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Biomaterials 24 (2003) 1771–1780
Biomaterials
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Microcapsules with improved mechanical stability for hepatocyte culture

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Received 19 August 2002; accepted 11 November 2002

Abstract

Packed-bed or fluidized-bed bioreactor filled with microencapsulated hepatocytes has been proposed as one of the promising designs for bioartificial liver assist device (BLAD) because of potential advantages of high mass transport rate and optimal microenvironment for hepatocyte culture. Recently, we have developed a microcapsule system for the encapsulation of hepatocytes. The microcapsules consist of an inner core of modified collagen and an outer shell of terpolymer of methyl methacrylate, methacrylate and hydroxyethyl methacrylate. Cells encapsulated in these microcapsules exhibit enhanced cellular functions. Improving the mechanical stability of the microcapsules to withstand the shear stress induced by high perfusion rate would be crucial to the success of BLAD applications. In this study, we investigated the effects of terpolymer molecular weight (M_w) on the mechanical property of these microcapsules and the differentiated functions of encapsulated hepatocytes. Six terpolymers with different M_w were synthesized using radical polymerization in solution by adjusting the reaction temperature and the initiator concentration. All the terpolymers formed microcapsules with the methylated collagen. While the terpolymer M_w had little effect on the capsule membrane thickness and permeability of serum albumin, the mechanical property of the microcapsules was significantly improved by the higher M_w of the terpolymer. Differentiated functions of the hepatocytes cultured in the microcapsules, including urea synthesis, albumin synthesis and cytochrome P450 metabolic activity, were not significantly affected by the terpolymer M_w.

Keywords: Microcapsule; Cell-encapsulation; Hepatocyte; Liver assist device; Bioartificial liver

1. Introduction

Hepatocytes are anchorage-dependent cells and sensitive to their surrounding microenvironment [1,2]. Liver tissue engineering in the form of hepatocyte transplantation or extracorporeal assist device requires a favorable culture microenvironment for the hepatocytes to maintain their viability and differentiated functions. A successful bioartificial liver assist device (BLAD) would also require a culture configuration amenable to an optimal bioreactor design. While hollow fiber bioreactor may excel in mass transfer considerations, the conventional hollow fiber materials fail in promoting hepatocyte attachment and maintaining the differentiated functions of hepatocytes [3,4]. A packed-bed or fluidized-bed bioreactor based on microencapsulated hepatocytes may provide an attractive alternative. Besides providing immunoprotection to the xenogenic hepatocytes, the microcapsule configuration offers a high surface area-to-volume ratio to facilitate maximal mass transport [5,6]. Moreover, it facilitates manipulation of the microenvironment surrounding the hepatocytes to maximize sustenance of hepatic functions via selection of suitable substrates for microencapsulation, addition of growth factor controlled release formulations, and inclusion of supporting cells for co-culture.

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0142-9612/03/$ - see front matter
doi:10.1016/S0142-9612(02)00580-X

Microcapsules formed by the complex coacervation of alginate and polycations have been extensively studied for hepatocyte encapsulation [7–10]. However, the results of hepatocyte function maintenance are less than ideal because alginate is a poor material for hepatocyte attachment and some polycations, such as poly-L-lysine, are toxic to hepatocytes. An alternative is poly(hydroxyethylmethacrylate-methylmethacrylate) microcapsule prepared by interfacial precipitation that allows the incorporation of collagen or Matrigel along with cells inside the microcapsules [11,12]. In this design, collagen or Matrigel serves as a good substrate for cell adhesion and culture, and the polyacrylate provides the structural stability. This microencapsulation process does involve the use of organic solvents such as dimethylsulfoxide and dodecane that are detrimental to cell viability.

Recently, we have developed a hybrid microcapsule prepared by complex coacervation between a modified biopolymer, methylated collagen, and a synthetic negatively charged terpolymer of methyl methacrylate (MMA), methacrylate (MAA) and hydroxyethyl methacrylate (HEMA) for hepatocytes culture [13]. The microcapsules consist of an inner core of modified collagen and an outer shell of terpolymer with tunable structure and property. The modified collagen is synthesized by partial methylation of the carboxyl side groups in collagen, and it carries net positive charges at neutral pH. This modified collagen serves as a polycation and has been shown to be a good substrate for hepatocytes. The resulting microcapsules show promise in BLAD design, with functions of the encapsulated hepatocytes improved over the 2D culturing conditions, and with semi-permeability adequate for immunoprotection nutrient transport. These microcapsules could be prepared under aqueous condition without the use of any organic solvent.

One of the main advantages of this microcapsule system is the tunable structure of terpolymer and collagen that allow for the optimization of the microcapsule properties. For example, the degree of collagen modification may affect its affinity to terpolymer and the resulting membrane stability; terpolymer structures may influence the membrane thickness, permeability, and mechanical properties, etc. This report focused on the effect of terpolymer molecular weight (M_w) on the mechanical property of these microcapsules and on the functions of the encapsulated hepatocytes. A series of terpolymers with different molecular weights were synthesized using radical polymerization in solution by adjusting the reaction temperature and the initiator concentration. Microcapsules containing primary rat hepatocytes were prepared using this series of terpolymers. Their membrane properties and mechanical properties were characterized, and cellular functions of the hepatocytes cultured in these microcapsules were evaluated and correlated with the terpolymer M_w.

