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Fabrication of a tunable hydrogel membrane for constructing indirect cell coculture system

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ABSTRACT: In this study, an improved indirect cell coculture system was constructed by using a polyelectrolyte complex membrane generated by alginate (A) and chitosan (C). Methodologies of characterizing thickness and permeability of flat AC membrane were first established due to the importance of these two parameters in determining intercellular distance and degree of contact between cocultured cells. Compared to reaction time, both alginate concentration and molecular weight (M_w) of chitosan play more dominant roles in determining the membrane thickness and diffusion coefficients. Moreover, cells in the alginate gel and on the AC membrane could maintain high cell viability. Thus, an improved indirect cell coculture system constructed by flat AC membrane was fabricated and characterized, which provides a robust tool to study the effect of intercellular distance and degree of contact between cocultured cells on cell–cell interactions. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43100.

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INTRODUCTION

Cell–cell interactions play pivotal roles in regulating normal tissue development, homeostasis as well as pathogenesis of organs, but much is yet to uncover to reach a full understanding of their complexity.[1] To address this issue, the establishment of relevant cell coculture system *in vitro* is essential, which potentially not only simulates the microenvironment *in vivo* more vividly but also recapitulates some biological event of tissue morphogenesis and functionality.[2]

According to the manners of contact between different cells, coculture system can be classified into three types: direct contact system, indirect contact system, and noncontact system[3–5] [Figure 1(A)]. Specifically, the direct contact culture system has been demonstrated to be more effective in exploring the interactions between different types of cells.[3,6] However, its application is severely hindered due to the difficulty in cell separating or purification.[7] Although the noncontact culture system provides an easy separation system through seeding the cells in upper and bottom compartment of the transwell-culture plate, respectively, the distance between those two types of cells (around 1 mm) has been shown to compromise the cell–cell interactions significantly, suggesting that data collected with this culture system be considered with caution.[3,8] By contrast, the indirect contact system seems to combine the advantages of both direct contact and noncontact system. It not only allows the cocultured cells to be effectively separated from each other, but also provides an optimized distance between them to maintain their interactions.[3,7,9]

A number of investigations on parenchyma–mesenchymal interactions have been conducted by using a flat porous membrane-based indirect cocultured system. For example, some commercially available porous membranes, such as polycarbonate (PC) and polyethylene terephthalate (PET), were used to culture human embryonic stem cells with feeder cells.[7] Although some progresses have been made in establishing the indirect contact system, it should be noted that most of the commercially available cell culture membranes used for the indirect contact culture system are still limited to be made from PC or PET.[10,11] In addition, their thickness usually is fixed at round 10 μm and their porosity is pretty low (1.6% porosity for 1 μm pores).[12] These not only lead to a fixed distance between the cocultured

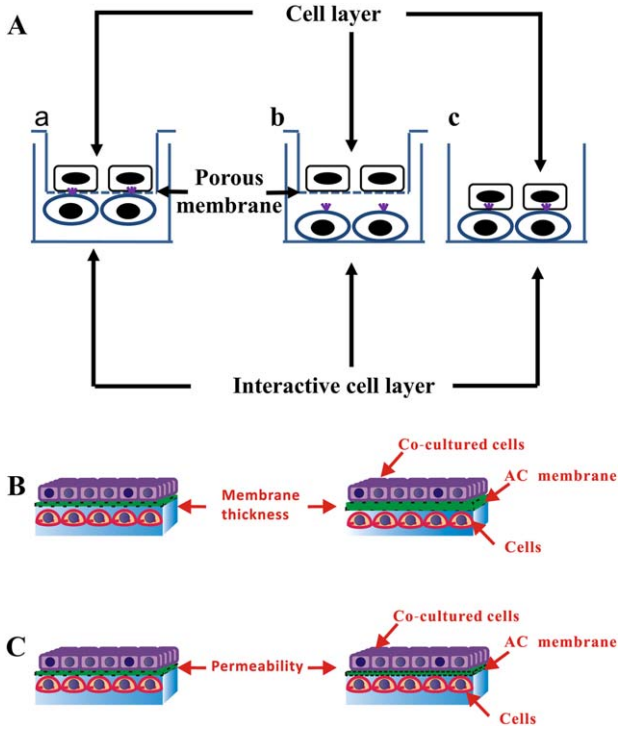

Figure 1. (A) Schematic of different coculture systems used to evaluate cell–cell interactions: (a) indirect contact system, (b) noncontact system, and (c) direct contact system. Schematic illustration of the AC membrane-based cell coculture system. (B) Thickness and (C) permeability of flat AC membrane were used to study the role of intercellular distance and degree of contact between cocultured cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells but also limit the degree of their contact.[8] Most importantly, few of the current indirect contact systems that are based on the porous PC or PET membranes are capable of providing a three-dimensional (3D) microenvironment for the cocultured cells. This is very important because it has been well demonstrated that 3D microenvironment plays a dominant role in influencing cellular genotype, phenotype, and even their functionality.[12]

