Electronic Supplementary Material

Biomimetic ECM coatings for controlled release of rhBMP-2: Construction and biological evaluation

Ying Huang[1#], Qiaojie Luo[1#], Guangyu Zha[1], Jianxiang Zhang[2], Xiaohui Li[2], Shifang Zhao[1], Xiaodong Li[1*]

[#] These two authors contributed equally to this article.

[1] Department of Oral and Maxillofacial Surgery, the Affiliated Stomatology Hospital, College of Medicine, Zhejiang University, Hangzhou, P. R. China.

[2] Department of Pharmaceutics, College of Pharmacy, Third Military Medical University, Chongqing 400038, China

[*] Correspondence to: X. D. Li, Department of Oral and Maxillofacial Surgery, the Affiliated Stomatology Hospital, College of Medicine, Zhejiang University, 395# Yan'an road, Hangzhou, P. R. China. Zip code: 310006.

E-mail address: cisarli@zju.edu.cn

Tel: +8657188208378

Fax: +8657187217433

1. *The sequences of primers for target genes.*

Table S1: Forward(F) and reverse(R) primers for target genes

Gene name	Amplicon (bp)		5'-3'prime sequence
OC	178	F	5'-AGCAGCTTGGCCCAGACCTA-3'
		R	5'-TAGCGCCGGAGTCTGTTCACTAC-3'
AKP-2	164	F	5'-TGCCTACTTGTGTGGCGTGAA-3'
		R	5'-TCACCCGAGTGGTAGTCACAATG-3'
Runx2	144	F	5'-CACTGGCGGTGCAACAAGA -3'
		R	5'-TTTCATAACAGCGGAGGCATTTC -3'
Osx	144	F	5' -ATGGCGTCCTCTCTGCTTG-3'
		R	5' -GTATGGCTTCTTTGTGCCTCCT-3'
GAPDH	150	F	5'-TGTGTCCGTCGTGGATCTGA-3'
		R	5'-TTGCTGTTGAAGTCGCAGGAG-3'

OC, osteocalcin; AKP-2, alkaline phosphatase 2; Runx2, runt-related transcription factor 2; Osx, osterix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2. *FT-IR of the HA derivatives*

HA-GRGDSPC-(**SH**): 1 mg/ml HA was reacted with 0.12 mg/ml GRGDSPC-(S-S)-CPSDGRG (the molar ratio of carboxylic groups in HA to GRGDSPC-(S-S)-CPSDGRG was about 20: 1.1) in a aqueous solution containing 1-Ethyl-3-3-dimethylaminopropyl carbodiimide (EDC, 40 mM) and N-hydroxysuccinimide (NHS, 10 mM) at 4˚C for 48 h, followed by dialysis against deionized water for 2 weeks. The HA-GRGDSPC-(S-S)-CPSDGRG-HA was then obtained by lyophilization. To reduce the disulfide linkages to mercapto groups, HA-GRGDSPC-(S-S)-CPSDGRG-HA (1 mg/ml) was reacted with dithiothreitol (DTT, 1 M) at room temperature for 4 h, followed by dialysis against deionized water for 1 week. HA-GRGDSPC-**(SH)** was then harvested by lyophilization.

HA-GRGDSP: 1 mg/ml HA was reacted with 0.1 mg/ml GRGDSP (the molar ratio of carboxylic groups in HA to GRGDSP was about 10: 1.1) in a aqueous solution containing EDC (40 mM) and NHS (10 mM) at 4˚C for 48 h, followed by dialysis against deionized water for 2 weeks. HA-GRGDSP was then obtained by lyophilization.

