Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Novel injectable porous poly(γ -benzyl-L-glutamate) microcarriers for cartilage tissue engineering: Preparation and evaluation

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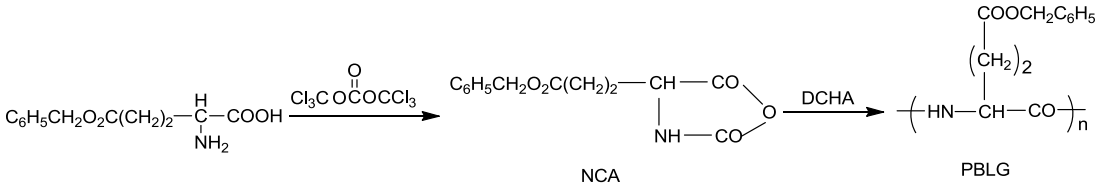
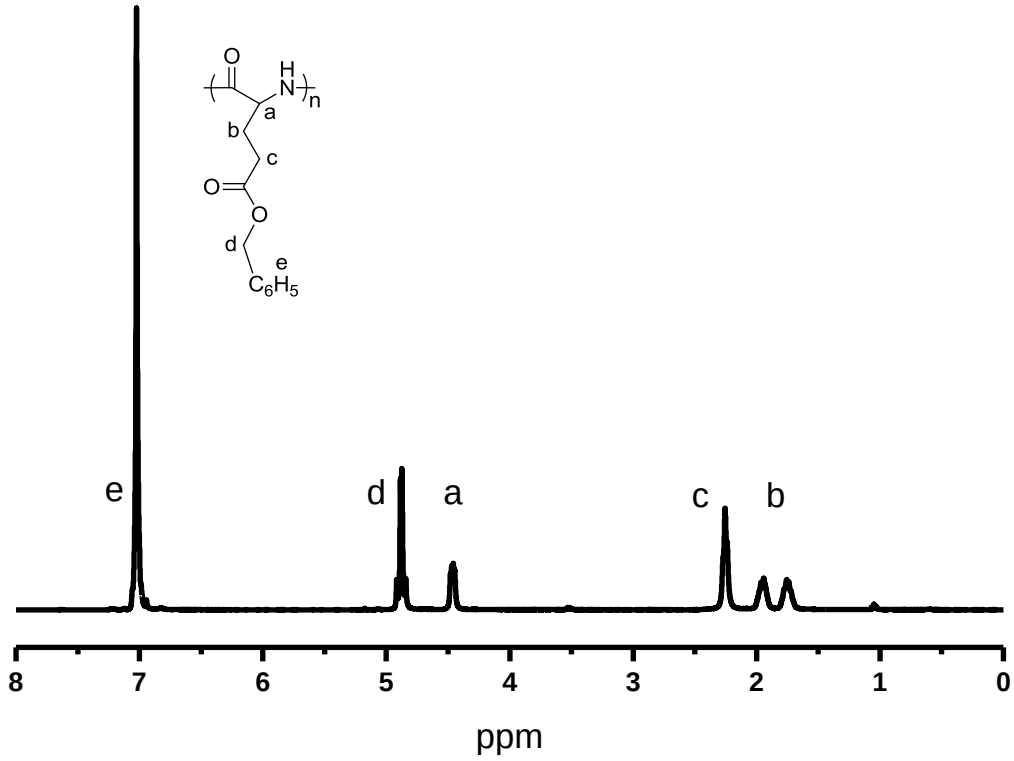
Part 1. Synthesis and characterization of poly(γ-benzyl-L-glutamate) (PBLG)



(A)



(B)

Figure S1. Synthesis and characterization of PBLG. (A) Synthetic route of PBLG using DCHA as an initiator. (B) ^{1}H NMR spectrum of PBLG.

Table S1. Molecular weight and molecular weight distribution of PBLG

Entry	Initiator	[M]/[I]a	Mn (GPC)b	Polydispersityc	Conv.(%)
1		10	102500	1.21	92
2		20	173400	1.24	91
3	DCHA	50	302100	1.33	99
4		100	459800	1.41	98
5		200	706200	1.36	95

a: [M]/[I] refers to the molar ratio of monomer to initiator.

b: Measured by GPC and calculated using polystyrene as standards and THF as eluent. The data refers to the average molecular weight of PBLG.

c: Determined by GPC (Mw/Mn)

