applied sciences

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Supplementary Materials

Composite Hydrogels with Simultaneous Release of VEGF and MCP-1 for Enhancing Angiogenesis for Bone Tissue Engineering Applications

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1. Materials and Methods

1.1. Materials

DL-lactide (LA), glycolide (GA) and monomethoxy poly(ethylene glycol) (mPEG, Mn = 550) were purchased from Aldrich. Dichloromethane (CH_2Cl_2), diethyl ether, polyvinyl alcohol (PVA), and stannous octoate ($Sn(Oct)_2$) was supplied by Sigma. Vascular endothelial growth factor (VEGF) was from ThermoFisher Scientific Co., Ltd., monocyte chemotactic protein-1 (MCP-1) was from Sino Biological Co., Ltd, and ELISA kits for VEGF and MCP-1 were purchased from Peprotech (Rocky Hill, NJ, USA). All other chemicals were purchased from China National Medicines Corporation Ltd (analytical grade).

1.2. Linear PLGA-mPEG Block Copolymers Preparation

The linear $PLGA_{1320}$-$mPEG_{550}$ with [LA]/[GA] ratio of 1 (CP-1) was prepared as below. Firstly, 3.3 g of mPEG-550 was dried in a three-necked flask under dry nitrogen atmosphere and stirring at 120 °C for 3 h. Then, 6.24 g of *DL*-Lactide and 1.68 g of glycolide were added into above flask, and the reaction mixture was heated under nitrogen atmosphere for 1h. After all the *DL*-Lactide and glycolide were melted, 0.0057 g of $Sn(Oct)_2$ were added and the reaction mixture was further heated at 150 °C for 10 h. The synthesized polymer was recovered by dissolving in dichloromethane followed by precipitation in ice cold diethyl ether (the volume of diethyl ether used was 10 times at least compare to dichloromethane). Finally, the precipitate was filtered out and dried 2 days under reduced pressure to obtain CP-1.

The linear $P(LA_3GA)_{1320}$-$mPEG_{550}$ with [LA]/[GA] ratio of 3 (CP-2) was prepared as below. Firstly, 3.3 g of mPEG-550 was dried in a three-necked flask under dry nitrogen atmosphere and stirring at 120 °C for 3 h. Then, 6.24 g of *DL*-Lactide and 5.04 g of glycolide were added into above flask, and the reaction mixture was heated under nitrogen atmosphere for 1h. After all the *DL*-Lactide and glycolide were melted, 0.02 g of $Sn(Oct)_2$ were added and the reaction mixture was further heated at 150 °C for 10 h. The synthesized polymer was recovered by dissolving in dichloromethane followed by precipitation in ice cold diethyl ether (the volume of diethyl ether used was 10 times at least compare to dichloromethane). Finally, the precipitate was filtered out and dried 2 days under reduced pressure to obtain CP-2.

1.3. Growth Factors Loaded Microspheres Preparation

The above-prepared CP-1 and CP-2 were used for growth factors loaded microspheres. The VEGF and MCP-1 loaded CP-1 microspheres, VEGF and MCP-1 loaded CP-2 microspheres, VEGF loaded CP-1 microspheres, MCP-1 loaded CP-1 microspheres, VEGF loaded CP-2 microspheres, and MCP-1 loaded microspheres were prepared by using double-emulsion method. The growth factors loaded microspheres were prepared by double emulsion method, the details as below in brief. The prepared CP-1 or CP-2 (100 mg) was dissolved in 4 mL dichloromethane (CH_2Cl_2), then 0.1 mL of growth factors aqueous solution was added. The concentration of growth factors was designed according to the initial loading with respect to copolymer weight. The colostrum was formed by using a homogenizer with 15000 r/min emulsification for 10 s. The first emulsion composite was added into the aqueous solution of 0.25 % 10 mL polyvinyl alcohol (PVA), emulsified for 10 s again at 7000 r/min. Then compound emulsion added in 100 mL 0.25% PVA water solution, stirred at 500 r/min for removing volatile CH_2Cl_2 for 48 h. Finally, the microspheres were centrifuged 3500 r/min, washed with distilled water three times, freeze-dried for 48 hours to obtained loaded microspheres. The initial loading of VEGF for VEGF loaded CP-1 microspheres and VEGF loaded CP-2 microspheres was 1200 ng/mg. The initial loading of MCP-1 for MCP-1 loaded CP-1 microspheres and MCP-1 loaded CP-2 microspheres was 1200 ng/mg. The initial loadings of MCP-1 and VEGF for VEGF and MCP-1 loaded CP-1 microspheres, VEGF and MCP-1 loaded CP-2 microspheres were both 800 ng/mg.

