

# Supporting Information

## One-pot Preparation of Polymer Microsphere with Different Porous Structure to Sequentially Release Bio-molecules for Cutaneous Regeneration

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### 1. Materials

PLGA copolymers with LA/GA ratio of 50:50 (Medisorbs, Mw = 64 kDa) was purchased from Alkermes Inc. Poly(vinyl alcohol) (PVA) (88 mol% hydrolyzed, Mw = 25,000) was obtained from Polysciences Inc. FITC labeled Bovine serum albumin (FITC-BSA), Rhodamine B, dichloromethane,  $\alpha$ -cyclodextrin, polyethylene glycol (Mw = 2000), interleukin-4 and melatonin were obtained from Sigma-Aldrich Inc. and used without further treatment.

### 2. Preparation of biomolecules loaded microsphere with embedding pores

Microspheres with embedding pores were fabricated by a modified double emulsion technique, where the  $\alpha$ -cyclodextrin/PEG solution was used to replace pure water as solvent to dissolve biomolecules in the first time emulsion. Briefly, 100  $\mu$ l biomolecules (melatonin or rhodamine B) solution containing 72.5 mg/ml of PEG 2000 and 6.56 mg/ml  $\alpha$ -cyclodextrin was emulsified in a 10 wt% PLGA solution in dichloromethane (DCM), using a probe sonicator at an output power of 15W (Virsonic 100, Cardiner, NY) for 20 s over an ice bath to form primary water-in-oil (w/o) emulsion. The w/o emulsion was gradually added into 250 ml aqueous PVA solution under stirring or sonication to form a water-in-oil-in-water (w/o/w) double

emulsion. The solution was stirred at room temperature for 3 h respectively to evaporate dichloromethane and then centrifuged to collect microspheres with embedding pores. The resultant microspheres were washed with distilled water three times and freeze dried.

### **3. Preparation of biomolecules loaded microsphere with open pores**

Microspheres with open pores were fabricated similar with microspheres with embedding pores, while the solvent evaporation time was increased to 24 h and the biomolecules (IL-4 or FITC-BSA) was loaded after the two-step emulsification. As to effectively load the targeted biomolecules, the as-synthesized microspheres were firstly well dispersed in biomolecules solution by stirring. After 24 h, the biomolecules loaded microspheres were collected from medium by centrifugation.

The amount of loaded rhodamine B or melatonin in embedding pore PLGA MS and FITC-BSA or IL-4 in open pore PLGA MS were determined by UV-Vis spectra and Elisa, respectively. The loading capacity and encapsulation efficiency were calculated by the following equations

$$\text{Loading capacity} = \frac{\text{Weight of cargo in PLGA MS}}{\text{Weight of cargo loaded PLGA MS}}$$

$$\text{Encapsulation efficiency} = \frac{\text{Weight of cargo in PLGA MS}}{\text{Initial weight of cargo}}$$

### **4. *In vitro* release of preloaded biomolecules from microspheres**

*In vitro* release profiles of various biomolecules from PLGA microspheres with embedding or open pores were determined as follows. 10 mg of microspheres were suspended in 2ml PBS (pH=7.4). The microsphere suspensions were incubated at 37°C with shaking. At designated times, 1ml release medium was collected by centrifugation and replaced with equal amount of fresh PBS. The released amount was measured by monitoring the payload absorption.

### **5. Formation of hydrogel patch containing two type porous microspheres**

6 mg sodium alginate was dissolved in 300 µl distilled water under continuous stirring. After that, the biomolecules loaded microspheres were added into the sodium alginate solution under continuous stirring. The suspension were then dropped onto the glass sheet, then another glass sheet was gently covered on the drop to form films after certain volume of calcium chloride solution (0.5 M) was added at same place. The film was aged at room temperature for 30 min to obtain calcium crosslinked film containing microspheres.

## **6. Sterilization**

The empty open pore PLGA MS and melatonin loaded embedding pore PLGA MS were pre-wetted in 70% ethanol (v/v) for 30 min to sterilize, while the IL-4 solution, alginate solution and Ca<sup>2+</sup> solution were all sterilized by 0.22 µm filter. Then these sterile components would consist of sterile hydrogel patch before *in vivo* experiments.

## **7. Immuno-fluorescence staining.**

Immuno-fluorescence staining was performed as described below. Briefly, the sections from tissue were fixed and rinsed. After rinsing, they were incubated overnight (at least 8 hours) at 4°C with the primary antibody for CD206 (1:200, Abcam, USA), respectively. After rinsing, the sections were incubated with fluorescence secondary antibody (Cell Signaling, USA) at room temperature for 1 hour. The nuclei were counterstained by Hoechst 33342 (Sigma-Aldrich, USA) for 10 min at room temperature. The results were examined under a confocal microscope (Olympus, Japan). The photographs were evaluated by Image-Pro Plus 6.0 (Media Cybernetics, USA) from three randomly selected views of each specimen, respectively.