Part 2. Structures and properties of various microspheres

2.1 Type of porogen

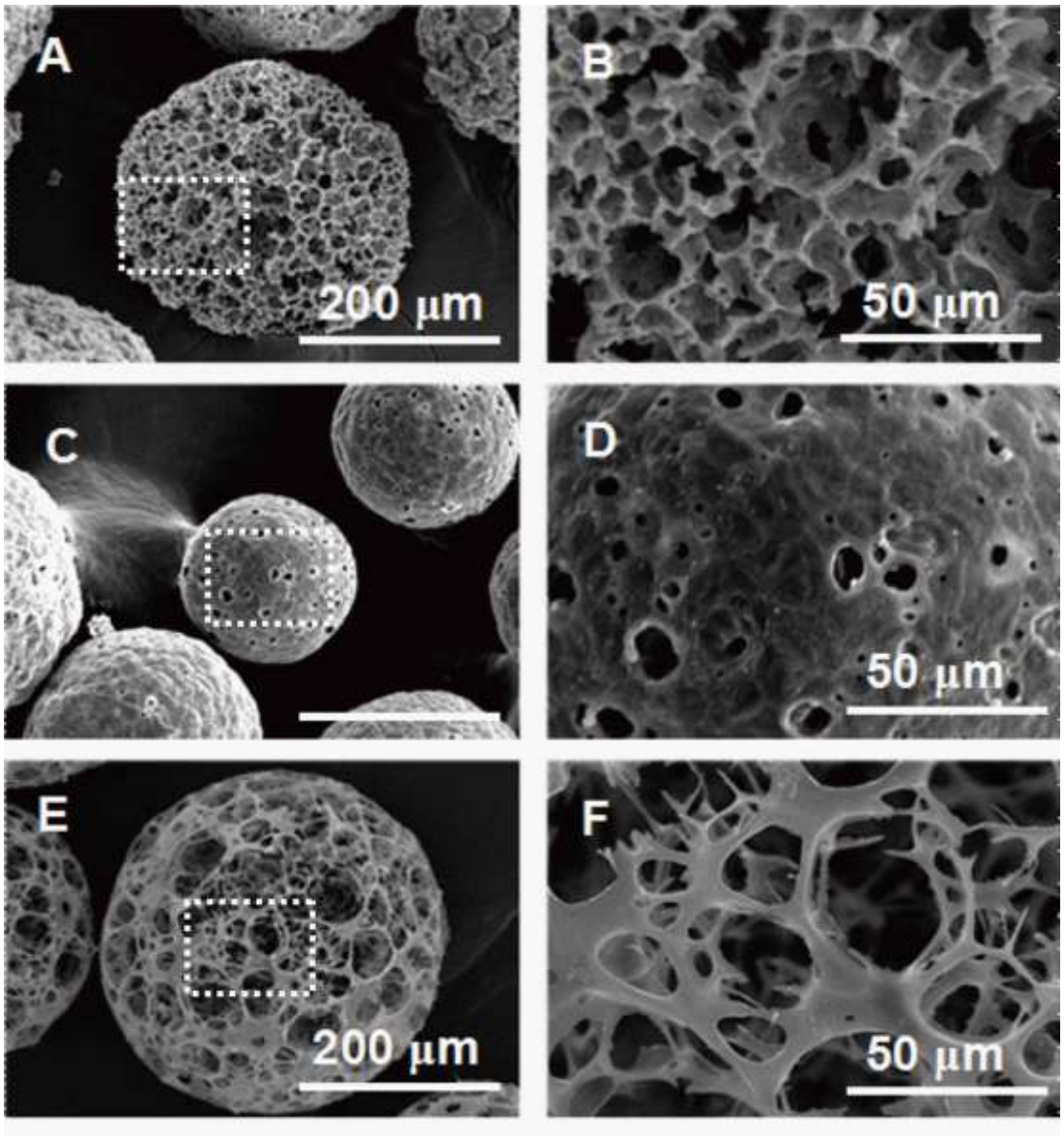
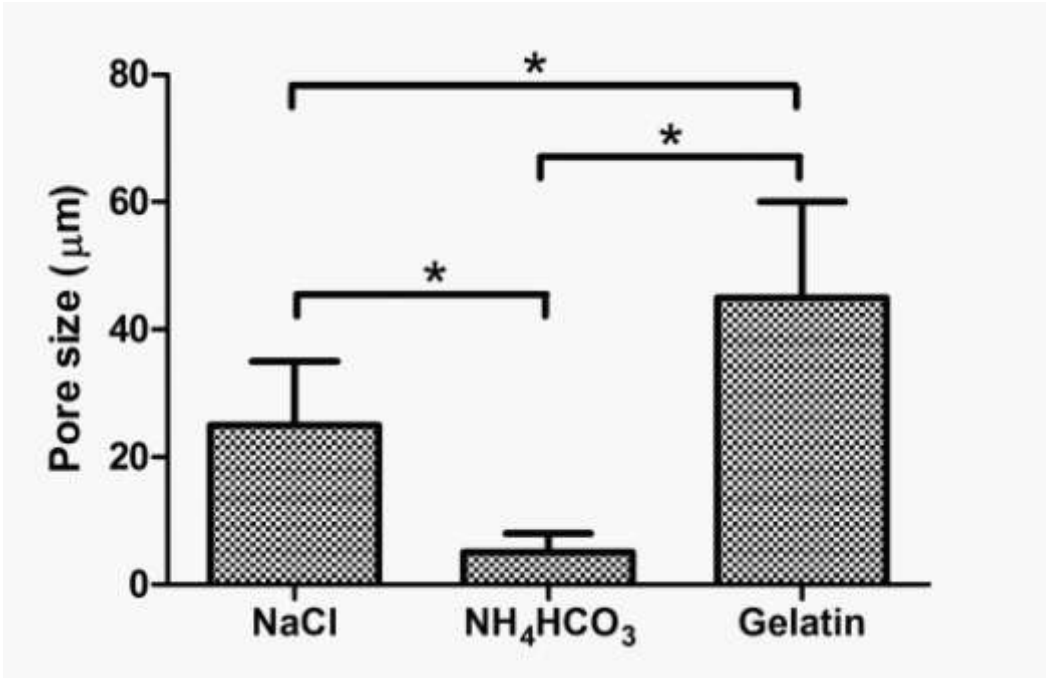
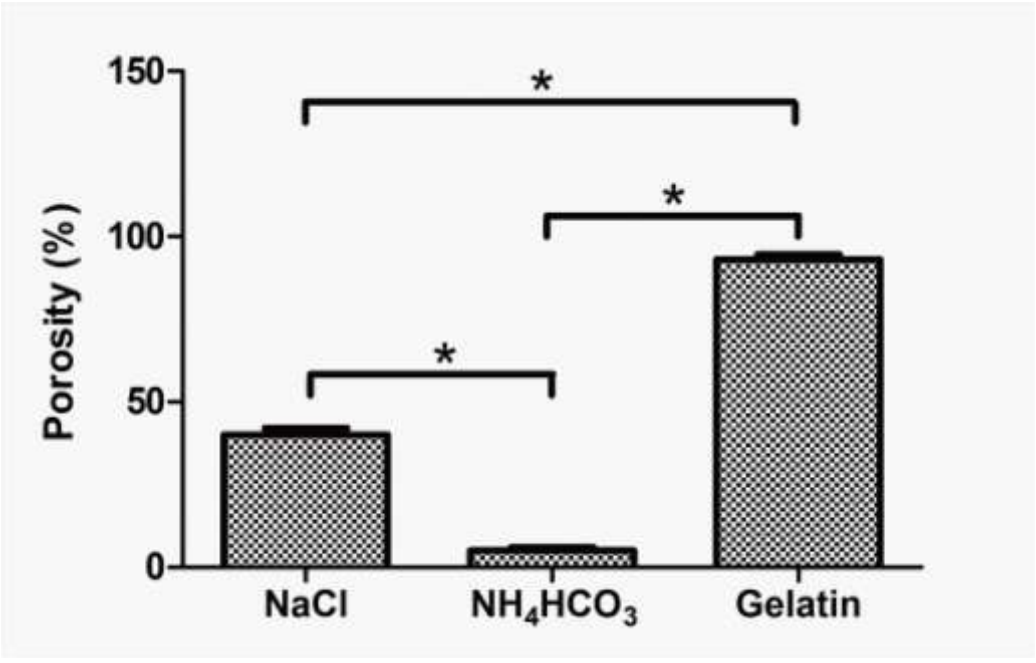


Figure S2 Scanning electron micrographs of porous PBLG microspheres fabricated at a porogen of: (A,B)NaCl; (C,D) NH_4HCO_3; (E,F)gelatin.



(A)



(B)

Figure S3 Graphs showing changes in (A)average pore size and (B) porosity of the porous microspheres prepared according to varying the type of porogen.