1.4. Composite Hydrogels Preparation

The star-shaped PLGA-mPEG block copolymer (star-P(LA_3GA)$_{1320}$-$mPEG_{550}$ with [LA]/[GA] ratio of 3) was synthesized according to our previous works [1]. The prepared star-shaped block copolymers were dissolved into simulation body fluids (40 wt%) to form copolymer solution at 10~20 °C. Then, the prepared growth factors loaded microspheres (total weight of microspheres respect star-shaped block copolymer solution weight is 5 wt%) added into copolymer solution, at the same time, VECs solution was also composited with into, then the composite solution was formed to composite hydrogels by increasing temperature to 37 °C. In this paper, three sequential delivery systems were designed to evaluate the vascularization, and designation of composite hydrogels with sequential release of VEGF and MCP-1 was shown in Table S1.

1.5. *The Sol-gel Transition Temperature Measurement of Composite Hydrogels*

The above prepared star-shaped block copolymers were dissolved into simulation body fluids to yield copolymer solution with different concentration and stored at 4 °C. Then, different ratio of microspheres was added into copolymer solution to prepare microspheres/copolymer solution. The viscosity of microspheres/copolymer solution was measured to illustrate the sol-gel transition by using a Brookfield Viscometer DV-III ultra with a programmable rheometer and a TC-502P temperature controlled in water bath. The viscosity of microspheres/copolymer solution was carried out using a HA7 spindle at 40 rpm from 10 to 60 °C in increments of 1 °C [4]. According the measured viscosity, the ratio of microspheres/copolymer for composite hydrogels and cells encapsulation was confirmed.

1.6. *VECs Viability Evaluation in Composite Hydrogels*

Fluorescence assay was used to evaluate the living cells in composite hydrogels. The VECs-laden composite hydrogels were removed from the culture media after 3 days and washed 3 times with PBS, fixed with 2.5% glutaraldehyde overnight. The samples were washed with PBS, then soaked in 0.1% Triton X-100 for 10 min, washed again in PBS. Subsequently, 1% bovine serum albumin (BSA) was added and kept for 30 min. The VECs were stained with 50 μg/mL phalloidin-FITC (Invitrogen) solution for 1 h at room temperature, followed by washing with PBS 3 times. Then 10 μg/mL 4, 6-diamidino-2-phenylindole (DAPI, Invitrogen) was added and incubated in dark at room temperature for 5 min, and then the samples were washed with PBS. Finally, the cytoskeleton and nucleus of BV2 cells was imaged using confocal laser scanning microscope (CLSM) (Leica TCS SP5 II, Braunschweig, Germany). The VECs in the composite hydrogels were quantitatively investigated by the CCK-8 assay after cultured for 1, 3, and 5 days. After removal of the culture media from cell culture plates, 300 μL fresh culture media and 30 μL CCK-8 kit solutions were immediately added and homogeneously mixed and then incubated for 4 h in a CO_2 incubator. Finally, 200 μL reaction solutions were put into 96-well plate. The optical density of each well at 450 nm was read by a microplate reader (SpectraMax 190, Molecular Devices, USA).