Therefore, in this study, we establish an improved cocultured system by using a polyelectrolyte complex (PECs) membrane, which was generally formed by combining oppositely charged polyelectrolytes together via ionic interaction.[13] A number of studies on two types of polyelectrolytes—alginate and chitosan—have been conducted in our laboratory, including material characterization,[14–18] improvement, and their biological application in cell encapsulation,[19–23] drug delivery,[24,25] and even tissue engineering.[26,27] Here, a flat AC membrane formed by biocompatible alginate and chitosan was adopted to establish an improved coculture system as described in the schematic diagram [Figure 1(B,C)]. The reason of using AC membrane as a model system is mainly due to its unique advantages as followings: first, cells entrapped in calcium alginate gel grow in a 3D microenvironment that better mimics the microenvironment *in vivo*; second, the thickness of the flat AC membrane are controllable, which could regulate intercellular distance between cocultured cells, so as to explore the effect of intercellular distance between cocultured cells; finally, the permeability of the flat AC membrane could also be regulated, which are good for studying the degree of contact between cocultured cells.

In this article, methodologies to characterize the thickness and permeability of the flat AC membrane were established. Meanwhile, effects of reaction time, alginate concentration, and M_w of chitosan on the thickness and permeability of the flat membrane were further investigated. Moreover, cell viability in the alginate gel and on the AC membrane was also investigated. It is expected to provide guidance for preparing flat AC membrane properly to establish indirect coculture system, so as to study the effects of intercellular distance and degree of contact between cocultured cells on cell–cell interactions.

EXPERIMENTAL

Materials

Sodium alginate was purchased from Qingdao Crystal Salt Bioscience and Technology Company Corporation (Qingdao, China), of which the viscosity at a concentration of 1.5% (w/v) in 0.9% (w/v) NaCl solution was 220 cP. The M_w and G/M ratio of alginate were determined as 430 kDa and 34/66, respectively. Chitosan samples were degraded from the raw materials provided by Yuhuan Ocean Biomaterials Corporation (Zhejiang, China) using gamma (γ) rays treatment (Key Laboratory of Nuclear Analysis Techniques, Chinese Academy of Sciences). M_w of the degraded chitosan sample was determined by gel permeation chromatography (GPC) which gave the values of 30, 50, and 65 kDa, respectively, and deacetylation degree (DD) is 96%. Fluorescein isothiocyanate (FITC, Sigma-Aldrich, St. Louis, MO)-labeled chitosan was prepared according to the method developed in our previous study.[28] Bovine serum albumin (BSA, $M_w = 66$ kDa) was purchased from Sigma-Aldrich Chemical Co.

Flat Calcium Alginate Gel Fabrication

Sodium alginate was dissolved in 0.9% (w/v) NaCl solution to form a final concentration of 1.5% (w/v). After being filtered by 0.22 μm filter, the solution was stored in a refrigerator (4°C) over night before use to facilitate deaeration. Sodium alginate solution at different concentrations (1.0% (w/v), 0.75% (w/v), and 0.5% (w/v)) was all prepared through diluting the 1.5% (w/v) alginate solution. To prepare the flat calcium alginate gel, 0.3 mL sodium alginate solution was first loaded onto a circular coverslip (diameter = 3.0 cm) gently, and then the coverslip was immersed into 1.1% (w/v) $CaCl_2$ solution for 30 min.

Flat AC Membrane Fabrication

To prepare flat AC membrane, the flat calcium alginate gel prepared above was put into a 6-well cell culture plate filled with 3.0 mL 0.5% (w/v) FITC-labeled chitosan (FITC-chitosan) or chitosan solution in each well. After being reacted for a certain time, a flat AC membrane was generated, followed by a rinsing with 0.9% (w/v) NaCl solution for 3 times.