2.2 Amount of gelatin

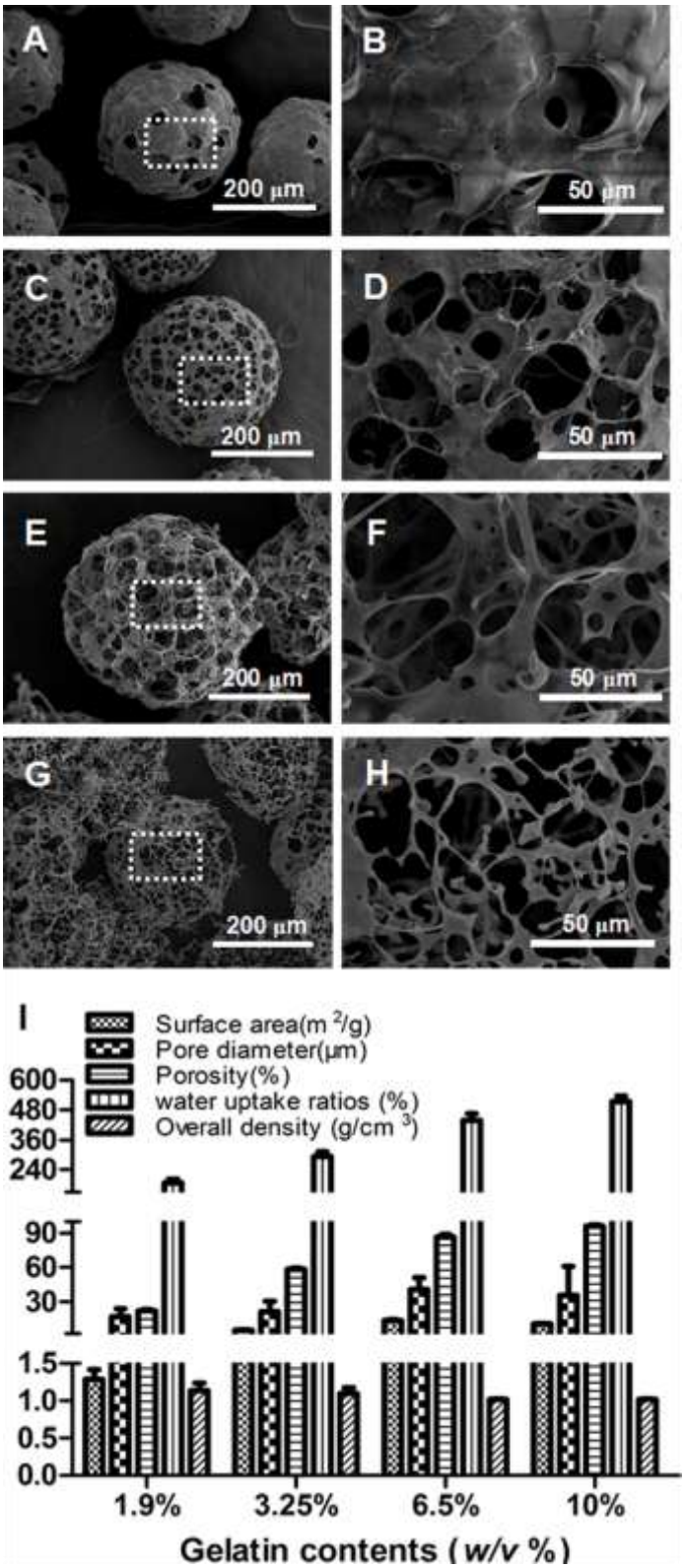


Figure S4 Scanning electron micrographs of porous PBLG microspheres fabricated at a gelatin concentration of: (A,B)1.9%; (C,D) 3.25%; (E,F)6.5%; (G,H)10%. (I)Effects of gelatin concentration on the surface area,pore size, porosity, water uptake ratios and overall density of the PBLG spheres.