1.7. Animal Experiments

The Japanese rabbits were used in this study because of the load-bearing similarity between rabbit and human beings. For all studies, rabbits were anesthetized with 2% pentobarbital sodium (30 mg/kg) via rabbit ear marginal vein, and the animals were kept in the Institute of Experimental Animal of Wuhan Hanyang Hospital, in accordance with the institutional guidelines for care and use of laboratory animals. The femoral head necrosis model was constructed by injecting anhydrous alcohol into the center of femur head, the final model was obtained at week 3. Then composite solution with different delivery system and VECs was injected into necrosis site, then the hydrogels was formed in 10 min, the hole was sealed with a medical bone wax, the wound was treated in a routine way of surgery. The animals were kept for normal observation in the first two weeks, such as wound infection, wound split and appetite loss, *etc*. Finally, at week 4 and 8, the rabbits were scanned by using X-ray firstly, then sacrificed, the biopsies of implants and the adjacent tissues were obtained, then fixed in formalin and embedded in paraffin for H&E and Masson's Trichrome staining. The vessels density and mean vessel diameter were quantitatively analyzed by using ImageJ software.

1.8. Statistical Analysis

Unless indicated, data were given as mean ± SD (n = 5). Statistics analysis of obtained results was carried out using a one-way analysis of variance (one-way ANOVA), and significance reported at a p value of <0.05 for 95% confidence. All statistical calculations were performed using GraphPad Prism software.

Table S1. The designation of composite hydrogels with sequential release of VEGF and MCP-1

Composite system	Hydrogels	Microspheres
Delivery System I	hydrogel [1]	VEGF and MCP-1 loaded CP-1 microspheres [2]
Delivery System II	hydrogel	VEGF loaded CP-1 microspheres with MCP-1 loaded CP-2 microspheres [3]
Delivery System III	hydrogel	MCP-1 loaded CP-1 microspheres with VEGF loaded CP-2 microspheres

[1] Star-shaped PLGA-mPEG block copolymer hydrogel; [2] CP-1 microspheres prepared by linear PLGA-mPEG block copolymer ($PLGA_{1320}$-$mPEG_{550}$ with [LA]/[GA] ratio of 1, CP-1); [3] CP-2 microspheres prepared by linear PLGA-mPEG block copolymer ($P(LA_3GA_1)_{1320}$-$mPEG_{550}$ with [LA]/[GA] ratio of 3, CP-2).