2. Materials and methods

2.1. Materials

Methacrylic acid (MAA), methyl methacrylate (MMA), hydroxyethyl methacrylate (HEMA), 2,2′-azobisisobutyronitrile (AIBN) were purchased form Sigma-Aldrich Chemicals Ltd. (Singapore). The monomers (MAA, MMA and HEMA) were purified by vacuum distillation, and AIBN was recrystallized from ethanol before use. Methanol, ethanol, 2-propanol and petroleum ether were purchased from Merck Pte. Ltd. (Singapore). Collagen (Type I, Vitrogen 100) was obtained from Cohesion Technologies (USA).

2.2. Terpolymer synthesis and characterization

Terpolymers were synthesized by radical polymerization in 2-propanol using AIBN as initiator. MMA, MAA and HEMA were fed in a fixed molar ratio of 50:25:25, and different initiator concentrations and reaction temperatures were used to modulate the molecular weight of terpolymers.

A 100-ml two-neck round bottom flask was charged with MMA (5.01 g, 50 mmol), MAA (2.15 g, 25 mmol), HEMA (3.25 g, 25 mmol) and 25 ml of 2-propanol. Under ice cooling and magnetic stirring, nitrogen was introduced into the solution for 0.5 h to remove oxygen before the desired amount of AIBN was added (see Table 1). The flask was transferred to an oil bath set at the desired temperature, and polymerization was performed under magnetic stirring for 16 h, after which the flask was chilled to room temperature. Fifty milliliters of ethanol was added to dissolve the residue. Terpolymer was precipitated by adding the above solution to a large excess of low-boiling-point petroleum ether. Precipitation was repeated once using the ethanol–petroleum ether solvent pair. The resulted terpolymer was dried under vacuum and dissolved in 1 N NaOH to convert the carboxyl acid groups to sodium carboxylate. The final terpolymer in sodium salt form was purified by dialysis against water for 2 days, followed by lyophilization. ^{1}H-NMR spectrum (D_2O, δ ppm, recorded on a Bruker ACF 300 FT-NMR spectrometer, 300 MHz): 0.6–1.15 (m, $C\underline{H}_3$ of all three types of repeating units), 1.5–2.1 (m, $C\underline{H}_2$ of all three units), 3.57 (s, $OC\underline{H}_3$ of MMA unit), 3.78 (s, $HOC\underline{H}_2$ of HEMA unit), 4.04 (s, $COOC\underline{H}_2$ of HEMA unit).

The compositions of terpolymers (MMA, MAA and HEMA contents) were calculated from ^{1}H-NMR spectra and the acid–base titration curves according to the reported method [14]. The molecular weights of this series of terpolymers were determined by gel permeation chromatography (GPC) using a Waters 2690 separation module (Waters Corporation, USA) equipped with a

Table 1
Molecular weights and intrinsic viscosities of terpolymers prepared under different conditions

Polymer	Reaction temperature (°C)	AIBN concentration (wt%)	M_w ($\times 10^5$)	M_n ($\times 10^5$)	Polydispersity (M_w/M_n)	Intrinsic viscosity[a] (dl/g)
Ter 1	70	1.0	1.68	0.92	1.83	0.215
Ter 2	70	0.5	3.2	1.32	2.42	0.353
Ter 3	60	1.0	3.44	1.55	2.22	0.351
Ter 4	70	0.25	6.66	2.51	2.65	0.478
Ter 5	60	0.5	8.03	3.07	2.62	0.587
Ter 6	60	0.25	10.6	4.14	2.56	0.940

[a] In sodium phosphate buffer (0.1 M, pH 7.4) with 0.15 M NaCl at room temperature.

serial combination of a Phenomenex polysep-GFC-P linear column (Phenomenex Inc., USA) and a Shodex Protein KW-804 column (Showa Denko K. K., Japan). Samples were detected with a multiangle light scattering detector (MiniDawn, Wyatt Technology, USA) and a differential refractive index detector (Optilab DSP interferometric refractometer, Wyatt Technology) at room temperature. Phosphate buffer (0.1 M, pH 7.4) with 0.15 M NaCl was used as the mobile phase at 0.5 ml/min flow rate.

Intrinsic viscosity of terpolymers was determined using a typical Ubbelhode viscometer at room temperature using the same phosphate buffer in GPC determination as solvent.

The acid–base titration curve of the terpolymers was conducted on a digital pH meter (Orion 420A, Orion Research Inc., USA). Fifty mg of terpolymer was dissolved in 25 ml of distilled water, and the pH of the solution was adjusted to approximately 12 by 0.1 M NaOH. The solution was then back titrated with 0.1 M standard HCl solution.

2.3. Hepatocytes isolation, collagen modification and microcapsule preparation

Hepatocytes were harvested from male Wistar rats weighing 250–300 g by a two-step in situ collagenase perfusion method. Viability of the hepatocytes was determined to be 90–95% by Trypan Blue exclusion assay. Modified collagen that carries net positive charges was prepared by esterification of collagen with methanol in 0.1 M HCl at 4°C for 6 days.

The microcapsules with or without hepatocytes were formed by the complex coacervation between terpolymers of different M_w's and the modified collagen using the reported method [13]. Briefly, freshly isolated hepatocytes were suspended in the modified collagen solution (1.5 mg/ml) in phosphate-buffered saline (PBS) and extruded from a 27.5-gauge needle into a 1% (w/v) terpolymer solution in PBS. The microcapsules were harvested by sedimentation methods, washed with PBS, and used for subsequent cell culture or physico-chemical characterization.