2.3 Concentration of PBLG

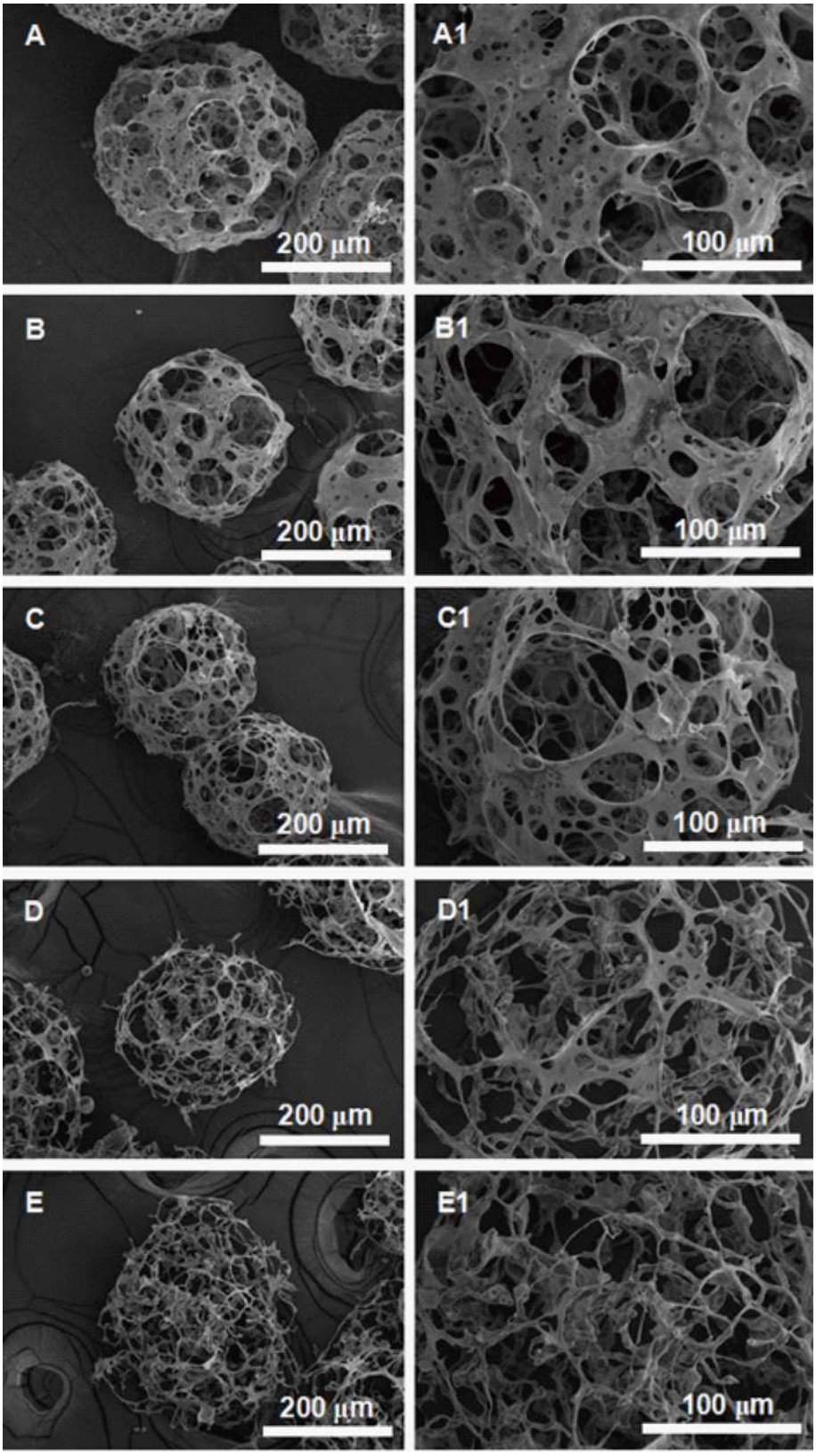
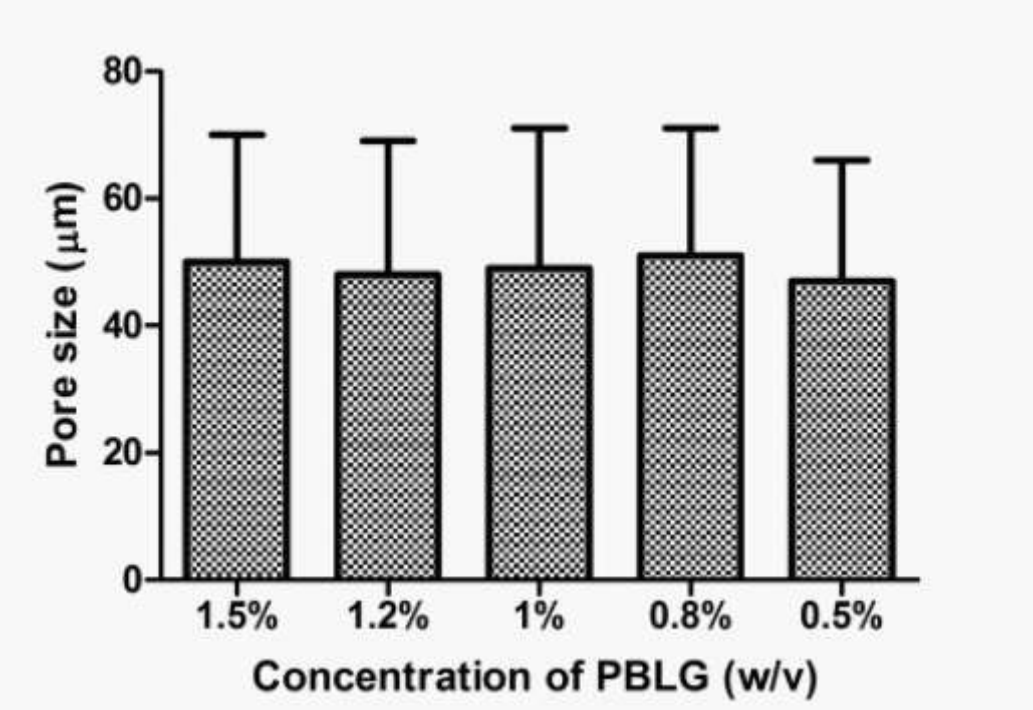
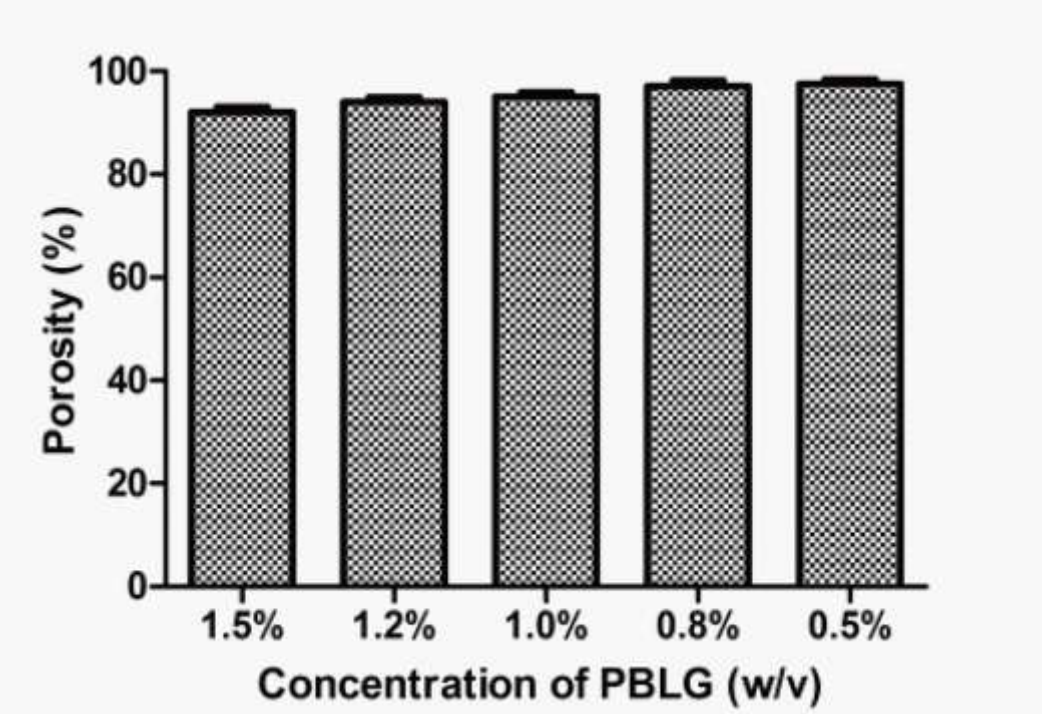


Figure S5 Scanning electron micrographs of porous PBLG microspheres fabricated at a PBLG concentration of: (A,A1)1.5%; (B,B1) 1.2%; (C,C1)1%; (D,D1)0.8%; (E,E1)0.5%.



(A)



(B)

Figure S6 Graphs showing changes in (A) average pore size and (B) porosity of the porous microspheres prepared according to varying PBLG concentrations in methylene chloride (w/v%).