Measurement of Flat AC Membrane Thickness

Thickness of flat AC membrane was measured by confocal laser scanning microscope (CLSM, TCS-Leica SP2, Germany) equipped with both blue (Ar 488 nm/5 mW), green (He/Ne 543 nm/1.2 mW) laser sources and an inverted microscope

(Leica, DMIRE2, Germany) using 100× objective and 0.5 μm Z-intervals.[29]

Measurement of Binding Amount of Chitosan

The binding amount of chitosan (m_b, μg/cm^2) was defined as the mass of chitosan per unit area of flat membrane. This was determined as measuring the concentration decrease of chitosan during the process of the membrane formation by using gel permeation chromatography (GPC) and calculated by the following equation described by Yu *et al.*[30]:

$$m_b = (C_0\text{-}C_n)V_t/S \quad (1)$$

where C_0 and C_n are the initial chitosan concentration and chitosan concentration in the supernatant after the membrane formation, respectively; V_t is the total volume of the chitosan solution; and S is the total surface area of the flat membrane.

BSA Diffusion

The diffusion through flat AC membranes was studied using BSA as a model protein. The flat hydrogel membranes described above were plated in a side-by-side diffusion cell with 10 mg/mL BSA solution in the donor chamber and 0.9% (w/v) NaCl solution in the receptor chamber. The solutions were stirred to provide uniform concentrations, and a water jacket was used to maintain a consistent temperature. Samples were collected every 2 h from the receptor chamber, and the same volume of 0.9% (w/v) NaCl was added to compensate the volume change after each sample collection. BSA concentration was measured by Bradford method.[31]

BSA diffusion rate (q) was calculated using the following equation:

$$q(\%) = C_d V_d / C_0 V_0 \times 100\% \quad (2)$$

where q is the BSA diffusion rate (or cumulative BSA diffusion), C_0 and C_d are the initial BSA concentration (10 mg/mL) and the BSA concentration diffusing in the receptor chamber, respectively, V_0 and V_d are the initial BSA volume added in the donor chamber and the volume of the receptor chamber, respectively.

The diffusion coefficient was calculated following the equation proposed by Gant *et al.*[32]:

$$Q_t = \frac{ADC_1}{l}\left(t - \frac{l^2}{6D}\right) \quad (4)$$

where Q_t is the total amount of BSA through the membrane until t, A is the hydrogel membrane area exposed to the donor or receptor chambers, D is the diffusion coefficient, C_1 is the BSA concentration in the receptor chamber, and l is the thickness of hydrogel membrane.

Scanning Electron Microscopy Observations

Scanning electron microscopy (SEM) was employed to investigate structure of alginate network at various alginate concentrations (1.5%, 1.0%, and 0.5%, w/v). Calcium alginate hydrogels prepared as described above were washed 3 times with deionized water and frozen at −70°C. The hydrogels were freeze-dried until the water had dehydrated. The hydrogels were mounted on a specimen stub with graphite paste, coated with palladium alloy, and observed under a scanning electron microscope (Supra 55 VP, Zeiss, Oberkochen, Germany).

Cell Culture

Human skin fibroblast cells (HSF, Kunming institute of zoology, Chinese Academy of Sciences) were cultured in Dulbecco's Modified Eagle's medium with high glucose (Thermo Scientific Hyclone), 10% (v/v) newborn calf serum (NCS, Invitrogen), and 1% (v/v) penicillin–streptomycin (pen/strep). To investigate the cell viability, HSF cells were seeded either in the alginate gel, or to the top surface of the AC membrane. For alginate gel cell culture, HSF cells (2×10^6 cells/mL alginate) were mixed with 1.5% alginate solution and immersed into 1.1% (w/v) $CaCl_2$ solution to form flat calcium alginate gel. Then the flat AC membrane was obtained by dipping alginate gel into 0.5% (w/v) chitosan (M_w = 50 kDa) solution for 10 min. For flat AC membrane cell culture, HSF cells were inoculated onto the top side of the membrane. The cells were incubated in a 37°C, 5% CO_2 in a 100% humidified incubator for 2 weeks.

Cell Viability

Cells seeded either in the alginate gel or on the top surface of the membrane were incubated with live/dead staining working solution composed of 2 μM calcein AM (Sigma-Aldrich) and 4 μM ethidium homodimer-1 (ED-1, Sigma-Aldrich) at 37°C for 1.5 h. The cells were then imaged using CLSM.

Statistical Analysis

All reported values were averaged (n = 3) and expressed as mean standard ± deviation. Statistical differences were determined by Student's t test or one-way analysis of variance. Differences were regarded as statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Reaction Time Affects the Thickness of Flat AC Membrane and BSA Diffusion Behavior

Different time points (10, 20, 30, and 60 min) were selected to investigate reaction time on the thickness of AC membrane, which was prepared by 1.5% (w/v) alginate solution and 0.5% (w/v) chitosan solution (M_w = 30 kDa). It showed that the thickness increased with the extension of reaction time [Figure 2(A)]. The thickness was significantly increased from 8.73 ± 0.050 μm (10 min) to 26.44 ± 1.81 μm (30 min), and no obvious difference was observed between the membrane thickness at 30 and 60 min. Consistent with these results, a significant increase in the amount of bound chitosan of the AC membrane (from 15.14 to 86.57 μg/cm^2) was observed with the extension of reaction time, but the amount of bound chitosan at 60 min showed significant higher than that at 30 min. The reason for the results could be found in Figure 2(D). GPC chromatograms of chitosan samples at 30 and 60 min showed that more chitosan molecules with lower M_w took part in the membrane formation during the process from 30 to 60 min.