2.4. Membrane property

Membrane thickness of the microcapsules was measured by a confocal microscope (FluoView 300, Olympus America Inc., USA) with the associated software. The permeability of the microcapsules was estimated by measuring the release of encapsulated FITC-labeled bovine serum albumin (FITC-BSA, Sigma-Aldrich Singapore) in 1.5-ml tubes at room temperature. FITC-BSA was dissolved in the modified collagen solution at a concentration of 2 mg/ml. One hundred μl of this solution was slowly extruded into 1 ml of terpolymer solution to form microcapsules. After washing with PBS, the microcapsules containing FITC-BSA were immersed in 1 ml of FITC-BSA solution (2 mg/ml) in PBS and allowed to equilibrate for 2 h at room temperature. The microcapsules were washed quickly and the release profile of FITC-BSA was obtained at a 5–15-min interval. The amount of FITC-BSA released in the supernatant was analyzed on a microplate spectrofluorometer (SPECTRAmax GEMINI XS, Molecular Devices Corp., USA).

Morphology of the membrane was determined by scanning electron microscope (JSM 5600, Jeol USA Inc., USA). The microcapsules were fixed with 3% glutaraldehyde on a coverglass coated with poly-L-lysine for 1 h, after which they were washed gently with 1 × PBS for 5 min. The microcapsules were then post-fixed with osmium tetraoxide for 1 h and dehydration was accomplished using a graded series of ethanol (25%, 50%, 75%, 95%, and 100%). The microcapsules were critical-point-dried for about 2 h, mounted onto an aluminum stub and sputter-coated with gold before viewing under SEM.

2.5. Mechanical property of microcapsules

Mechanical property of the microcapsules was determined by a shear force test. Around 60 microcapsules with diameter ranging from 300 to 500 μm were added in the well of a 12-well plate (Nunc, USA). The microcapsules were equilibrated in 2 ml of Hepato-ZYME culture medium for 1 day. The plate was then

fixed on an orbital shaker (Model 420, Forma Scientific, USA) and agitated with a frequency of 250 rpm at 37°C. The percentage of intact microcapsules was counted and recorded at various time points. Images of microcapsules at different time point were taken with a digital camera for visual comparison.

2.6. In vitro culture and functional analysis of the microencapsulated hepatocytes

Microencapsulated hepatocytes (equivalent to 0.1×10^6 cells) were cultured in 1.0 ml HepatoZYME serum-free medium (GIBCO Laboratories, USA) in a 12-well tissue culture plate (Nunc) under humidified atmosphere with 5% CO_2. The culture medium was supplemented with 0.1 μM dexamethasone, 10 nM insulin, 20 ng/ml epidermal growth factor, and 100 units/ml penicillin and 100 mg/ml streptomycin. The medium was refreshed daily. The collected medium was centrifuged in 14,000 rpm for 10 min and the supernatant was stored at −20°C for albumin assay.

2.7. Albumin determination by ELISA

The albumin concentration in the culture medium collected at various time points during the culture period was determined by a competitive enzyme linked immunosorbent assay (ELISA) according to the protocol reported by Friend et al. [15]. Briefly, samples were serially diluted, and peroxidase conjugated rabbit antibody against rat albumin (ICN Biochemicals, USA) was added to a final concentration of 0.6 μg/ml. After incubation at 37°C for 2 h, 100 μl aliquots of each sample were transferred to 96-well Maxisorp plates (Nunc-Immuno^{TM}, Nunc), which were pre-coated by incubating with 100 μl/well of rat albumin at 0.2 μg/ml in PBS overnight at 4°C, and washed three times with 0.05% Tween-20 in PBS before use. The samples were incubated in the wells at room temperature for 2 h in a humidified chamber. Subsequently, the plates were washed three times with 0.05% Tween-20 in PBS and filled with 100 μl/well 1-Step Turbo TMB-substrate (Pierce, USA). Plates were incubated at room temperature in the humidified chamber for 30–45 min and reaction was stopped by adding 100 μl of 2 N H_2SO_4. The optical density (OD) at 450 nm of the solution in each well was determined on a microplate reader (Model 550, Bio-Rad Laboratories, USA). Rat albumin concentrations of the samples were calculated from the ODs using rat albumin as standards.

2.8. Urea synthesis assay

To assess the urea synthesis function of the encapsulated hepatocytes, the culture medium was replaced with fresh medium containing 1 mM NH_4Cl. The microcapsules were incubated in this medium for 90 min, before the medium was again replaced with the normal medium. The collected medium was tested for urea production using a colorimetric kit from Sigma Diagnostic (Sigma-Aldrich).

2.9. Assay for cytochrome P450 activity

Cytochrome P450 activity of the microencapsulated hepatocytes at the 4th day of the culture was evaluated by the *O*-dealkylation activity using 7-ethoxyresorufin as a substrate. 7-Ethoxyresorufin is the substrate for the cytochrome P450IA1 [16]. Briefly, a small quantity of microcapsules was incubated in the medium that contained 39.2 μM 7-ethoxyresorufin for 2 h. The microcapsules were then imaged under the confocal microscope (FluoView 300) with a rhodamine filter within 2 h.

3. Results and discussion

3.1. Synthesis and characterization of terpolymers

Terpolymers were synthesized by the free radical solution polymerization using 2,2′-azobisisobutyronitrile (AIBN) as an initiator and 2-propanol as solvent (Fig. 1). In free radical polymerization, polymer molecular weight could be adjusted by a number of experimental parameters including reaction temperature, concentrations of monomers and initiator, initiator efficiency, addition of chain transfer agents, etc. In this study, we focused on reaction temperature and initiator concentration. Theoretically lower temperature would yield higher molecular weight, but should still be high enough to initiate the chain growth process with reasonable initiator efficiency. Therefore, the polymerization temperature was set at 60°C or 70°C. The initiator concentration is another important parameter for controlling the polymer M_w. Kinetic chain length is proportional to $[I]^{-1/2}$, where $[I]$ represents initiator concentration. Here, the initiator amount was set to 0.25%, 0.5% and 1% (w/w) of the total monomer amount. With different combinations of these two parameters, we have obtained a series of six polymers with different M_w's shown in Table 1.