2.4 Stirring rate

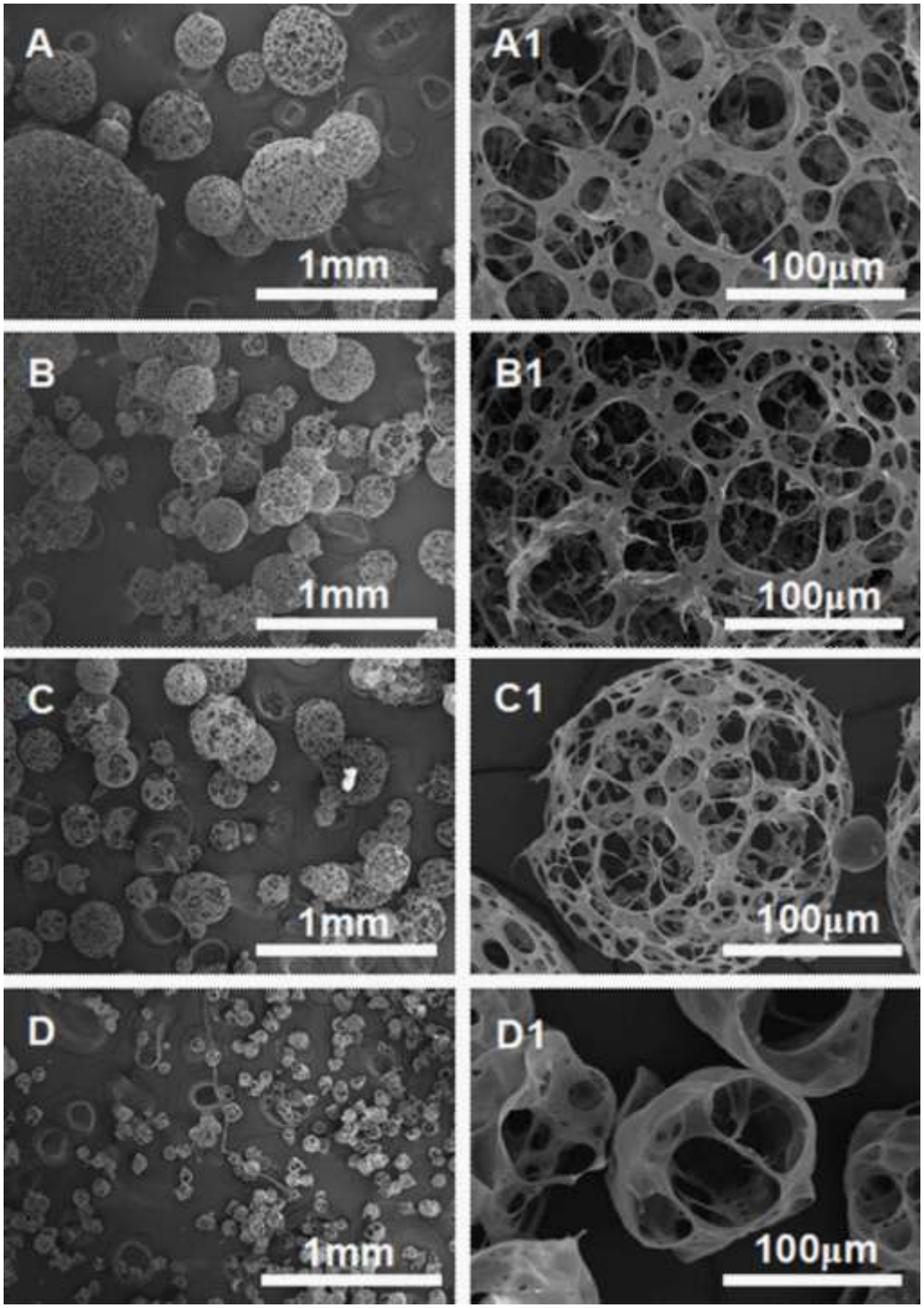
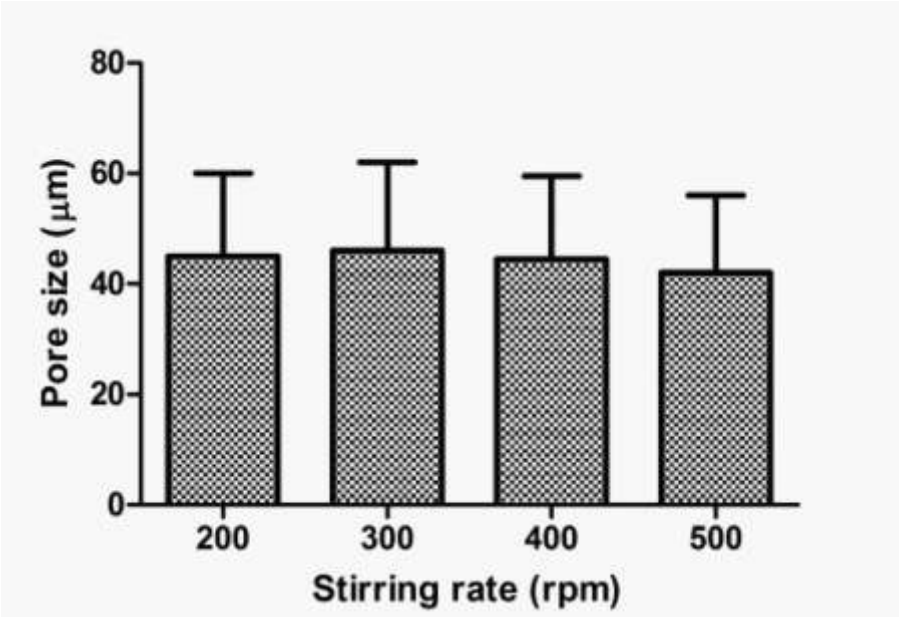
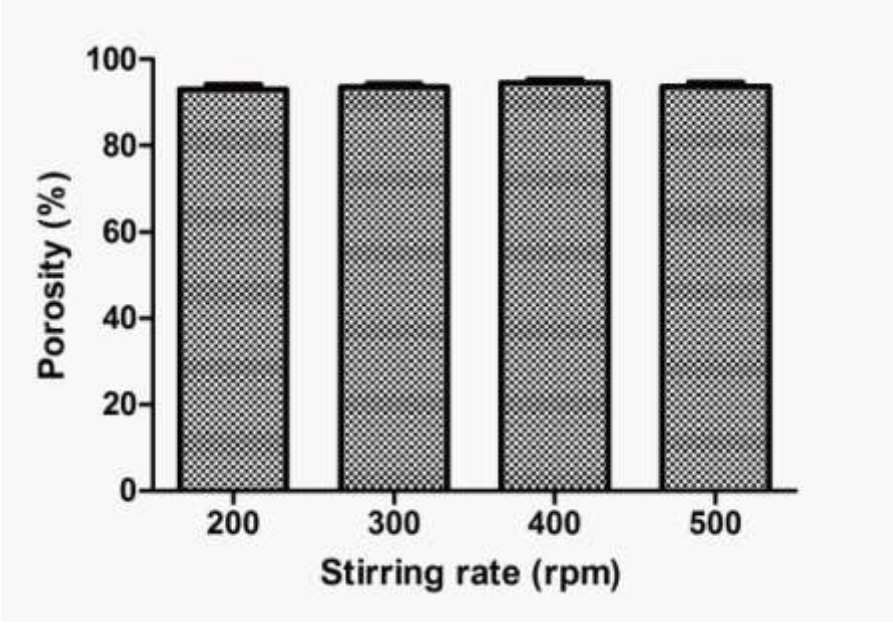


Figure S7 Scanning electron micrographs of porous PBLG microspheres fabricated at a stirring rate of: (A,A1)200rpm; (B,B1) 300rpm; (C,C1)400rpm; (D,D1)500rpm.

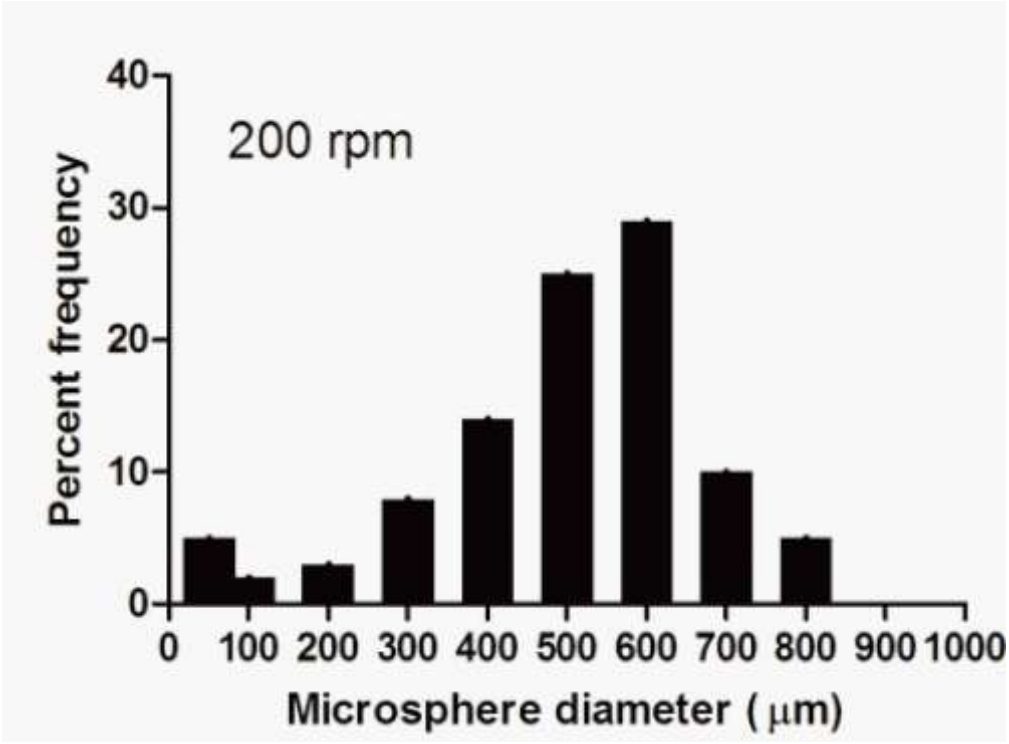


(A)