To study the effect of reaction time (or membrane thickness) on the BSA diffusion behavior, we first evaluated the influence of reaction time on BSA diffusion rate. The flat membranes were prepared, as described above. As shown in Figure 2(B), the BSA diffusion rate declined with the extension of reaction time. The diffusion rate of BSA at 10 min was significantly higher

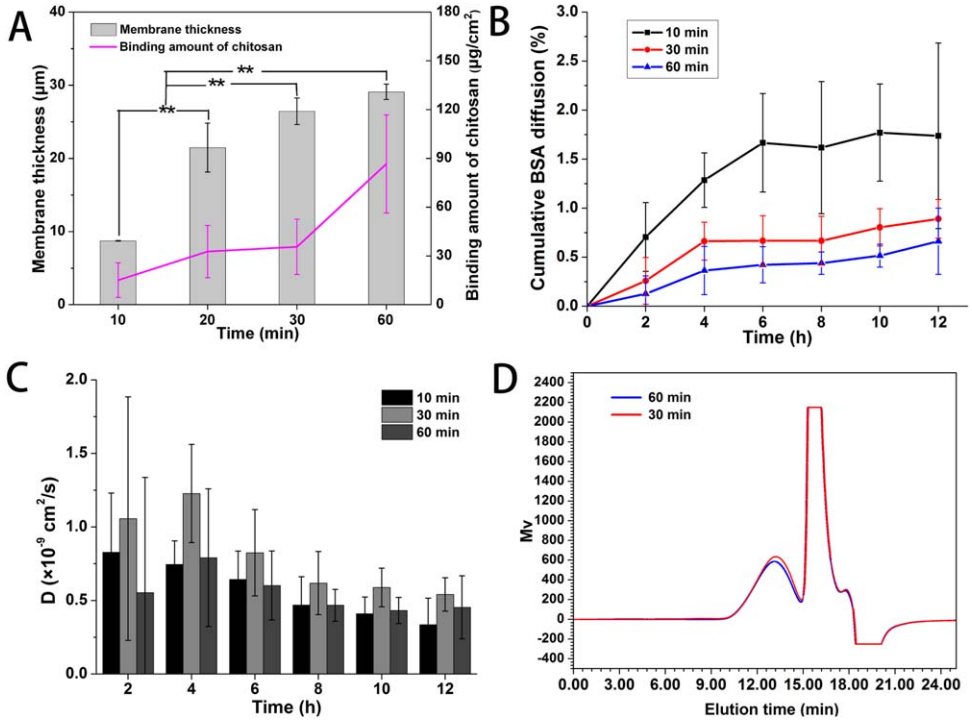

Figure 2. Effect of reaction time on (A) flat AC membrane thickness, (B) BSA diffusion rate, and (C) diffusion coefficients ($n = 3$, $^{**}p < 0.01$). (D) GPC chromatograms of chitosan sample at the reaction time of 30 and 60 min. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

than that of 30 and 60 min at different diffusion time points ($^{**}p < 0.01$). And no significant difference was noted between the two time points (30 and 60 min) ($^{\#}p > 0.05$). Combing with flat AC membrane thickness, this suggested that increase of membrane thickness causes a reduction in the BSA diffusion rate. In addition to BSA diffusion rate, diffusion coefficients through the hydrogel membranes at different reaction time were further evaluated. As shown in Figure 2(C), no significant difference of BSA diffusion was found in the AC membranes with different thickness even though the diffusion time was prolonged up to 12 h, indicating an ignorable role of membrane thickness in affecting the permeability of the AC membrane.