The MWs of the terpolymers were characterized by gel permeation chromatography (GPC). A combination of a multi-angle light scattering detector and a refractive index detector was used to measure the absolute MWs. Fig. 2A and B show a typical set of GPC chromatograms of this series of terpolymers. The terpolymers are polyelectrolytes whose hydrodynamic radii depend on polymer concentration, pH, or ionic strength of the solvent. Suitable eluent buffer with high ionic strength is typically needed to reduce intramolecular interactions

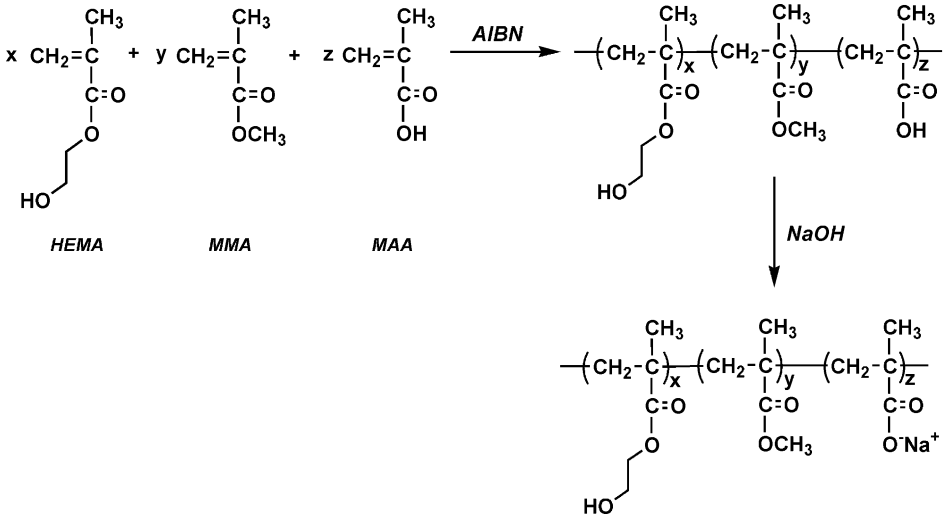

Fig. 1. The synthetic scheme of MMA–MAA–HEMA terpolymer.

and interaction between polymer and column. A phosphate buffer (0.1 M, pH 7.4) with 0.15 M NaCl was found to be a suitable elution buffer for these polymers, as indicated by the aggregation-free chromatograms. The almost linear decrease of terpolymer's molecular mass (determined by MALS) vs. retention volume (Fig. 2B) indicated that the mechanism of terpolymer separation in the column is mainly based on the hydrodynamic radius or M_w of the terpolymers. Based on the chromatograms, the weight (M_w) and number (M_n) average molecular weights of the terpolymers were calculated and shown in Table 1. The Mw of this series of terpolymers ranged from 1.68×10^5 to 1.06×10^6. Both lower temperature and lower initiator concentration were in favor of obtaining terpolymers with higher MW. The highest MW was obtained (Ter 6, $M_w = 10.6 \times 10^5$, $M_n = 4.14 \times 10^5$) at 60°C and with 0.25 wt% AIBN concentration.

The intrinsic viscosities of the terpolymers were also measured to correlate with the MW measurements. The same buffer (0.1 M PBS + 0.15 M NaCl) was used as the solvent to minimize the polyelectrolyte effect, particularly at the low polymer concentration. Satisfactory linear regression was obtained in the plot of the reduce viscosity vs. polymer concentration, from which the intrinsic viscosity of the polymer was calculated, and shown in Table 1. A good correlation between the intrinsic viscosity [η] and terpolymer Mw was found as follows (Fig. 2C):

$$[\eta] = 4.019 \times 10^{-5}(M_w)^{0.712}.$$

The composition of the terpolymer is another parameter that directly affects the physicochemical properties of the microcapsule membrane. In our previous studies [13], we have optimized the feed ratio of the three monomers as 50:25:25 (MMA:MAA:HEMA) to achieve suitable membrane permeability and cellular functions. This ratio was kept constant in this study in order to maintain similar membrane permeability and pore size. The final composition of the terpolymer was determined by its proton-NMR spectrum and acid–base titration curve. The results (Table 2) suggest that the reaction temperature and initiator concentration had little impact on the composition and ionization degree of the polymers at pH 7.4, although these parameters controlled the MWs of the terpolymers. Therefore the following discussion will concentrate on the relationship between the microcapsule properties and the M_w effect of the terpolymers.

3.2. Microcapsule formation

Microcapsules were prepared by complex coacervation of four different terpolymers (Ter 1, Ter 3, Ter 5 and Ter 6) and modified collagen, respectively. The terpolymer concentration was fixed at 1.0% (w/v) and collagen concentration 1.5 mg/ml. Microcapsules were formed readily in all cases. By gross observation, microcapsules were formed at a faster rate with the higher M_w-terpolymers. This suggested that terpolymer with higher M_w complexed with methylated collagen more efficiently.

