliminary tests, 20 pieces of hydrogel discs (diameter: 6 mm, height: 1 mm) were used in each of the

Table I. Formulations for Effectiveness of Feed Compositions on Pore Size and Morphology of CS and PEGM Based Hydrogels

Sample code	% CS[a]	Distilled water (mL)	Amount of PEG (M_n: 2000) (g)	$NaHCO_3$ (g)	DPAC (g)	UV
PEG6	1	-	0.2	-	0.001	✓
PEG7	-	1.5	0.05	-	0.001	✓
PEG8	1	-	-	-	0.002	✓
PEG9	-	1.5	-	-	0.002	✓
PEG10	-	1.5	-	0.001	0.002	✓
PEG11	1	1.5	-	0.001	0.002	✓

[a] The concentration (w/v %) of CS in 2 vol % aqueous acetic acid solution. 1.5 mL of CS solution and 0.18 g PEGM with molecular weight of 4000 were used.

antibacterial activity tests. Therefore, the CS content of hydrogels was approximately adjusted to 40, 80, and 160 mg in 10 mL Nutrient Broth by using 1%, 2% and 4% (w/v) CS solution in hydrogel synthesis. In the preliminary experiments, the antibacterial activity of swollen hydrogels was found to be higher than in the dry hydrogels. Antibacterial activity tests on some of the selected hydrogels were also carried out. Details on antibacterial activity method are provided below.

Two strains, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as test microorganisms. The strains were obtained from the Mugla Sitki Kocman University Culture Collection. The antibacterial activity of CS/PEGM semi-IPN hydrogel discs was evaluated using the optical density (O.D.) method.[28] For the antibacterial tests, three groups of hydrogels were set as follows: CS/PEGM semi-IPN hydrogel discs (20 pieces) in 10 mL Nutrient Broth (Difco, Michigan, USA) (Group I), Nutrient Broth without bacteria (Group II) and Nutrient Broth with bacteria (Group III). The pH level of all groups was initially adjusted to 6.5 using a diluted HCl solution. *E. coli* was incubated in a shaker at 30°C and *S. aureus* was incubated in a shaker at 37°C for 24 hours. The O.D. of the medium was measured at 550 nm after 24 hours. The bacterial

Table II. The Feed Compositions of CS/PEGM Semi-IPN Hydrogels

Sample code	Mw of PEGM (g/mol)	% CS[a]	Amount of PEG 2000 (g)	DPAC (g)	$NaHCO_3$ (g)	APS[b] (mL)	TEMED[c] (mL)	UV	Yield[d] (%)	EWC[e] (g/g)
UV initiated/$NaHCO_3$										
UV/SC/8000	8 000	1	-	0.002	0.001	-	-	✓	62.3	13.3
UV/SC/8000-2	8 000	2	-	0.002	0.001	-	-	✓	-	-
UV/SC/8000-4	8 000	2	-	0.002	0.01	-	-	✓	-	-
UV/SC/2000	2 000	1	-	0.002	0.001	-	-	✓	72.4	6.2
UV/SC/2000-2	2 000	2	-	0.002	0.001	-	-	✓	-	-
UV/SC/2000-4	2 000	2	-	0.002	0.01	-	-	✓	73.6	5.5
Chemical initiated/PEG2000										
CH/PEG/ 8000	8000	1	0.25	-	-	0.1	0.1	-	35.2	10.2
CH/PEG/8000-2	8 000	2	0.25	-	-	0.1	0.1	-	-	-
CH/PEG/2000	2 000	1	0.25	-	-	0.1	0.1	-	77.9	4.4
CH/PEG/ 2000-2	2 000	2	0.25	-	-	0.1	0.1	-	61.7	4.0
CH/PEG/ 2000-2-0.5	2 000	2	0.5	-	-	0.1	0.1	-	-	-
CH/PEG/ 2000-4	2 000	4	0.25	-	-	0.1	0.1	-	-	-
CH/PEG/400-2	400	2	0.25	-	-	0.1	0.1	-	-	-
Chemical initiated/$NaHCO_3$										
CH/SC/2000-2	2000	2	-	-	0.012	0.1	0.1	-	45.5	3.83
CH/SC/400-2	400	2	-	-	0.012	0.1	0.1	-	-	-