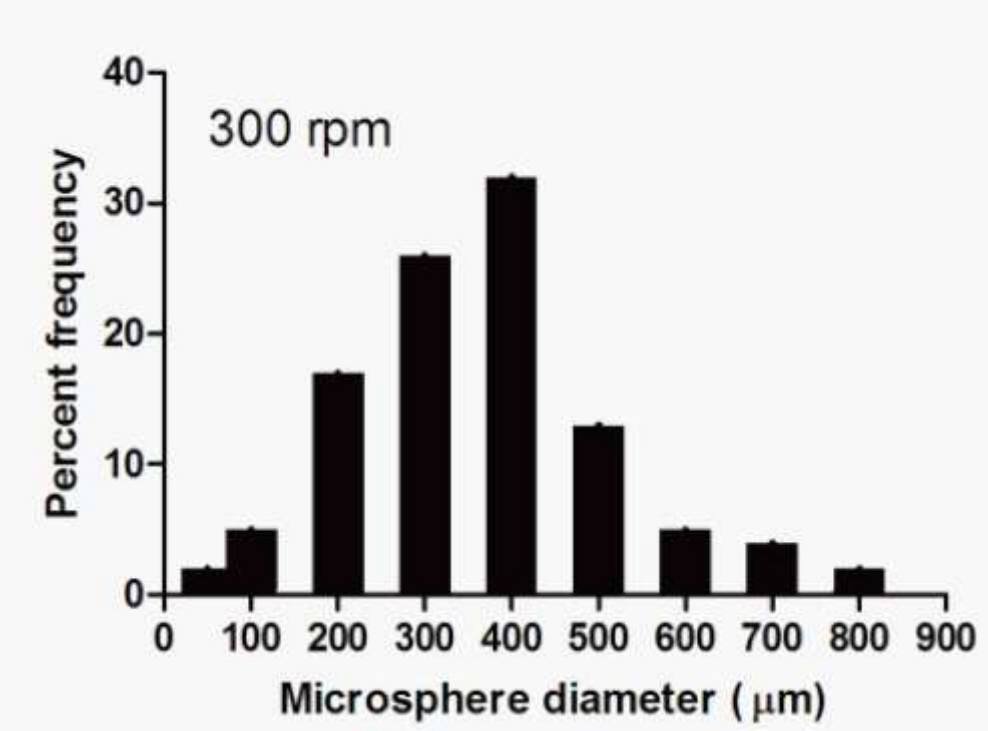


(B)

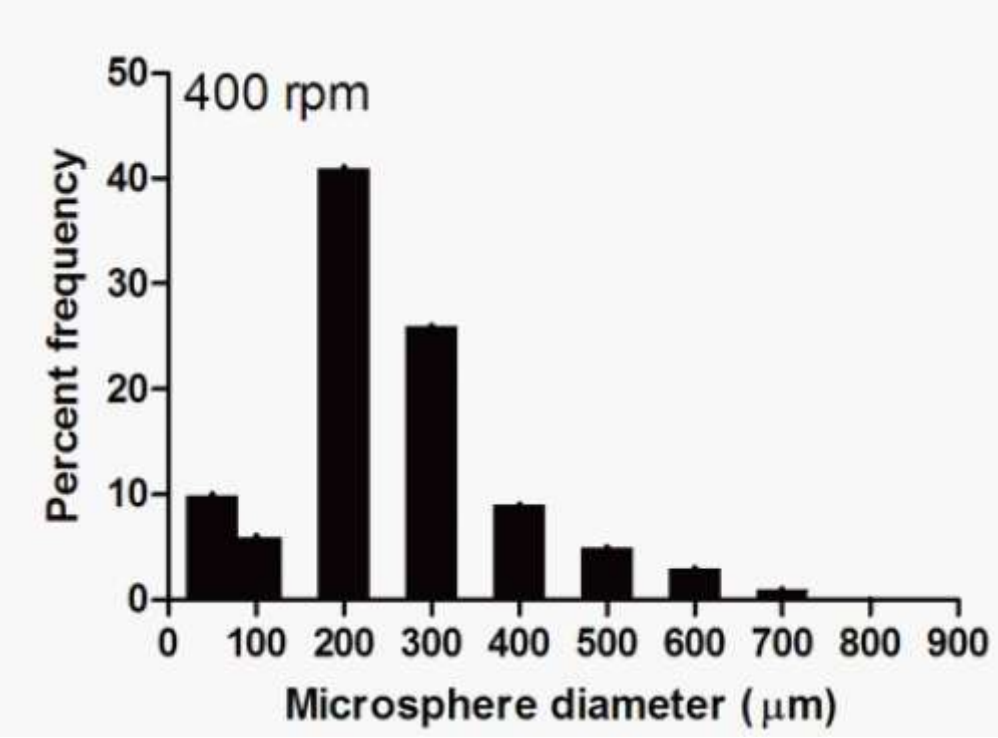
Figure S8 Graphs showing changes in (A)average pore size and (B) porosity of the porous microspheres prepared according to varying stirring rate.


200 rpm
Percent frequency
40
30
20
10
0
0 100 200 300 400 500 600 700 800 900 1000
Microsphere diameter (μm)