3.3. Membrane properties

Although the rate of microcapsule formation increases with M_w, it does not affect the thickness of the resultant capsule membrane. As shown in Fig. 3, microcapsules prepared from terpolymers with different M_w's had similar membrane thickness, around 2 μm as determined by confocal microscopy. SEM images of the freeze-dried microcapsules revealed that the membrane

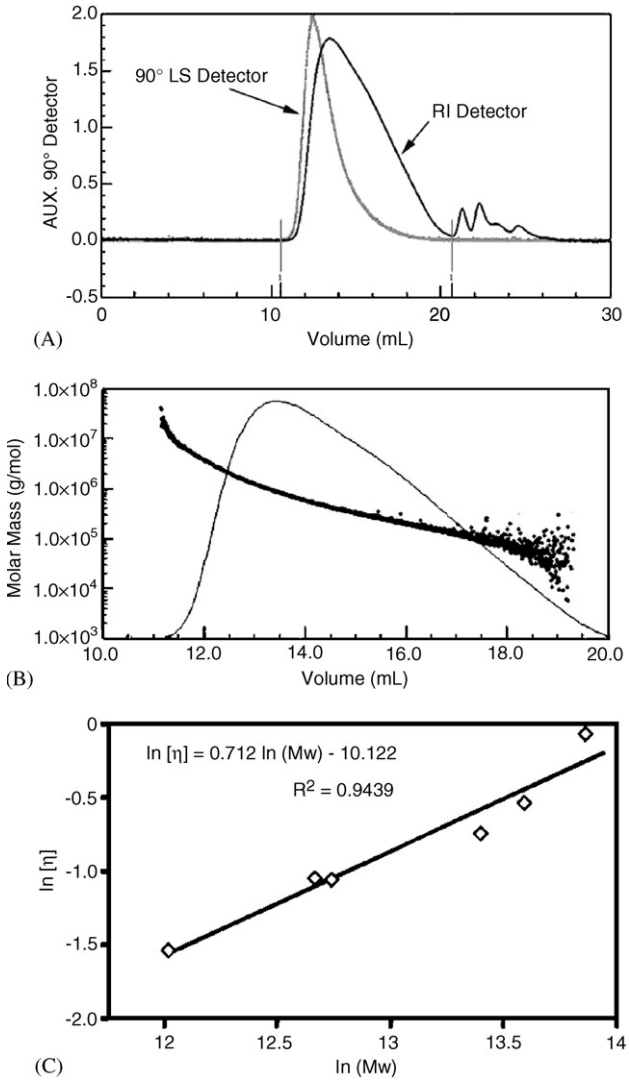

Fig. 2. Characterization of the molecular weight of terpolymer: (A) Chromatograph of Ter 6; (B) molecular weight distribution of Ter 6 and molar mass vs. elution volume; (C) correlation between terpolymer molecular weight and intrinsic viscosity measured in sodium phosphate buffer (0.1 M, pH 7.4) with 0.15 M NaCl at room temperature.

pore size of capsules made from higher M_w-terpolymer was slightly smaller than that of capsules made from terpolymer with lower M_w (Fig. 4). Nevertheless, such a difference in pore size did not have a significant impact on the permeability of BSA. FITC-BSA was encapsulated into capsules prepared from different terpolymers and the release rate of the marker protein was followed and compared. The diffusivity of FITC-BSA through different capsule membranes were comparable. About 80% of FITC-BSA was released from the microcapsules in about 15 min, reaching 100% within an hour (Fig. 5).

3.4. Mechanical stability

Mechanical stability of the microcapsule is an important parameter, since it not only facilitates

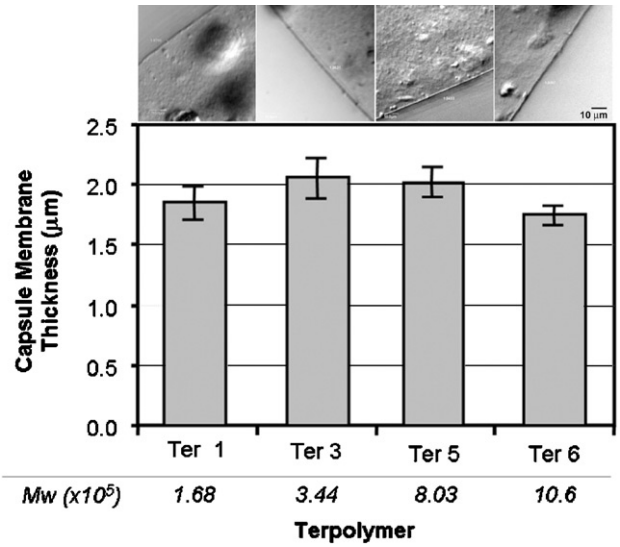

Fig. 3. Membrane thickness of different microcapsules prepared from terpolymer Ter 1 ($M_w = 1.68 \times 10^5$); Ter 3 ($M_w = 3.44 \times 10^5$); Ter 5 ($M_w = 8.03 \times 10^5$) and Ter 6 ($M_w = 10.6 \times 10^5$), and their confocal microscopy images.

Table 2
The overall composition and ionization degree at pH 7.4 of terpolymers prepared under different conditions as defined in Table 1

Polymer	Composition[a] (mol%)			Ionization degree[b] at pH 7.4 (%)
	MAA	HEMA	MAA	
Ter 1	34.10	24.11	41.79	90.3
Ter 2	35.11	24.34	40.56	85.9
Ter 3	36.62	25.02	38.36	88.5
Ter 4	33.72	25.49	40.79	87.5
Ter 5	36.40	25.10	38.49	91.4
Ter 6	34.69	25.12	40.19	88.2
Average	35.11 ± 1.19	24.86 ± 0.53	40.03 ± 1.35	88.63 ± 1.97

[a] The overall composition was determined by ^{1}H-NMR spectra and acid–base titration.
[b] The ionization degree was estimated by acid–base titration curve as described in experimental section.