[a] The concentration (w/v) of CS in 2 vol % aqueous acetic acid solution. 1.5 mL of this solution and 0.18 g PEGM were used in formulations.
[b] 10% Ammonium persulphate in water (w/v).
[c] 10% TEMED in water (v/v).
[d] Yield = $(W_h/W_m) \times 100$ where W_h and W_m represent dry weight of purified hydrogel and sum of CS and PEGM content of hydrogel preparation medium at the initial of hydrogelation, respectively.
[e] EWC = $(W_s - W_d)/W_d$ where W_s and W_d represent the weights of swollen and dry samples, respectively.

growth was illustrated by the O.D. and each operation was carried out using aseptic techniques.[29]

Percentage of bacterial reduction was calculated according to following equation:

$$\text{Bacterial Reduction}(\%) = [Z - X/Z - Y] \times 100$$

where X, Y, and Z represent the absorbances of Group I, Group II and Group III at the end of 24 hours, respectively.

Elastic Modulus Measurements

The shear moduli (G) of gels were determined by measuring the uniaxial deformation ($\pm$0.001 mm) under compression using a displacement transducer (E725, RDP Electronics, Grove St. Wolverhampton, UK) and by measuring the force ($\pm$0.0001 N) applied to the surface of swollen cylindrical gel using a force transducer (Sensotec Load Cell, RDP Electronics, Grove St. Wolverhampton, UK) as described in the literature.[30] Swollen gel cylinders were uniaxially compressed between a Petri dish and a Teflon plate, each parallel to the other. The swollen sample size was 8 mm in length (L_o) and 20 mm in diameter (D_o).

RESULTS AND DISCUSSION

The Effect of CS and PEG on the Pore Size of CS/PEGM Semi-IPN Hydrogels

To discuss the effect of CS and pore-forming agent PEG2000 on the pore size of hydrogels, their interior structure is observed with SEM photographs provided in Figure 2 as (a), (b), (c), (cx), and (d), respectively.

It is clearly seen in Figure 2(a) that the hydrogel composed of only PEGM (without CS, PEG2000 and $NaHCO_3$ pore-forming agent) contains no porosity. The presence of CS in the formulation without pore former led to the formation of a less porous structure [Figure 2(b)] with irregular pore size distribution. This result can be explained with pore formation as a result of phase separation occurred by physically or chemically crosslinking of PEGM with itself or CS. Extraction of water solubilized and disentangled PEGM molecules between CS/PEGM semi-IPN hydrogel network during purification may have forced the creation of a porous structure in addition to the pore-forming behavior of CS. Because of the insolubility of CS in water at a state of neutral pH and its high molecular weight, it cannot diffuse hydrogel during purification as presented in Figure 1. This result was proven through UV absorption experiments, but not presented here in order to limit the paper's volume. It is clearly seen from Figure 2(b) that the presence of CS caused the formation of porosity in the hydrogel. PEGM hydrogel without CS synthesized in the presence of only PEG2000 pore former has very small and well-distributed pores [Figure 2(c,cx)] as consistent with the finding of Salerno *et al.*[31] Hydrogel (PEG6) composed of both PEGM and CS with PEG2000 has large, regular and gallery type pores throughout the whole hydrogel matrix [Figure 2(d)] after purification presenting a synergetic effect on porosity. The solubility effect of PEG on CS and PEGM led to a well distributed phase separation and also the construction of thin matrix walls containing CS/PEGM semi-IPN hydrogel after purification. Purification causes the removal of PEG pore formers packed between separated phases and the synchronistical formation of large pores.

A SEM photograph of hydrogel synthesized using pore-former $NaHCO_3$ and PEGM without CS are provided in Figure 2(e). A small number of pores with distributed pore size were created by releasing of CO_2 gas packed between cross-linked PEGM chains during purification. This hydrogel has larger pores than hydrogel produced with PEGM and PEG pore former without CS [Figure 2(c)]. The pore morphology of the hydrogel in Figure 2(e) has changed into a hydrogel that has large distributed pores [Figure 2(f and g)] by adding CS in synthesis formulation. Because of the pore-forming actions of CS and its high molecular weight, which decreases the probability of evolved CO_2 escaping, a more porous hydrogel with a thicker wall was obtained after purification.