Alginate Concentration Affects the Thickness of Flat AC Membrane and BSA Diffusion Behavior

To characterize the effect of alginate concentration on the thickness of AC membrane and BSA diffusion behavior, alginate solutions at different concentration [1.5% (w/v), 1.0% (w/v), and 0.75% (w/v)] were selected to prepare flat AC membranes with 0.5% (w/v) chitosan (M_w = 50 kDa) for 30 min. Figure 3(A) showed that membrane thickness increased significantly (from 11.21 ± 3.19 to 48.39 ± 1.57 μm) with the decreased concentration of alginate solutions [from 1.5% (w/v) to 0.75% (w/v)]. Similarly, the amount of bound chitosan increased dramatically (from 24.97 to 176.00 μg/cm^2) with the decline of alginate concentration from 1.5% (w/v) to 0.75% (w/v). This phenomenon might be explained as following: it has been demonstrated that concentration of alginate determines the loose degree of the resultant 3D alginate gel network. Alginate gel generated from a lower concentration of alginate solution is much looser than that formed by a relatively higher concentration of alginate. The porous structure of the calcium alginate gels was assessed by SEM [Figure 4(A–C)] and the pore size was calculated according to the morphology. The result showed that the average pore size of alginate gel sample prepared with 0.75% (w/v) alginate was 195.2 ± 20.7 μm, larger than 75.9 ± 17.0 μm prepared with 1.0% (w/v) and 43.3 ± 17.9 μm prepared with 1.5% (w/v) alginate. Thus, more chitosan molecules could be able to diffuse into the relatively looser gel network that formed by a lower concentration of alginate more easily, leading to the increase of membrane thickness and the amount of bound chitosan. To assess the effect of alginate concentration on the BSA diffusion behavior, the flat membranes fabricated by different alginate concentration were first prepared. In view of the influence of membrane thickness on BSA diffusion rate, different AC membranes but with the same thickness (11.2 μm), which were prepared by controlling the reaction time, were made at alginate concentration of 1.5% (w/v), 1.0% (w/v), and 0.75% (w/v), respectively. The reaction time was 30 min [1.5% (w/v)], 6 min [1.0% (w/v)], and 3 min [0.75% (w/v)], respectively. Figure 3(B) showed that the BSA diffusion rate dramatically increased with the alginate concentration decreased ($^{**}p < 0.01$). Figure 3(C) also showed that the diffusion coefficients increased from 0.22 ± 0.048 to 5.25 ± 1.05 cm^2/s with the decreased alginate concentration [from 1.5% (w/v) to 0.75% (w/v)] at 12 h. These results indicate that the average pore size of AC membrane generated by low alginate concentration is much larger than that of high alginate concentration, which was in consistent with previous report that the pore size decreases with the increase of alginate concentration assessed by Cyro-SEM.[34]

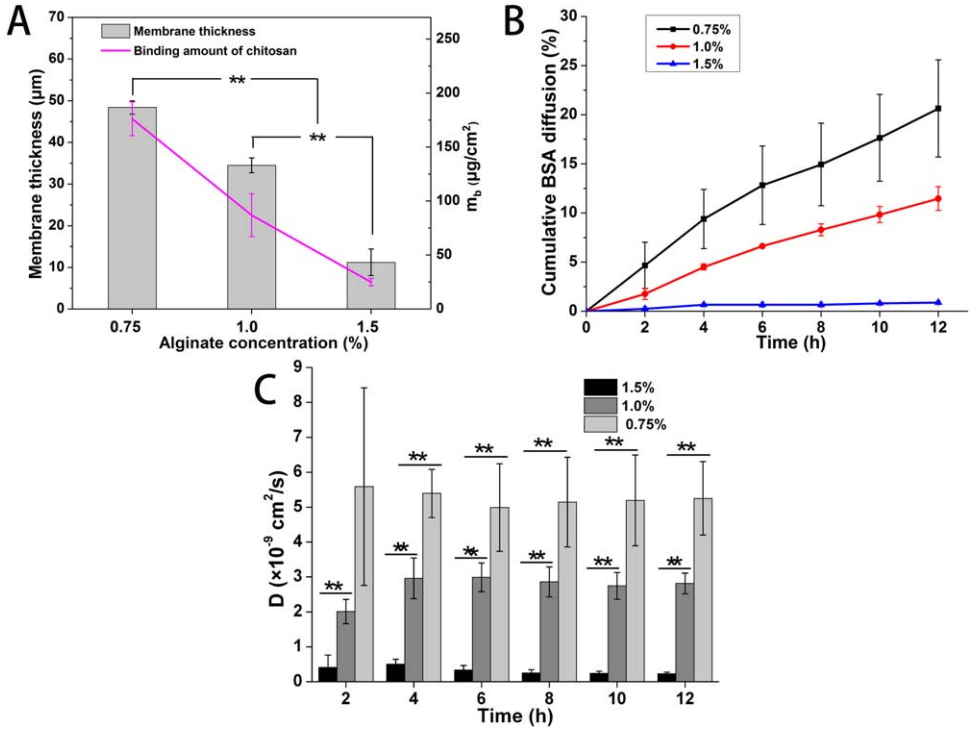

Figure 3. Effect of alginate concentration on the (A) flat AC membrane thickness, (B) BSA diffusion rate, and (C) diffusion coefficients ($n = 3$, ** $p < 0.01$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Molecular Weight of Chitosan Affects the Thickness of Flat AC Membrane and BSA Diffusion Behavior