## **8. RNA extraction and real-time RT-PCR of mRNA.**

The total RNA was extracted by Trizol reagent (Invitrogen, USA) according to the manufacture's protocol from skin. 1000ng total RNA was reverse transcribed to cDNA using a PrimeScript RT reagent kit (TaKaRa, Japan). Real-time RT-PCR analysis was performed using the SYBR Premix Ex Taq II kit (TaKaRa, Japan) and tested by CFX96TM Real-time RT-PCR System (Bio-Rad, USA). β-actin was used as the internal control for quantitation of the target mRNA.

## **9. Full-thickness cutaneous wound model construction.**

A total of twenty-four 8-week-old female C57BL/6 mice were randomized to four groups. Each mouse was anesthetized as previously described[1]. Briefly, each mouse was anesthetized intraperitoneally with 1% sodium pentobarbital, and the skin was prepared. Then the full-thickness wound including the panniculus carnosus muscle was made on the mid back. After that 1-cm diameter punch biopsy instrument was placed with moderate force onto the dorsum of the mouse to create an impression of the circumference. Our previous work indicated that the effective concentration of melatonin and IL-4 for cells were 2.3 ng/ml and 20ng/ml, respectively[2, 3]. Considering the loss during fabrication, diffusion and the dilution effect of the body fluid, we choose the hydrogel (20 mg sodium alginate per 1 ml water) containing 5 times amount of these molecules (11.5 ng/ml for melatonin and 100ng/ml for IL-4) to maintain the local concentration for effective tissue regeneration. Next, the middle of the outline region of skin was sharply excised along the outline with a pair of scissors.

The excised tissue was full-thickness skin in depth, leaving subcutaneous dorsal muscle exposed after excision. The gels were placed into the dorsal wound. The wound was then covered by two layers of Vaseline gauze with discontinuous suture onto the marginal recipient mice skin of the defect area by 4-0 silk suture.

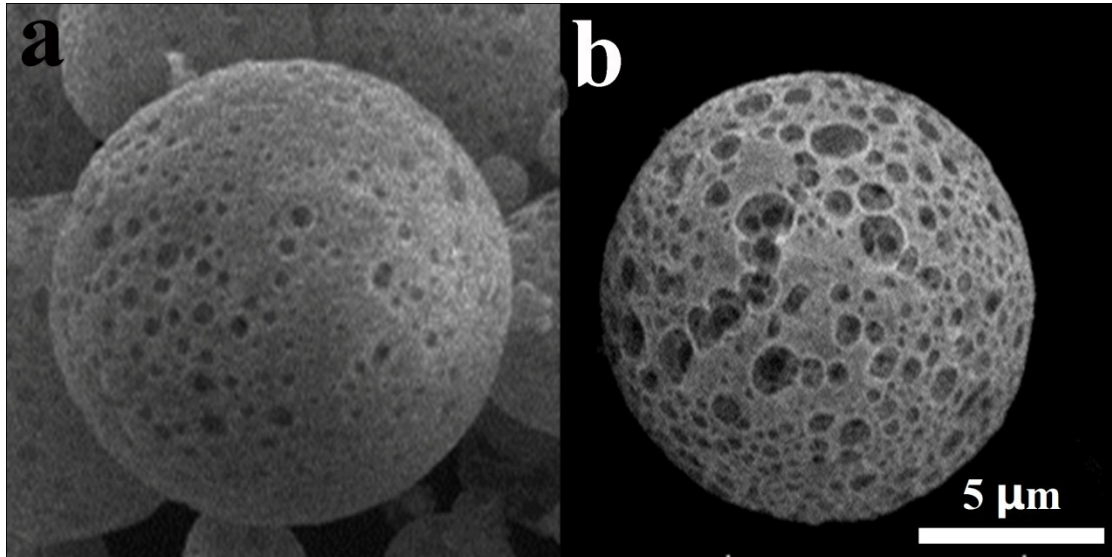
## 10. Wound healing rate measurement

To determine the rate of wound healing in the wound area, the wounds were imaged by digital camera after surgery at 3-10 days. Photographs were uploaded to the computer platform and were analyzed using the Image-Pro Plus 6.0 (Media Cybernetics, USA). All photographs were taken with the experimental mouse placed adjacent to a metric ruler that was used for distance calibration and standardization, allowing subsequent quantitative analysis. The percentage of wound closure was calculated as follows:  $(\text{area of original wound} - \text{area of actual wound}) / \text{area of original wound} \times 100\%$