Effect of Preparation Parameters on the Antibacterial Activities of Hydrogels

In order to study the effect of the molecular weight of PEGM and the amounts of CS and pore-former agents PEG2000 and $NaHCO_3$ on antibacterial activity, CS/PEGM semi-IPN hydrogels were prepared by using PEGM macromonomer with various molecular weights (400, 2000, 8000, and 20,000 g/mol) produced by us.

Since hydrogels prepared with PEGM (M_n: 20,000) was in a jelly form due to their low cross-link densities, its antibacterial activity results and SEM photographs were not presented. Antibacterial tests were performed under three main hydrogels groups on the basis of an initiation system and type of pore former. These can be classified as UV initiated/$NaHCO_3$, chemical initiated/PEG2000 and chemical initiated/$NaHCO_3$ hydrogel groups. All formulations and antibacterial results of CS/PEGM hydrogels and their SEM photographs are provided in Tables II and III and Figure 3, respectively. In these tables, UV, SC, CH, PEG (400, 2000, and 8000) were used to represent UV initiated, sodium bicarbonate pore former, chemical initiated, PEG pore former, PEGM (M_n: 400), PEGM (M_n: 2000) and PEGM (M_n: 8000), respectively.

While the UV/SC/8000 and UV/SC/8000-2 hydrogels synthesized with PEGM (M_n: 8000 g/mol) did not show any antibacterial activity on *S.aureus* according to the antibacterial activity results of the UV initiation/$NaHCO_3$ system, they presented weak antibacterial activity on *E.coli*. However, UV/SC/2000 and UV/SC/2000-2 synthesized with PEGM (M_n: 2000 g/mol) presented antibacterial activity on both bacterial strains. The gallery type pores in UV/SC/2000 series [Figure 3(b,d,f)] are seen slightly more open and connected than those in UV/SC/8000 [Figure 3(a, c, e)]. Increase in depth and size of pores in UV/SC/2000 hydrogel caused a rise in antibacterial activity. Although UV/SC/2000 has half the chitosan content of UV/SC/2000-2, it has more antibacterial activity. As summarized in Table III, UV/SC/2000 has pore properties of medium size, deep, regular and gallery type pores [Figure 3(b)]. But, UV/SC/2000-2 has fewer, irregular and overlapped pores [Figure 3(d)]. The differences in pore properties affect the contact surface of hydrogel with the bacteria. The high contact surface properties of UV/SC/2000 resulted in more antibacterial activity. This result revealed the importance of pore properties as well as CS content. This is supported by an interesting result obtained