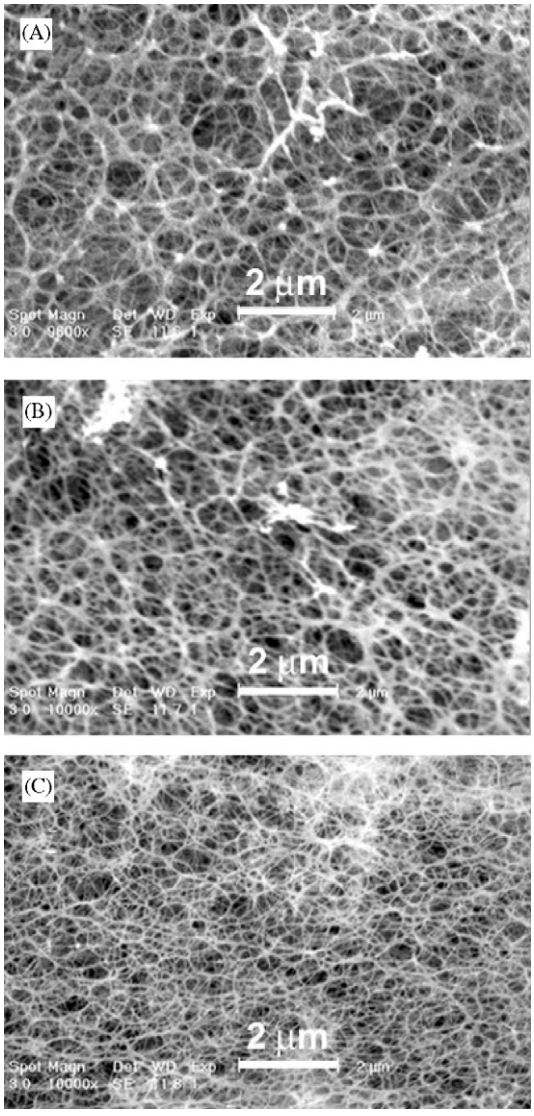

Fig. 4. SEM images of freeze-dried microcapsule membranes prepared from A: Ter 1 ($M_w = 1.68 \times 10^5$); B: Ter 3 ($M_w = 3.44 \times 10^5$); C: Ter05 ($M_w = 8.03 \times 10^5$).

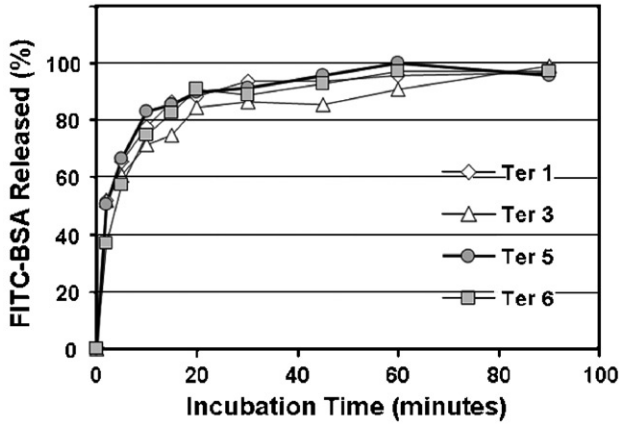

Fig. 5. Release of FITC-BSA from different microcapsules prepared from Ter 1 ($M_w = 1.68 \times 10^5$); Ter 3 ($M_w = 3.44 \times 10^5$); Ter 5 ($M_w = 8.03 \times 10^5$) and Ter 6 ($M_w = 10.6 \times 10^5$).

production and handling, but most importantly determines the applicability of the microcapsules in a packed-bed or fluidized-bed bioreactor under shear stress. To evaluate the stability of the microcapsules under shear condition, about 60 microcapsules were put into 1 ml of PBS inside the well of a 12-well-plate and agitated on an orbital shaker at 250 rpm and 37°C. The numbers of broken capsules were counted and used as an indication of the mechanical stability of the microcapsules. Fig. 6A and B showed the fraction of broken capsules at different time points. Thirty-five percent of microcapsules formed from Ter 1 ($M_w = 1.68 \times 10^5$) ruptured after 3 h of shaking. At the same time point, the rupture percentage of microcapsules prepared from Ter 3 ($M_w = 3.44 \times 10^5$) was only 5.2%, further dropping to 1.7% for capsules made from Ter 5 ($M_w = 8.03 \times 10^5$). No capsules was ruptured for Ter 6 with the highest M_w ($M_w = 10.6 \times 10^5$) after 3 h of shaking. After 12 h of shearing under this condition, all the microcapsules made from Ter 1 and Ter 3 were ruptured; whereas microcapsules prepared from Ter 5 had a rupture percentage of 22.4%. At this time point, microcapsules prepared from Ter 6 still remained intact.

Since the microcapsules prepared from different terpolymers had a similar membrane thickness, this improved mechanical stability of the capsules made with higher M_w-terpolymer was most likely due to the higher membrane strength, a benefit of the chain entanglement effect.