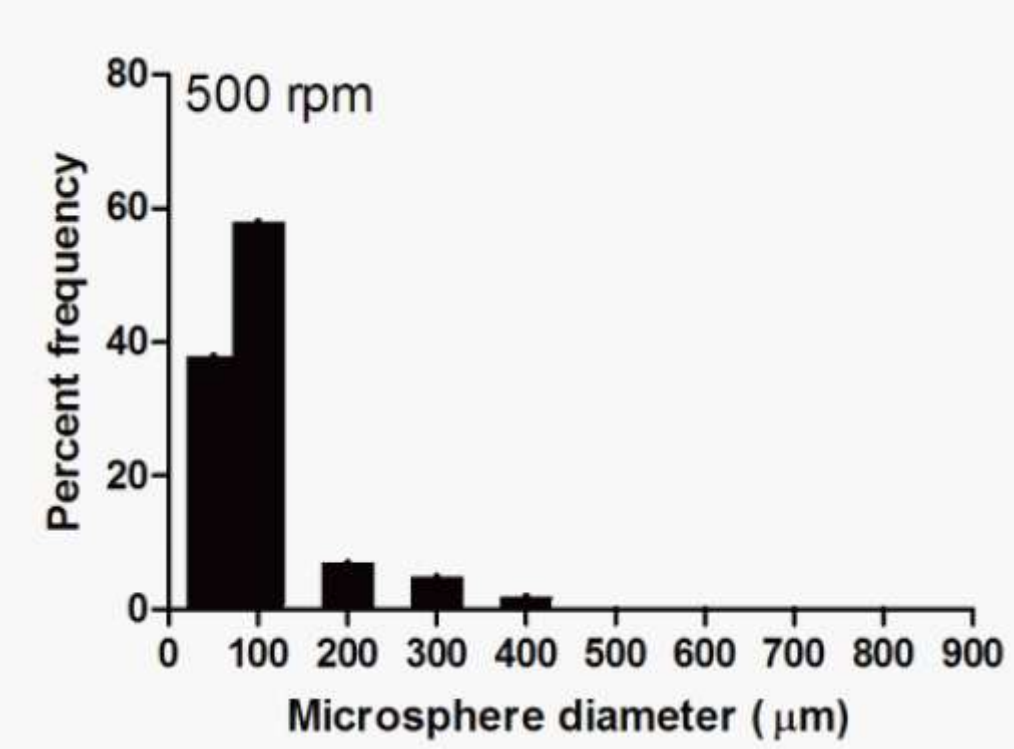
(A)


300 rpm
Percent frequency
40
30
20
10
0
0 100 200 300 400 500 600 700 800 900
Microsphere diameter (μm)

(B)



(C)



(D)

Figure S9 Graphs showing changes in average diameter of the porous microspheres prepared according

to varying stirring rate of (A) 200rpm; (B) 300 rpm; (C) 400rpm; (D) 500 rpm.

Part 3. Biological performance of PBLG porous microsphere for cartilage regeneration

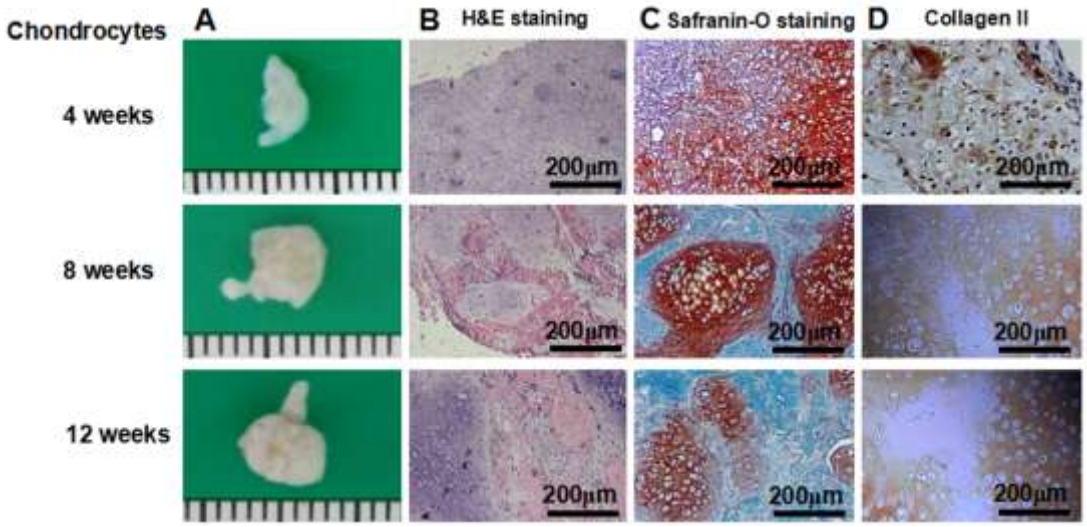


Figure S10 In vivo generation of cartilage tissue with injection of chondrocytes alone after 4, 8 and 12 weeks of injection: (A) Gross view of generated cartilage tissue subcutaneously in mice; (B) H&E staining; (C) Safranin-O staining; (D) immunohistochemical staining.

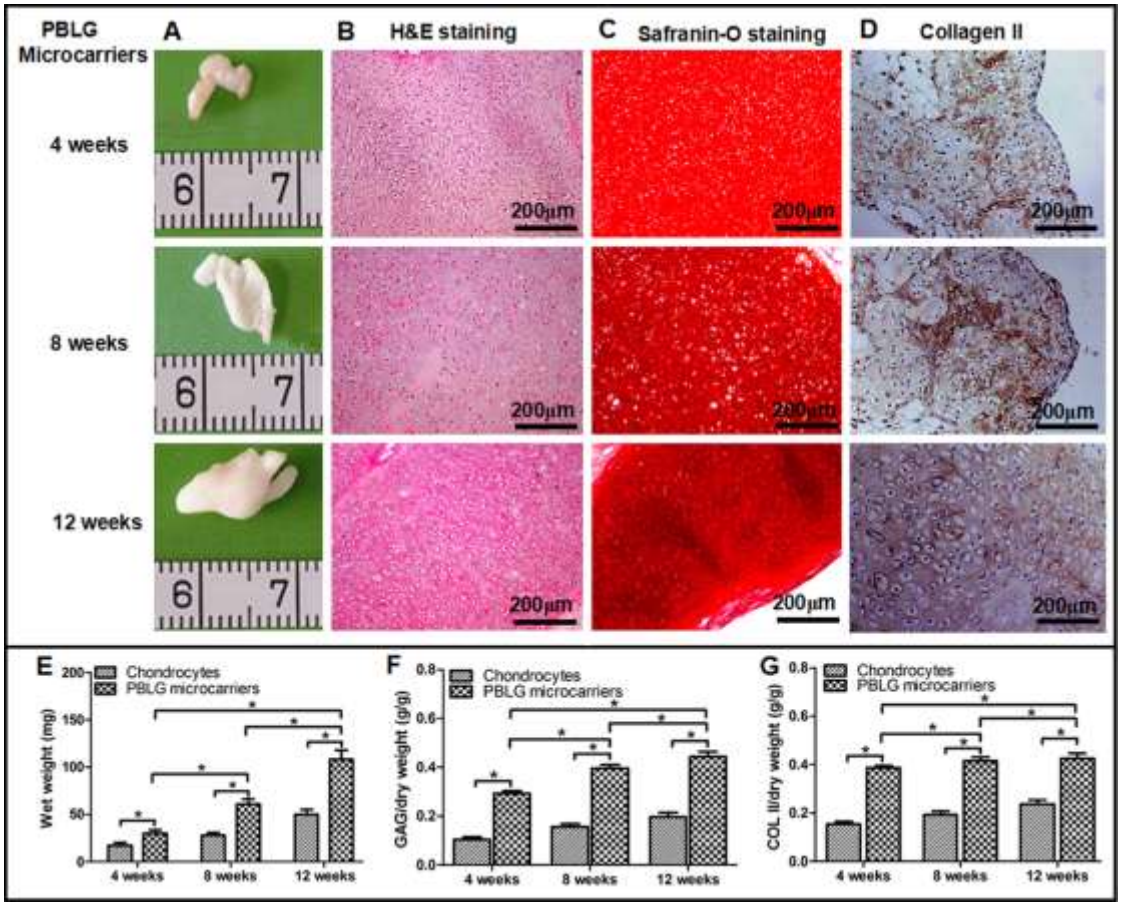


Figure S11 (A-D) In vivo generation of cartilage tissue with injection of chondrocytes loaded PBLG microspheres after 4, 8 and 12 weeks of injection: (A) Gross view of generated cartilage tissue subcutaneously in mice; (B) H&E staining; (C) Safranin-O staining; (D) immunohistochemical staining. (E-G) Comparison of PBLG microsphere as carriers and chondrocytes for cartilage regeneration in vivo: (E) Wet weight of harvested tissue (**P*< 0.01, n = 8 for each group). (F) Content of GAG and (G) collagen type II determined with comparison to dried weight of harvested engineered cartilage (**P*< 0.01, n = 5 for each group).