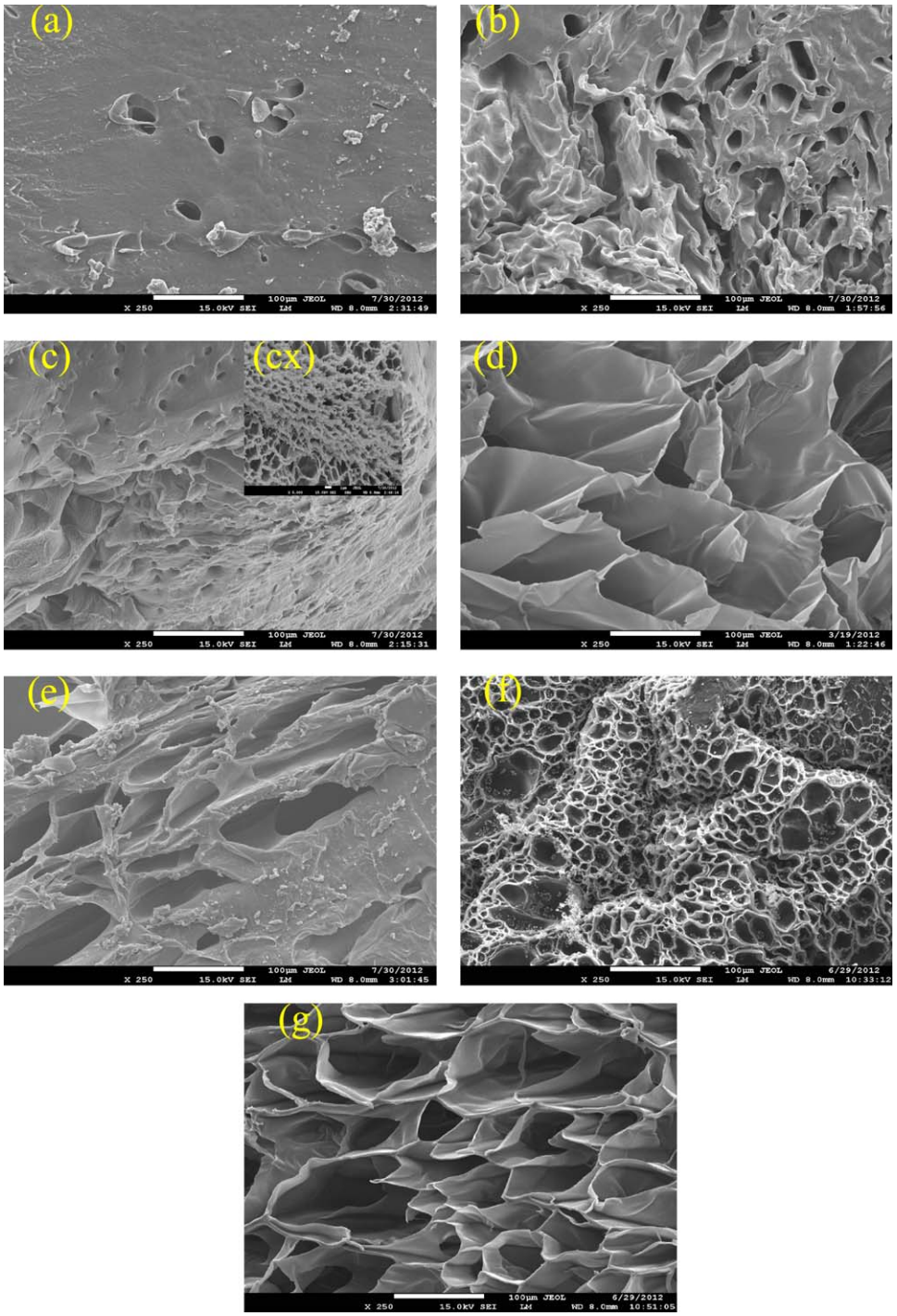

Figure 2. The effect of PEG and $NaHCO_3$ pore-forming agents and CS on the pore size of PEGM-based hydrogels.(a) PEG9 (b) PEG8, (c) PEG7, (cx) PEG7 at high magnification, (d) PEG6, (e) PEG10, (f) non-purified PEG11 and (g) purified PEG11 (scale bar represent 100 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with UV/SC/2000-4 [Figure 3(f)] hydrogel that exhibited the highest antibacterial activity only on *E. coli*. Since the negative charge on the cell surface of Gram-negative bacteria *E. coli* was higher than Gram-positive bacteria, more interaction between CS containing hydrogel surface and the Gram-negative bacteria *E. coli* occurs resulting in the cell wall destruction of *E. coli, more* than *S.aureus*. To be more detailed, the rod-shaped structure (approximately 2 μm in length) of *E. coli* may have led to a suitable alignment for contact with the interior morphology of UV/SC/2000-4 hydrogel having a larger pore size (approximately 50–200 μm) and relatively connected pore structure near boundary of hydrogel and higher available surface area for electrostatic interaction than UV/SC/2000-2 and UV/SC/8000-4 [Figure 3(d,e)]. Hence, CS macromolecules on UV/SC/2000-4