3.5. Hepatocyte functions

It is important to investigate how the increased MW of terpolymers, although yielding improved mechanical stability of the microcapsules, would influence the hepatocyte functions. For this evaluation, four batches of microcapsules containing primary rat hepatocytes were prepared from four terpolymers with different M_w's: Ter 1, Ter 3, Ter 5 and Ter 6 (see Table 1 for their M_w's) and the same methylated collagen. These microcapsules were cultured in HepatoZYME serum free medium and hepatocyte functions including urea synthesis, albumin secretion and cytochrome P450 activity were analyzed at various time points (shown in Figs. 7–9).

The albumin secretion levels of the hepatocytes in all four types of microcapsules were comparable during the first three days of the culture. However, albumin secretion had a significant decrease at the later time points (days 5 and 7). Albumin secretion was best maintained for hepatocytes encapsulated with the lowest M_w-terpolymer (Ter 1) (Fig. 7). The analysis for urea synthesis function revealed that hepatocytes encapsulated by different terpolymers generally exhibited similar level of urea synthesis function, although microcapsules prepared from Ter 6 (with the highest M_w) expressed

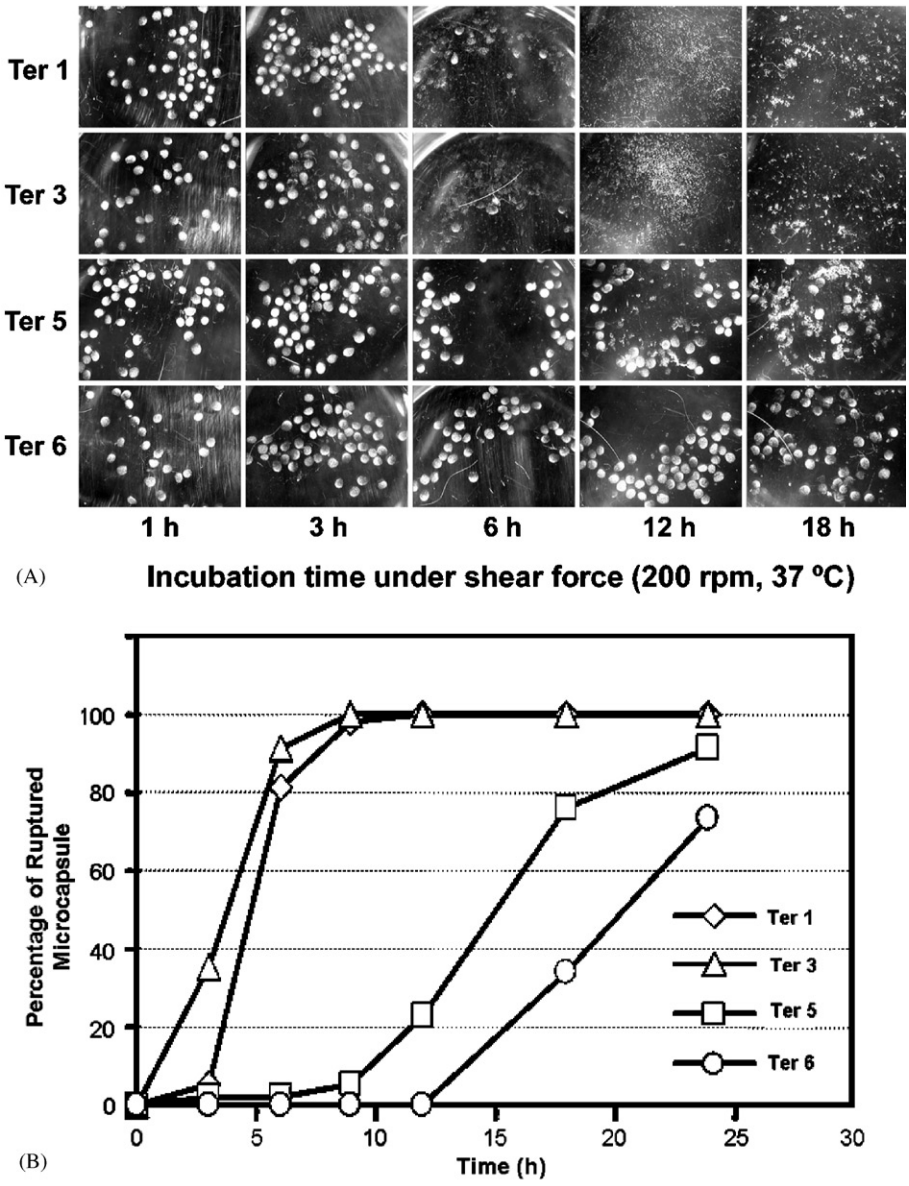

Fig. 6. Mechanical stability of different microcapsules prepared from Ter 1 ($M_w = 1.68 \times 10^5$); Ter 3 ($M_w = 3.44 \times 10^5$); Ter 5 ($M_w = 8.03 \times 10^5$) and Ter 6 ($M_w = 10.6 \times 10^5$): (A) Images of microcapsules incubated at 37°C in PBS at different time points under defined shear condition; (B) percentage of ruptured microcapsules under the same condition.