To evaluate the effect of chitosan molecular weight on the membrane thickness and BSA diffusion behavior, a series of chitosan solutions prepared with the chitosan powders with different M_w (30, 50, and 65 kDa) were employed to form different AC membranes at 1.5% (w/v) alginate concentration. And our results showed that an increase of chitosan M_w ranging from 30 to 65 kDa could lead to a significantly decreased thickness of the flat AC membranes [Figure 5(A)]. Thus, we hypothesize that only the chitosan molecules with a smaller M_w, but not those molecules with higher M_w, took part in the reaction process of the membrane formation, which might be explained by their distinct capability of diffusion into the 3D gel network.

With respect to the evaluation of BSA diffusion behavior, chitosan solutions with M_w of 30 and 65 kDa were adopted for the AC membranes preparation at 1.0% (w/v) alginate concentration, respectively. Considering that the membrane thickness is related to BSA diffusion rate, here, different AC membranes (with different M_w of chitosan molecules) but with a same thickness (10 μm) was prepared through adjusting reaction time. Figure 5(B) showed that the diffusion rate of BSA was significantly lower in the AC membrane with 65 kDa chitosan when compared to that of the AC membrane formed by 30 kDa chitosan after 2 h (*$p < 0.05$). Similarly, the diffusion coefficients in the AC membrane with 65 kDa were also significantly lower than that of the AC membrane formed by 30 kDa chitosan [Figure 5(C)]. This could be explained as follows: the larger molecular weight of chitosan means the larger steric hindrance, which made the resistance of BSA diffusion into the hydrogel membrane increased, when the membrane thickness was constant. These observations are consistent with our previous findings on alginate–chitosan microcapsules.[35] These results indicate that the pore size of the former (AC membrane with 65 kDa chitosan) is smaller than that of the latter.

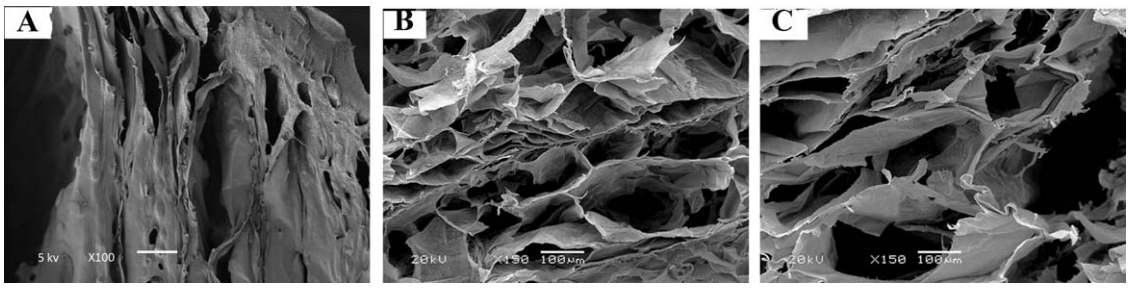

Figure 4. SEM images of gel structure made by (A) 1.5% (w/v) alginate, (B) 1.0% (w/v) alginate, and (C) 0.75% (w/v) alginate.