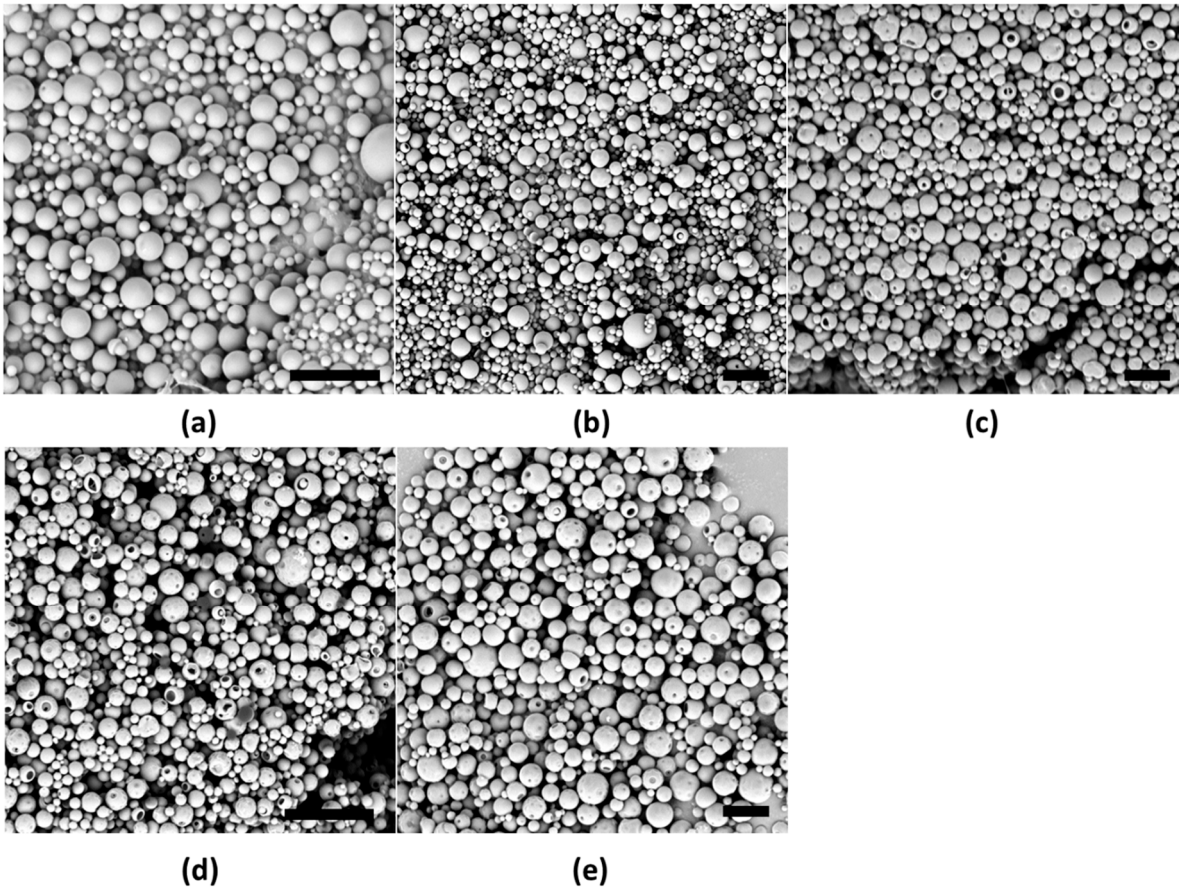

Figure S1. SEM images of VEGF and/or MCP-1 loaded CP-1 and/or CP-2 microspheres. (**a**) VEGF and MCP-1 loaded CP-1 microspheres; (**b**) VEGF loaded CP-1 microspheres; (**c**) MCP-1 loaded CP-1 microspheres; (**d**) VEGF loaded CP-2 microspheres; (**e**) MCP-1 loaded microspheres. Scale bar: 10 μm.