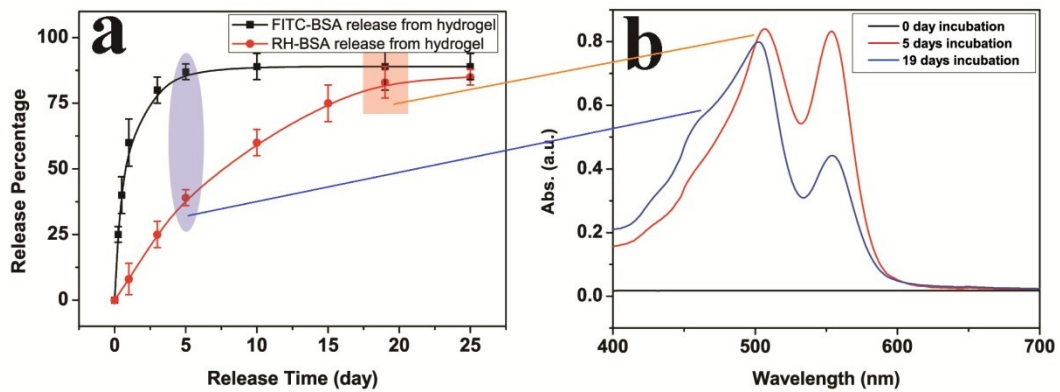
## 11. Characterization

The overall morphology of the microspheres was examined using scanning electron microscopy (SEM) (Hitachi S3200, Tokyo, Japan) after gold coating of the microsphere samples on a stub. The ultraviolet-visible (UV-vis) spectra were measured with diluted aqueous solution in a 2 mm thick quartz cell using a SHIMADZU UV-2401 PC spectrophotometer. The primer sequence for gene expression was listed below.

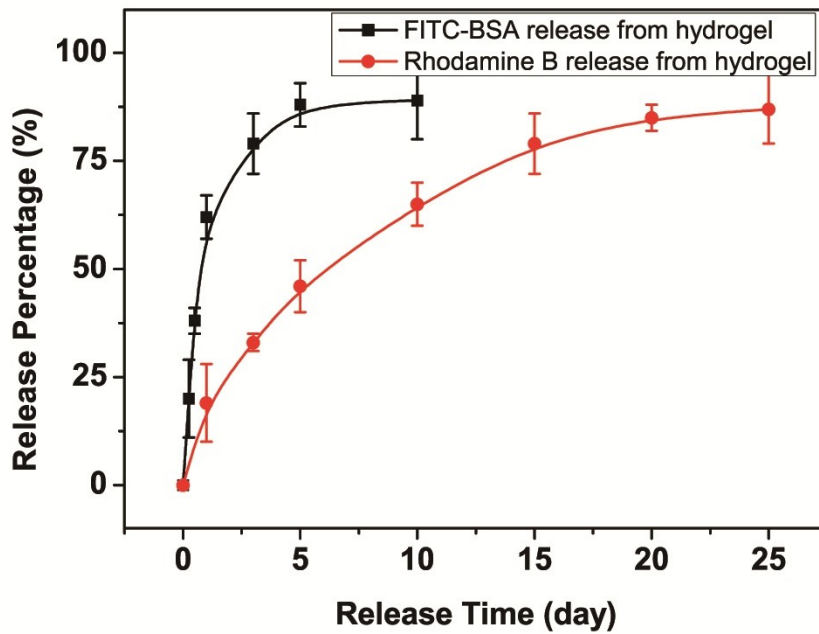
Gene Name	Gene Primer sequence
IL-10	Forward 5'- AGGCGCTGTCATCGATTTCT -3'
	Reverse 5'- ATGGCCTTGTAGACACCTTGG -3'
IL-13	Forward 5'- GCTCTTACTGACTGGCATGAG -3'
	Reverse 5'- CGCAGCTCTAGGAGCATGTG -3'
AngiogenesisII	Forward 5'- CCAACTCCAAGAGCTCGGTT -3'
	Reverse 5'- CGGTGTTGGATGACTGTCCA -3'
VEGF	Forward 5'- GTGCAGGCTGCTGTAACGAT -3'
	Reverse 5'- GACCCTTTCCTTTCCTCGAA -3'
β-actin	Reverse 5'-TGGCACCCAGCACAAATGAA-3'
	Reverse 5'- CTAAGTCATAGTCCGCCTAG-3'