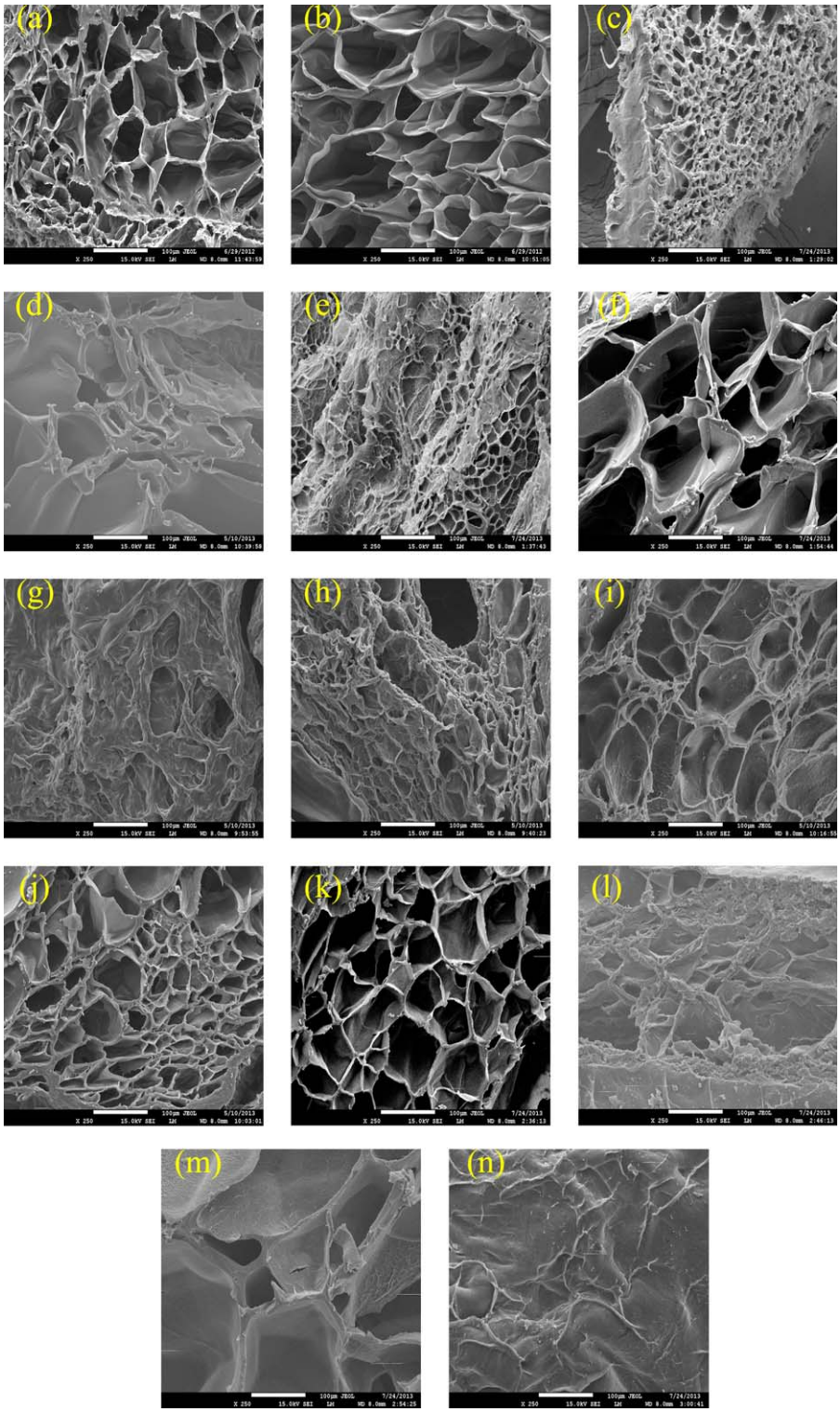

Figure 3. SEM photographs of CS/PEGM semi-IPN hydrogels used in antibacterial tests. (a) UV/SC/8000, (b) UV/SC/2000, (c) UV/SC/8000-2, (d) UV/SC/2000-2, (e) UV/SC/8000-4, (f) UV/SC/2000-4,(g) CH/PEG/8000, (h) CH/PEG/2000, (i) CH/PEG/8000-2, (j) CH/PEG/2000-2, (k) CH/PEG/2000-2-0.5, (l) CH/PEG/400-2, (m) CH/SC/2000-2,(n) CH/SC/400-2 (scale bar represent 100 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hydrogel surface had a higher interaction probability with *E. coli* and as a result of that a higher number of *E. coli* was inhibited. On the other hand, *S. aureus* has small (approximately 0.5 μm) and spherical dimensions. Antibacterial activity for *S. aureus* can occur with different mechanism mainly depending on the hydrophobic interaction with hydrogel surface. Since the

Table III. CS/PEGM Semi-IPN Hydrogels Pore Properties and Their Antibacterial Activity Results