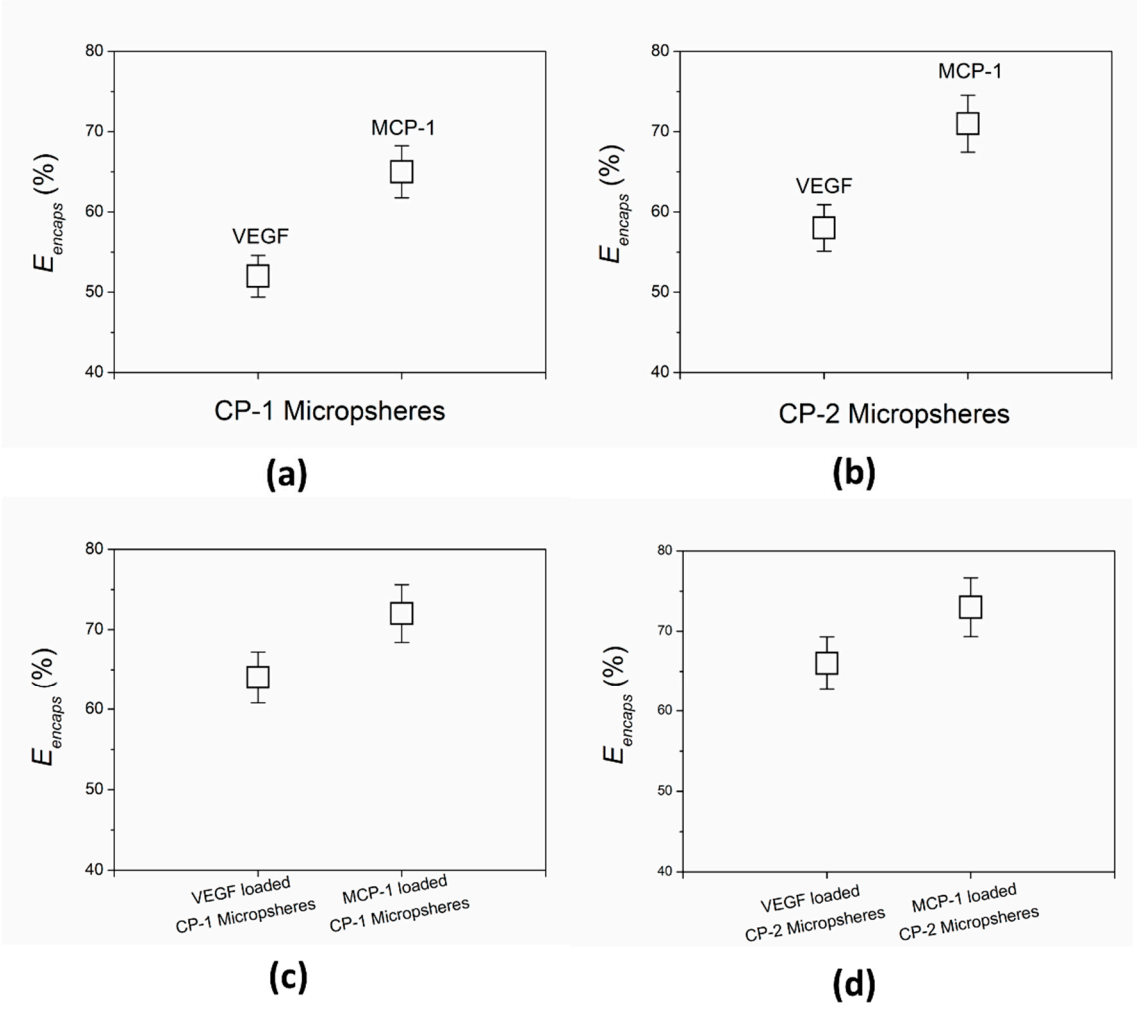

Figure S2. The encapsulation efficiency (E_{encaps}) of VEGF and/or MCP-1 in CP-1 and CP-2 microspheres tested by using ELISA kits. (**a**) E_{encaps} of VEGF and MCP-1 in VEGF and MCP-1 loaded CP-1 microspheres; (**b**) E_{encaps} of VEGF and MCP-1 in VEGF and MCP-1 loaded CP-2 microspheres; (**c**) E_{encaps} of VEGF and MCP-1 in VEGF loaded CP-1 microspheres and MCP-1 loaded CP-1 microspheres respectively; (**d**) E_{encaps} of VEGF and MCP-1 in VEGF loaded CP-2 microspheres and MCP-1 loaded CP-2 microspheres respectively.

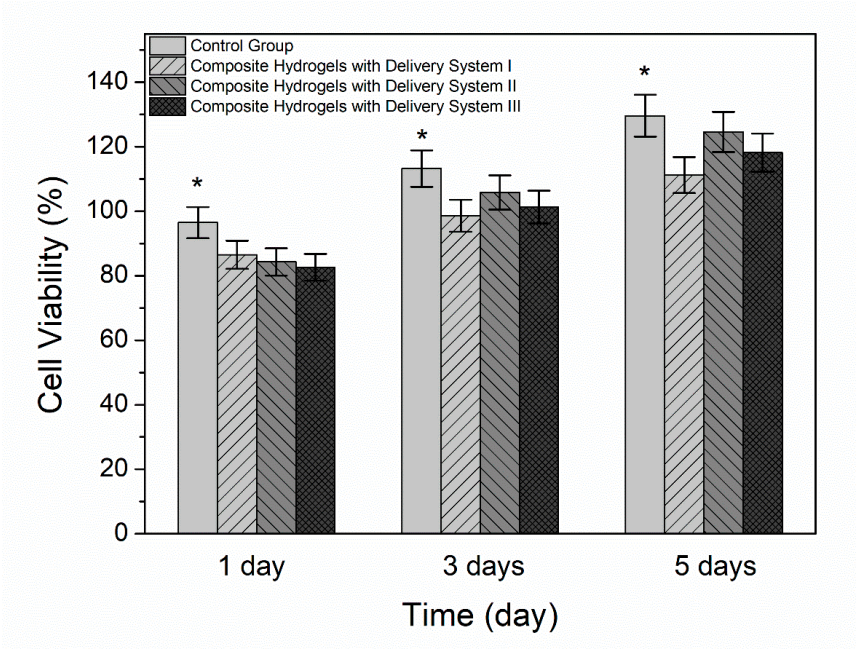

Figure S3. CCK-8 assay of VECs cultured with composite hydrogels for 1, 3 and 5 days. The error bars represent standard deviations with n = 7 for each sample. *$p < 0.05$.

References

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