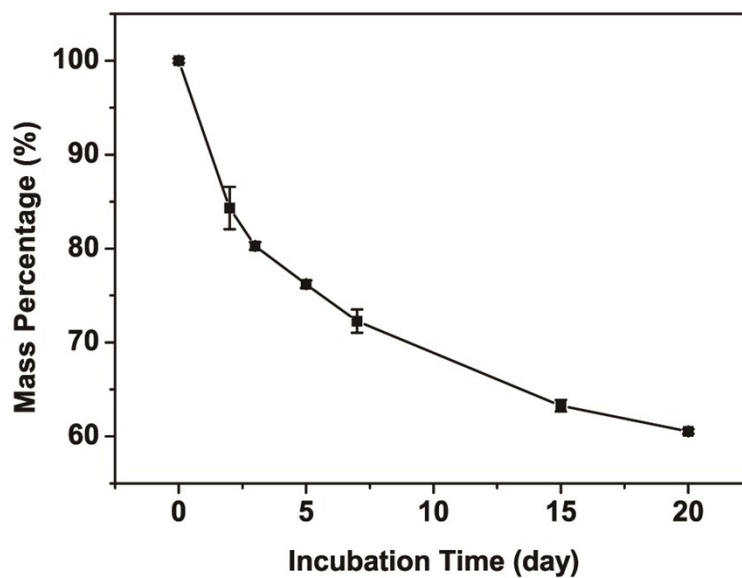
**Figure S1.** The scanning electron microscope images of the microspheres prepared by 5h (a) and 15h (b) incubation.



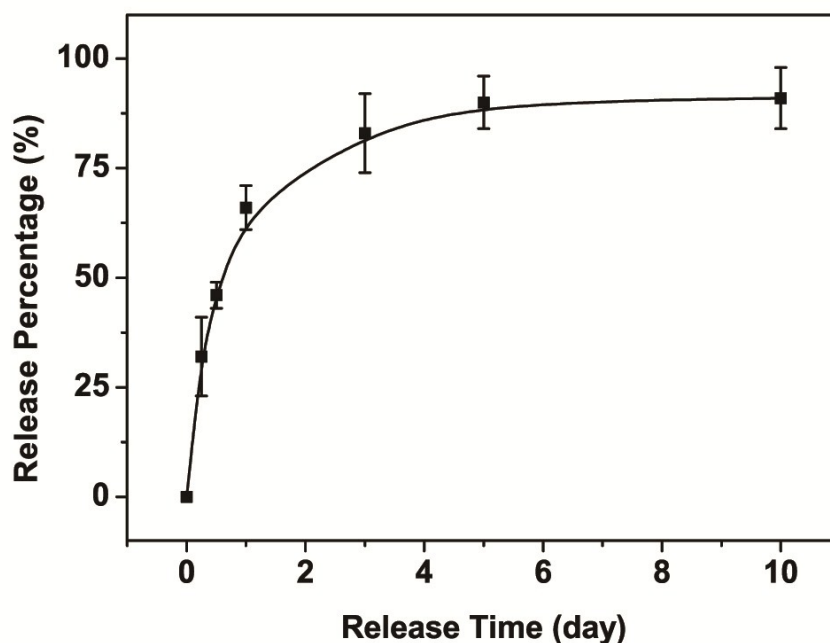
**Figure S2.** a) The percentage of FITC-BSA and RH-BSA released from the hydrogel containing two types of PLGA MS versus the incubation time in PBS. b) Corresponding UV-vis spectrum of the leaching liquor from the FITC-BSA and RH-BSA co-loaded hydrogel at different time point.



**Figure S3.** The percentage of FITC-BSA (model growth factor) and rhodamine B(model drug) released from the hydrogel containing two type of PLGA MS versus the incubation time in PBS.



**Figure S4.** Time dependent weight loss of the microspheres embedded hydrogel against incubation time in PBS buffer.



**Figure S5.** The percentage of IL-4 released from the open pore PLGA MS versus the incubation time in PBS.

**Table S1.** Cargo loading capacity and encapsulation efficiency of embedding pore and open pore PLGA MS

Samples	Cargoes	Loading capacity	Encapsulation efficiency
Embedding pore PLGA MS	rhodamine B	11.5wt%	66.8wt %
	melatonin	10.9 wt %	63.1wt %
Open pore PLGA MS	FITC-BSA	25.5 wt %	78.4 wt %
	IL-4	27.3 wt %	82.6 wt %

[1] G. Hu, P. Liu, J. Feng, Y. Jin, Transplantation with Bone Marrow Stromal Cells Promotes Wound Healing Under Chemotherapy through Altering Phenotypes, *Int. J. Biol. Sci.* 7(7) (2011) 912-926.

[2] Y. Shuai, L. Liao, X. Su, Y. Yu, B. Shao, H. Jing, X. Zhang, Z. Deng, Y. Jin, Melatonin Treatment Improves Mesenchymal Stem Cells Therapy by Preserving Stemness during Long-term In Vitro Expansion, *Theranostics* 6(11) (2016) 1899-1917.

[3] X.-T. He, X. Li, Y. Yin, R.-X. Wu, X.-Y. Xu, F.-M. Chen, The effects of conditioned media generated by polarized macrophages on the cellular behaviours of bone marrow mesenchymal stem cells, *Journal of cellular and molecular medicine* (2017).