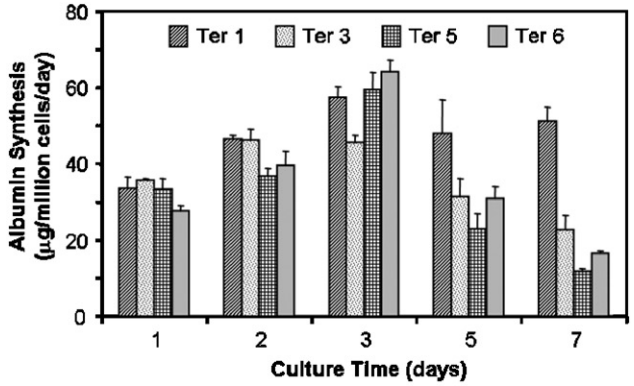

Fig. 7. Albumin secretion level of hepatocytes cultured in different microcapsules prepared from Ter 1 ($M_w = 1.68 \times 10^5$); Ter 3 ($M_w = 3.44 \times 10^5$); Ter 5 ($M_w = 8.03 \times 10^5$) and Ter 6 ($M_w = 10.6 \times 10^5$).

slightly higher level of urea synthesis than other groups on the first day of the culture (Fig. 8). Fig. 9 showed a typical confocal fluorescence image of hepatocytes in one capsule, which had been cultured for 4 days. This images were captured after exposing the microcapsules to 7-ethoxyresorufin for 2 h. The product resorufin transformed by hepatocyte P450IA1 was shown in red in the image. The fluorescence intensity was proportional to the enzyme activity of P450IA1. The images suggested that cells in all four different batches of microcapsules had comparable P450 enzyme activity. Taken together, the functions of hepatocytes encapsulated by different terpolymers had similar functional profiles when cultured in HepatoZYME medium, except that the albumin secretion function was best maintained

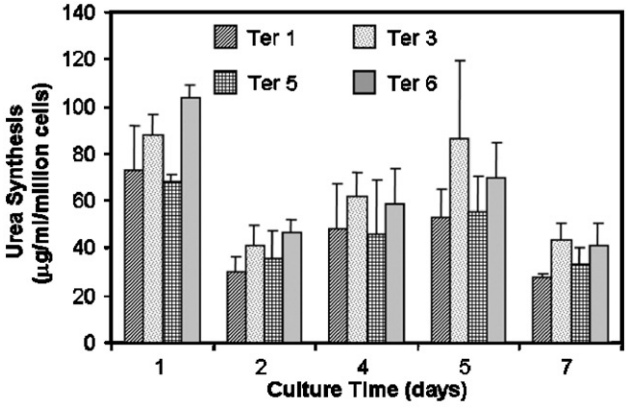

Fig. 8. Urea synthesis level of hepatocytes cultured in different microcapsules prepared from Ter 1 ($M_w = 1.68 \times 10^5$); Ter 3 ($M_w = 3.44 \times 10^5$); Ter 5 ($M_w = 8.03 \times 10^5$) and Ter 6 ($M_w = 10.6 \times 10^5$).

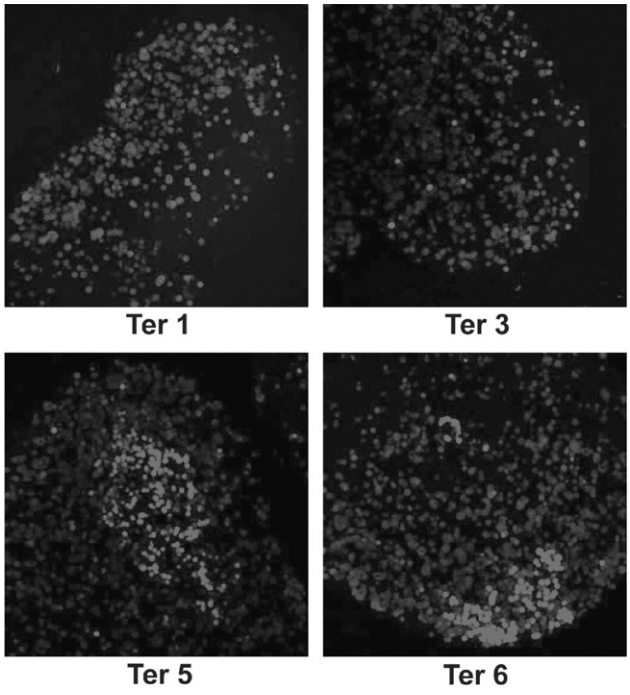

Fig. 9. Cytochrome P450IA1 activity of microencapsulated hepatocytes on the 4th day of culture determined by the EROD assay and revealed by confocal fluorescence microscopy. The microcapsules were prepared from Ter 1 ($M_w = 1.68 \times 10^5$); Ter 3 ($M_w = 3.44 \times 10^5$); Ter 5 ($M_w = 8.03 \times 10^5$) and Ter 6 ($M_w = 10.6 \times 10^5$).

in microcapsules made with lower M_w-terpolymer. The underlying mechanism for this difference is unclear at this point.

In summary, this microcapsule system prepared by complex coacervation between the methylated collagen and the synthetic MMA–MAA–HEMA terpolymer represented a promising candidate for the encapsulation of hepatocytes for potential applications in packed-bed or fluidized-bed BLAD. The physicochemical properties of the microcapsules could be optimized by adjusting parameters related to methylated collagen and terpolymer. By examining a series of microcapsules prepared with terpolymers of different M_w's, this study demonstrated that the formation of microcapsules is not affected by the M_w of terpolymer, nor the thickness of the microcapsule membrane and BSA permeability. On the other hand, the mechanical stability of the microcapsules under shear condition was significantly improved by higher terpolymer M_w. The differentiated functions of the hepatocytes in the microcapsules, including urea synthesis, albumin secretion and cytochrome P450 activity, were not significantly influenced by changing the molecular weight of the terpolymers.

Acknowledgements

This work is supported by Agency for Science, Technology and Research (A*STAR) of Singapore and Johns Hopkins Singapore.

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