Sample code	Pore properties		CS content of hydrogels (mg)	Bacterial reduction (%)	
	Approx. size range (μm)	Morphology		*S. aureus*	*E. coli*
UV initiated/$NaHCO_3$					
UV/SC/8000	25–100	Small, regular, gallery type and disconnected	40	-	8.9
UV/SC/8000-2	5–25	Small, regular and disconnected	80	-	4.5
UV/SC/8000-4	5–25	Small, fewer, distributed and disconnected	80	6.3	0.3
UV/SC/2000	50–150	Medium, deep, regular and gallery type	40	25.3	15.6
UV/SC/2000-2	50–100	Fewer, overlapped, gallery type, irregular disconnected	80	7.7	0.5
UV/SC/2000-4	50–200	Large, regular and connected	80	-	98.5
Chemical initiated/PEG2000					
CH/PEG/ 8000	No porosity	Rough surface	40	-	38.2
CH/PEG/8000-2	25–100	Shallow, distributed and disconnected	80	-	1.3
CH/PEG/2000	10–50	Medium, distributed and partially connected	40	82.8	7.9
CH/PEG/ 2000-2	50–100	Deep, medium and distributed	80	100	100
CH/PEG/ 2000-2-0.5	50–100	Shallow, medium and regular	80	-	5.3
CH/PEG/ 2000-4	ND[a]	ND[a]	160	-	-
CH/PEG/400-2	10–200	Too rough, distributed, partially connected and irregular	80	81.2	83.2
Chemical initiated/$NaHCO_3$					
CH/SC/2000-2	25–200	Very high size distribution, irregular, partially connected	80	99.8	100
CH/SC/400-2	No porosity	Smooth surface	80	-	8.9
Chemical initiated/PEG2000 (dry form)					
[b]CH/PEG/2000-2	ND[a]	ND[a]	80	39.4	-
[b]CH/PEG/400-2	ND[a]	ND[a]	80	52.1	-

[a] SEM images was not taken.
[b] The hydrogels used in antibacterial tests were in dry form, (-) no antibacterial activity.

contact area of *S. aureus* with CS containing hydrogel surface was very less in the case of large pore size, for instance UV/SC/2000-4, this hydrogel did not present any antibacterial activity on *S. aureus*. However, short distance between walls of neighboring pores with medium pore size, for instance UV/SC/2000, enable touching of spherical *S. aureus* bacteria with CS containing surface at more points that can effect hydrophobic interaction and also increase the antibacterial effect on *S.aureus*.[32] The same is also valid for UV/SC/8000 series (UV/SC/8000 and UV/SC/8000-2) has antibacterial activity only on *E. coli*. Since small, regular, gallery type but discontinuous pore morphology of PEGM (M_n: 8000) hydrogels [Figure 3(a,c,e)] have less contact surface area, this area can be used partially in electrostatic interaction between the negative charge of the *E. coli* and the positive charge of the hydrogel that resulted in more antibacterial activity for this bacteria. For these reasons, hydrogels UV/SC/2000 has deep and medium pore size and also a higher pore surface area that might present slight antibacterial activity in both bacterial strains (Table III).

Antibacterial activity results of chemical initiated/PEG2000 system are provided in Table III and Figure 3. PEGM (M_n: 8000) series showed slight antibacterial activity only for *E.coli*, similar to the results for the same hydrogel series obtained with UV/$NaHCO_3$ (Table III). As explained above, small surface area and discontinuous pores [Figure 3(g,i)] in PEGM (M_n: 8000) series caused less contact area of the CS containing hydrogel surfaces with *S. aureus*. With PEGM (M_n: 2000) hydrogels [Figure 3(h,j)], increasing in pore size and formation of partially connected pores caused a sharp increase in the antibacterial activity for both bacterial strains as seen in Table III. However, CH/PEG/2000 hydrogel that has less CS presented higher antibacterial activity on *S. aureus* relative to *E. coli*. However, increasing CS content (CH/PEG/2000-2) caused the highest amount of antibacterial activity on both bacterial strains. This result can be attributed to the formation of pores in different pore sizes and morphologies as well as high CS content. Although, CH/PEG/2000 hydrogel [Figure 3(h)] contains partially connected and distributed pore sizes between small and medium, CH/PEG/2000-2 hydrogel [Figure 3(j)] has more contact surface area meaning a higher surface interaction probability for both bacterial strains. Additionally, it is critical result that the two-fold increment in PEG content for PEGM (M_n: 2000) series caused

the formation of CH/PEG/2000-2-0.5-coded hydrogel carrying big and disconnected pores [Figure 3(k)] and also did not present enough antibacterial activity.