Part 4. Supplementary Methods

Synthesis of PBLG

γ-Benzyl-L-glutamate (BLG) was purchased from Sigma-Aldrich and used without further purification. BLG N-carboxyanhydrides (BLG-NCA) was synthesized with slight modification according to our previous works [1]. Dicyclohexylamine was purchased from Aladdin Reagent and purified by distillation before use. 1,4-Dioxane and diethyl ether were purchased from Sinopharm Chemical Reagent, and the 1,4-dioxane was further distilled before use. Other chemicals were analytical grade and used as received.

PBLG was synthesized through ring-opening polymerization of BLG-NCA in 1,4-dioxane using dicyclohexylamine as initiator (Figure S1) [2]. To tune the degradation rate, the average molecular weights of the PBLG were tailored through controlling the molar ratio of monomer and initiator [M]/[I], as listed in Table S1. PBLG with a molecular weight of 302100 synthesized at a feed [M]/[I] of 50 was used for the rest of the studies. In brief, BLG-NCA (2.0 g, 7.6 mmol) was dissolved in 60.0 mL of dry 1,4-dioxane in a flame-dried flask, and then 1.52 mL of 0.10 M dicyclohexylamine in 1,4-dioxane solution was added under vigorous stirring ([M]/[I] = 50/1). After stirring for 3 days at 25 °C, the mixture was precipitated into an excessive diethyl ether (2/1, v/v) mixture. The obtained product was further washed twice with diethyl ether and dried under vacuum at room temperature for 24 h.

Polymer characterization

The ^{1}H NMR spectrawere obtained using an Inova 400 NMR spectrometer at room temperature using $CDCl_3$as the solvent. Tetramethylsilane (TMS) was used as the internal reference.

The molecular weight and molecular weight distributionwere measured on a Waters 440 gel permeation chromatograph at 35^{o}C. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1.0 mL/min, and standard polystyrenes were used to calibrate the molecular weights.

Preparation of PBLG microspheres by ammonium bicarbonate

PBLG microcarriers were prepared using a gas foaming method in a (W_1/O/W_2) double emulsion[3]. Briefly, 3.5 mL of 10% (w/v) ammonium bicarbonate aqueous solution was homogenized in 20 mL of methylene chloride containing a concentrations of 1 wt% PBLG using PowerGen 700 homogenizer (Fisher Scientific Co.) at 14,000 revolutions/min (rpm) for 3 min. The primary emulsion was then stirred in 200 mL of 0.1% (w/v) aqueous PVA solution with an overhead propeller at room temperature for 4 h. The microspheres formed were washed 3 times with distilled water and then freeze-dried.

Preparation of PBLG microspheres by NaCl

An aqueous solution (3.5 mL) containing a concentrations of 5% NaCl was emulsified in a methylene chloride solution (20 mL) of 1 wt% PBLG solution (0.2 g) using a homogenizer at 14000 (rpm) for 3 min. The primary emulsion was then stirred in 200 mL of 0.1% (w/v) aqueous PVA solution at room temperature for 12 h. The microspheres formed were washed 3 times with distilled water and then freeze-dried.

Preparation of PBLG porous microspheres by gelatin with varying concentration of PBLG

An aqueous solution (3.0 mL) of 6.5 wt% gelatin was emulsified in a methylene chloride solution (20

mL) containing various concentrations PBLG using a homogenizer at 14000 (rpm) for 3 min. This emulsion was then mechanically stirred in a PVA (0.1 wt%, 100 mL) solution at room temperature for 3 min to form a double emulsion. The double emulsion was immediately immersed in an ice-cold PVA (0.1 wt%, 1000 mL) solution and were gently stirred (400 rpm) 24 h to remove the methylene chloride. To remove the residual gelatin, the obtained beads were gently stirred in a warm water bath (40 °C) for 5 h. The microspheres were washed 3 times with distilled water and then freeze-dried.

Preparation of PBLG porous microspheres by gelatin with varying stirring rates

An aqueous solution (3.0 mL) of 6.5 wt% gelatin was emulsified in a methylene chloride solution (20 mL) of 1 wt% PBLG solution (0.2 g) using a homogenizer at 14000 (rpm) for 3 min. This emulsion was then mechanically stirred in a PVA (0.1 wt%, 100 mL) solution at room temperature for 3 min to form a double emulsion. The double emulsion was immediately immersed in an ice-cold PVA (0.1 wt%, 1000 mL) solution and were stirred at a stirring rate of 200, 300, 400 and 500 rpm for 24 h to remove the methylene chloride, respectively. To remove the residual gelatin, the obtained beads were gently stirred in a warm water bath (40 °C) for 5 h. The microspheres were washed 3 times with distilled water and then freeze-dried.

Microsphere characterization

The surface morphologyof microspheres was observed using SEM (JXA-840, JEOL, Japan). The spheres were coated with gold using a sputter coater (DeskII, Denton vacuum Inc). During the process of gold coating, the gas pressure was 50 mtorr, and the current was 40 mA. The coating time was 120 s. Samples were analyzed at 10 kV.

The pore diameter and porosity of PBLG microspheres were measured with a mercury porosimeter (PoreMaster-60, USA).

The size distribution of PBLG porous microspheres was measured using a Coulter Multisizer 3 (Beckman Coulter, Inc., Fullerton, CA). The microspheres were wetted with ethanol and dispersed in an Isoton II Diluent.

Part 5. Supplementary Reference

[1] Xiao CS, Zhao CW, He P, Tang ZH, Chen XS, Jing XB. Facile synthesis of glycopolypeptides by combination of ring-opening polymerization of an alkyne-substituted N-carboxyanhydride and click "glycosylation". Macromol Rapid Comm 2010;31:991 – 7.

[2] Han JD, Ding JX, Wang ZC, Yan SF, Zhuang XL, Chen XS, Yin JB. The synthesis, deprotection and properties of poly(γ-benzyl-l-glutamate). Sci China Chem 2013;56:729 – 38.

[3] Kim TK, Yoon JJ, Lee DS, Park TG. Gas foamed open porous biodegradable polymeric microspheres. Biomaterials 2006;27:152-159.