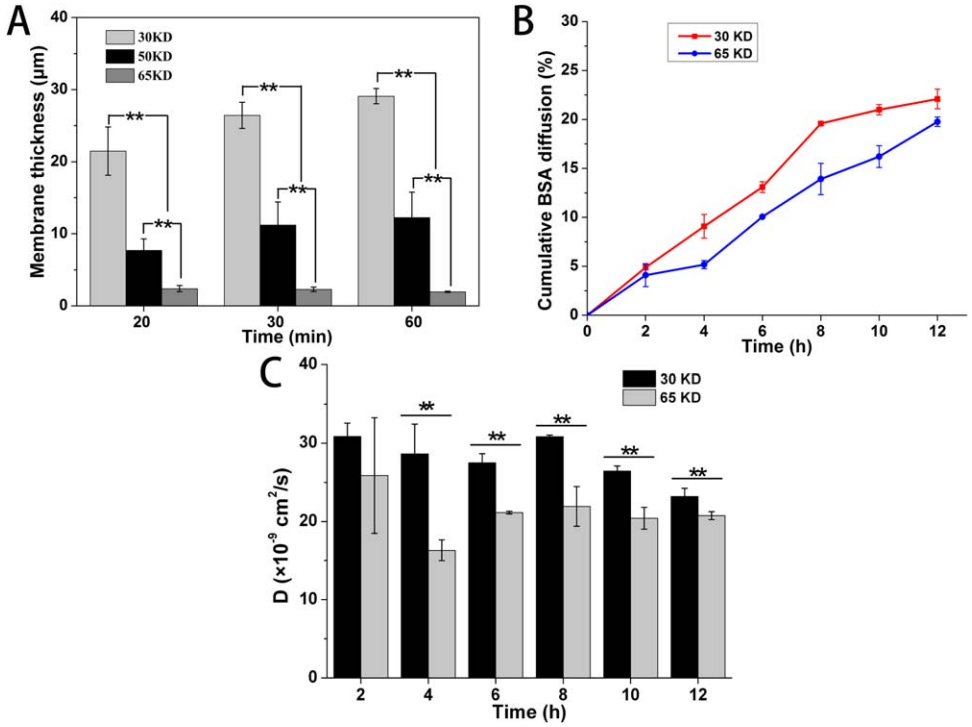

Figure 5. Effect of molecular weight of chitosan on (A) flat membrane thickness, (B) cumulative diffusion BSA rate, and (C) diffusion coefficients ($n = 3$, $^{**}p < 0.01$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Controllable Preparation of the Flat AC Membrane

The commercially porous membranes used to indirect cell coculture system have thickness of around 10 μm, so the distance between the two cocultured cells is fixed. Moreover, the porosity of the membrane is pretty low. Therefore, the effect of intercellular distance and degree of contact between cocultured cells on cell–cell interactions could not be explored by using commercially porous membranes. In contrast, the thickness of porous AC membrane for indirect cell coculture system could be tightly controllable. We could fabricate flat AC membrane

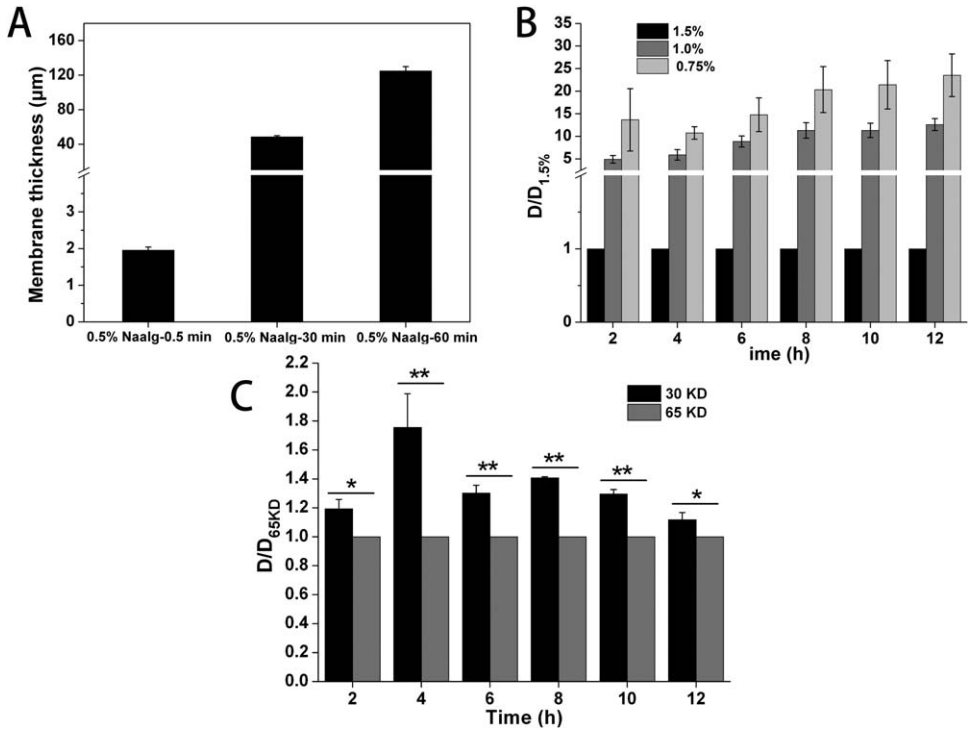

Figure 6. Preparation of tunable flat AC membranes with different (A) thickness and (B) permeability by adjusting alginate concentration and (C) molecular weight of chitosan ($n = 3$, $^{*}p < 0.05$, and $^{**}p < 0.01$).