Hydrogel produced a low PEGM (M_n: 400) molecular weight and high CS content (CH/PEG/400-2) has very rough, partially interconnected pores and distributed pores sizes [Figure 3(l)]. It has also antibacterial activity on both bacterial strains between CH/PEG/2000-2 and CH/PEG/8000-2. In summary, the order of antibacterial activity of 80 mg CS containing hydrogels on the basis of the PEGM molecular weight can be CH/PEG/2000-2>CH/PEG/400-2>CH/PEG/8000-2, depending on pore morphology. Pores with medium-pore size or pore size distribution about this size as well as interconnectivity caused high antibacterial activity. This is an important result of this study, showing the effect of PEGM molecular weight on antibacterial activity.

For chemical initiated/$NaHCO_3$ hydrogel group, the effect of PEGM molecular weight on the antibacterial activity is provided in Table III. The SEM photographs of this group are presented in Figure 3. Hydrogel CH/SC/2000-2 [Figure 3(m)] has a high antibacterial activity effect on both *E. coli* and *S. aureus*. The pore size and morphology of this hydrogel are similar but has a very high level of size distribution containing medium and large pores together confirming higher and appropriate inner surface area for antibacterial activity on both bacterial strains than UV/SC/2000-4 [Figure 3(f)]. Thus, CH/SC/2000-2 showed antibacterial activity for both bacterial strains, which supports our discussion about antibacterial activity–structure relationship for CH/PEG/2000-2 and CH/PEG/400-2.

Another important result supporting our theory was obtained with CH/SC/400-2 hydrogel. This hydrogel hasn't got any substantial surface area or porosity [Figure 3(n)]. Because of less surface area, the electrostatic effect only has a slight antibacterial activity on *E. coli*.

Although the hydrogels (^{d}CH/PEG/2000-2 and ^{d}CH/PEG/400-2) that have the best antibacterial activity on both of the bacterial strains was in the swollen form, they presented antibacterial activity only on *S. aureus* in the dry form (Table III). This can be clarified by the hydrophobic activities of hydrogels. During the antibacterial tests, the combination of these dry hydrogels with bacteria in its nutrient medium caused the swelling of the hydrogel and increased the contact area of bacteria with hydrogel surface, simultaneously. Initially, the surface of the hydrogel is highly hydrophobic and suitable for hydrophobic interaction with *S. aureus*, but not suitable for electrostatic interaction with *E. coli*. Since the swelling of these hydrogels was attained approximately within 20 minutes at a pH 6.5 level, this slow swelling rate caused existing of predominantly hydrophobic surface during the swelling process and the antibacterial effect on *S. aureus*. In other words, the ionization rate of amine groups as a result of swelling was not sufficient to prevent *E. coli* growth and the pH level of the culture media increased over a neutral pH level of 7 with the growing bacteria resulting in no antibacterial activity on *E. coli*.

In this study, 100% bacteria reduction on both bacterial strains was provided by CS carrying CH/PEG/2000-2 hydrogels providing 8 mg/mL CS content in an antibacterial solution at a pH 6.5 level. This value is higher than in other studies where it was obtained at 0.5 mg/mL with pure CS powder in acidic medium where the pH level was not presented[33] and at 0.06 mg/mL with pure CS nanoparticles[34] at the pH 6.5 level. Since CS was entangled with PEGM chains in CS-PEGM semi-IPN structure and also there was discontinuous pore formation probability inside of a hydrogel, all CS molecules may not have been used effectively in this study. In addition, the pH level of antibacterial test medium is 6.5 and very close to a neutral pH level. It is well known that the antibacterial activity of CS is strongly affected by the pH level of medium and it should be lower than 7 to result in antibacterial activity[35] for Gram-negative bacteria. The pH level of the medium that contains hydrogels with low CS was increased from 6.5 to 8.5 at the start of the incubation period. Thus, our hydrogels presented antibacterial activity only with hydrogels containing a high amount of CS, which is consistent with literature findings. The hydrogel morphology with the highest amount of pores was obtained with 2% CS in our study. Another important result obtained with hydrogels containing 4% CS (CH/PEG/2000-4) did not present antibacterial activity (Table III).[36] Additionally, CS with deacetylation degree (>85%) was found most suitable for antibacterial activity. However, this value is approximately 76% in our study and caused a decrease in antibacterial activity in comparison to those published in the literature.

Table IV. The Effect of Molecular Weight of PEGM and Production Conditions on the Elastic Modulus of Hydrogels

Sample code	M_w of PEGM (g/mol)	Elastic modulus (kPa)
UV/SC/8000	8000	1.23 ± 0.057
UV/SC/2000	2000	2.2 ± 0
UV/SC/2000-4	2000	8.16 ± 0.057
CH/PEG/ 8000	8000	0.66 ± 0.11
CH/PEG/2000	2000	1.3 ± 0
CH/PEG/2000-2	2000	1.13 ± 0.057
CH/SC/2000-2	2000	6.03 ± 0.11

The Effect of PEGM Molecular Weight on the Elastic Modulus of CS/PEGM Semi-IPN Hydrogels

Elastic moduli of some hydrogels with considerable antibacterial activity are provided in Table IV. A decrease in the PEGM molecular weight in UV initiated/$NaHCO_3$ (UV/SC/8000 and UV/SC/2000) and chemical initiated/PEG2000 (CH/PEG/8000 and CH/PEG/2000) systems caused increase in elastic modulus from 1.23 to 2.2 kPa and from 0.66 to 1.3 kPa, respectively. This result can be attributed to the high flexibility of PEGM (M_n: 8000) relative to PEGM (M_n: 2000). Since hydrogels produced with PEGM (M_n: 400) (CH/PEG/400-2 and CH/SC/400-2) are brittle even under very low loads, their elastic moduli was not tested.

In the case of CH/PEG/2000 and CH/PEG/2000-2, their elastic moduli were found approximately equal to 1.3 and 1.13 kPa,

respectively. Polymerization yield of these hydrogels decreased with CS content and found at 77.9% and 61.7%, respectively. Since the increase in CS content caused the dilution of PEGM in polymerization medium and also decreased in its possibility for addition to chain reaction, the cross-linking degree of hydrogel decreased. However, it is expected that the cross-linking density should increase the CS content. These two opposite effects led the elastic modulus to remain constant. However, the increase in $NaHCO_3$ amount in both UV and chemical initiation system caused the formation of hydrogels (UV/SC/2000-4 and CH/SC/2000-2) with high elastic modulus. Large and interconnected pore morphology of these hydrogels and also their wall thickness can be seen in Figure 3(f,m). Probably, thicker walls relative to CH/PEG/2000-2 [Figure 3(j)] caused strong and less flexible hydrogel formation.

Elastic modulus of hydrogels (Table IV) is lower than minimal acceptable value of 400 kPa for bone tissue engineering.[36–38] However, these hydrogels can be proposed for wound dressing applications.[39]

CONCLUSIONS

In this study, CS/PEGM semi-IPN hydrogels with antibacterial activity on both Gram-negative and Gram-positive bacteria were prepared in the presence of porogens ($NaHCO_3$ and PEG). The hydrogels prepared with PEGM (M_n: 2000) in medium size (around 100 μm) or pore size distributed around this size and relatively interconnected pores displayed high antibacterial activity on *E. coli* and *S. aureus*. APS/TEMED redox initiation system with PEG or $NaHCO_3$ pore formers is the most suitable initiator for the preparation of hydrogels in comparison to UV initiation system. In this study, the macropore forming ability of CS with PEGM macromonomer in the presence of PEG and $NaHCO_3$ was presented for the first time. Also this is the first detailed study to demonstrate the relationship between pore properties and antibacterial activity. One of the most important results obtained in this study is the presence of antibacterial activity for both bacteria were detected in hydrogels carrying pores with the medium distributed pore sizes and relatively connected pores.

Antibacterial and compressive strength results revealed that hydrogels synthesized with PEGM (M_n: 2000) and 2% CS content with proper morphology has possibility for application in tissue engineering for example, wound dressing, soft tissue, vascular and stem cell growth. The hydroxyl and amino groups of CS also provide several possibilities for the derivatization or grafting of desirable bioactive groups.

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