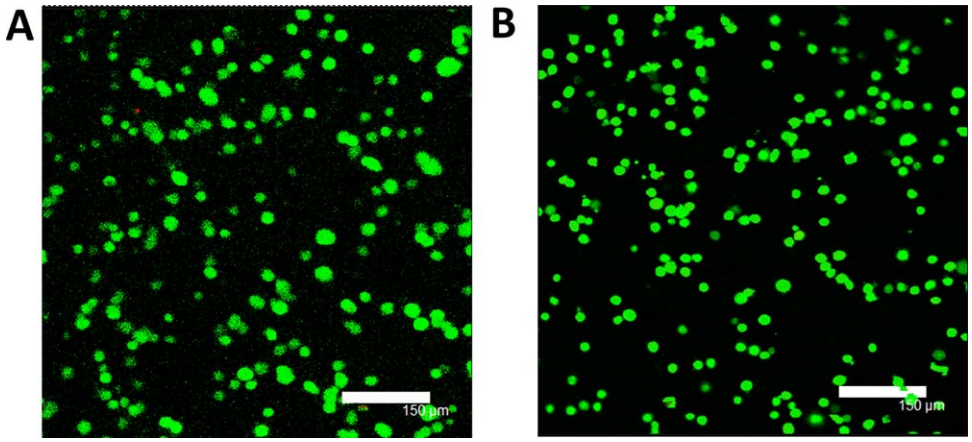

Figure 7. Cell viability (A) in the alginate gel and (B) on the AC membrane detected by live/dead staining. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

down to 2 μm, up to 125 μm, and <5% variation in membrane thickness by adjusting alginate concentration, chitosan M_w, and reaction time [Figure 6(A)]. Thus, the effect of intercellular distance between two cocultured cells could be studied by the new indirect coculture system.

For permeability of the AC membrane, we achieved different pore sizes of membranes by adjusting alginate concentration and M_w of chitosan. Figure 6(B) showed that the ratio of the diffusion coefficient of the membrane generated by 1.0% (w/v) alginate concentration ($D_{1.0\%}$) to that of 1.5% (w/v) alginate concentration ($D_{1.5\%}$) at 12 h was 12.60 and the value of $D_{0.75\%}/D_{1.5\%}$ was 23.52 at 12 h. And Figure 6(C) presented that the ratio of the value of the diffusion coefficient of the membrane incorporated by 30 kDa chitosan to that of 65 kDa chitosan was 1.4 at 8 h. These results suggested that compared to membrane thickness, alginate concentration was a much more important factor to affect diffusion coefficients. Therefore, the effects of degree of contact between cocultured cells could be studied by the flat AC membrane through preparing the hydrogel with different concentration of alginate and incorporating chitosan molecules with different molecular weight.

Cell Viability in the 3D Alginate Gel and on the Flat AC Membrane

Cell viability in the alginate gel and on the AC membrane was evaluated by Calcein-AM/ED-1 staining, where only the live cells exhibit green. After 13 days in culture, the majority of HSF cells in the alginate gel [Figure 7(A)] and on the flat AC membrane [Figure 7(B)] exhibited good viability, suggesting that both alginate gel and flat AC membrane provided a suitable microenvironment for the cells in the alginate gel and on the AC membrane. Compared to the current indirect coculture system based on PC or PET membrane, the improved indirect coculture system are capable of providing a three-dimensional (3D) microenvironment for the cocultured cells that better mimic the microenvironment *in vivo*.

CONCLUSIONS

To study the effect of intercellular distance and degree of contact between cocultured cells on cell–cell interactions, an improved indirect coculture system was constructed in this study using a polyelectrolyte complexes membrane generated by alginate and chitosan. Through establishment methodologies of characterizing flat AC membrane thickness, we are able to fabricate and characterize flat AC membrane down to 2 μm and up to 125 μm in membrane thickness, and <5% variation in membrane thickness. We achieved flat AC membrane with different permeability by calculating the diffusion coefficients of the membrane. Moreover, cells in the alginate gel (3D microenvironment) and on the AC membrane could maintain high cell viability. Previously, the commercially porous membranes used to indirect coculture system have thickness of around 10 μm, so the distance between the cocultured cells is fixed. And the porosity is pretty low, which leads to low contact degree of the cocultured cells. In addition, the current indirect coculture systems are not capable of providing a three-dimensional (3D) microenvironment for the cocultured cells. We also investigate the effects of reaction time, concentration of alginate, and M_w of chitosan on the properties of the flat membrane thickness and permeability, respectively. Our data support that all the three factors above influence the membrane thickness. Compared to the reaction time, alginate concentration and M_w of chitosan play more dominant roles in determining the membrane thickness and diffusion coefficients. Taken together, a porous hydrogel membrane, flat AC membrane was developed in this study, which could provide a robust tool for the indirect coculture investigation.

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