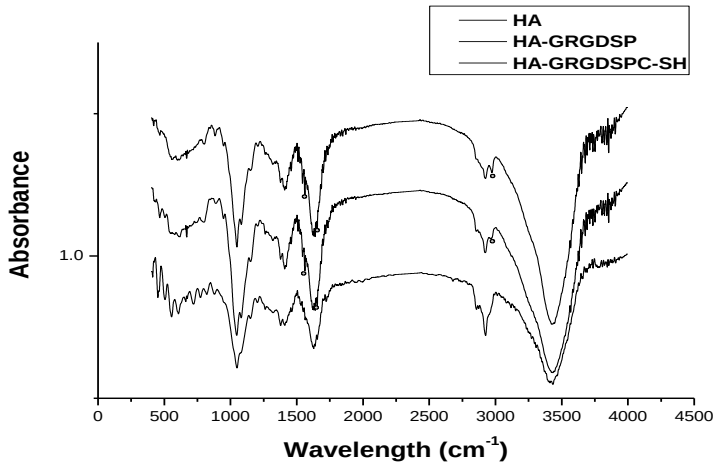


Figure S1. FTIR spectra of HA, HA-GRGDSP and HA-GRGDSPC-SH. Compared with the FT-IR spectrum of HA, three new peaks at 1530, 1600 and 3030 cm^{-1} appeared in the spectra of HA-GRGDSP and HA-GRGDSPC**-(SH)**. Due to the extreme weak absorbance of mercapto groups, there was no difference between the absorption curves of HA-GRGDSP and HA-GRGDSPC**-(SH**).

3. Detailed assay procedures of ALP activity, cytoplastic total protein concentration and OC production.

3.1 Alkaline phosphatase activity

The activity of intracellular alkaline phosphatase (ALP) was measured with a commercial phosphatase substrate kit (Wako, Japan). In brief, after gentle removal of culture medium and washing with PBS, cells were lysed using CelLytic buffer (CelLyticTM M, sigma) for 15 min at 4℃. 20 μl cell lysate mixed with 100 μl p-nitrophenylphosphate disodium in 96-well plate was shaken for 60 s and then incubated at 37°C for 15 min. Then, 80 μL stop solution was added to each well to terminate the reaction. After that, the 96-well plate was shaken for another 60 s and read at 405 nm with a spectrophotometer (Sunrise-Basic TECAN, Austria). The p-nitrophenol concentration of each sample can be calculated according to the standard curve. Thus, one unit of ALP activity can be defined as release of 1 nmol p-nitrophenol per min at pH 9.8 at 37℃ and calibrated by per unit total cellular protein.

3.2 Cytoplastic total protein concentration

Cellular protein content was detected by a BCA (bicinchoninic acid) Protein Assay Kit (Pierce, USA) at the same time with ALP activity assay. Briefly, 25 μl aliquots of cell lysate mixed with 200 μl of BCA working reagent in microplate were incubated at 37℃ for 30 min. The resulting optical densities were measured at 562 nm with a spectrophotometer. Bovine serum albumin was used to generate a standard curve.

3.3 Osteocalcin release

Osteocalcin was measured from cell culture medium using Mouse Osteocalcin EIA Kit (Biomedical Technologies Inc., USA) according to the manufacturer's protocol. Briefly, 25 μl of the cell culture medium was added into wells of a 96-well EIA plate, followed by the addition of 100 μl osteocalcin antiserum and the mixtures was incubated at 4℃ for 18-24 h. The plate was washed thoroughly, and the wash buffer was removed completely. Then, 100 μl streptavidin-horseradish reagent was added into all wells and incubated at room temperature for 30 min. The plate was washed again and 100 μl substrate was immediately added into all wells after the mixing of 50 μl TMB solution and 50 μl hydrogen peroxide solution. The plate was incubated in the dark for 15 min at room temperature. After adding 100 μl stop solution, the plate was read at 450 nm on a spectrophotometer (Sunrise-Basic TECAN, Austria). OC concentration of each sample was obtained from standard curve and calibrated by total protein amounts. Cells cultured on the Ti discs were lysed by CelLytic buffer (Sigma) and the total cellular protein was detected using BCA Protein kit described above.

4. *The representative SFM images of AE-Ti group.*

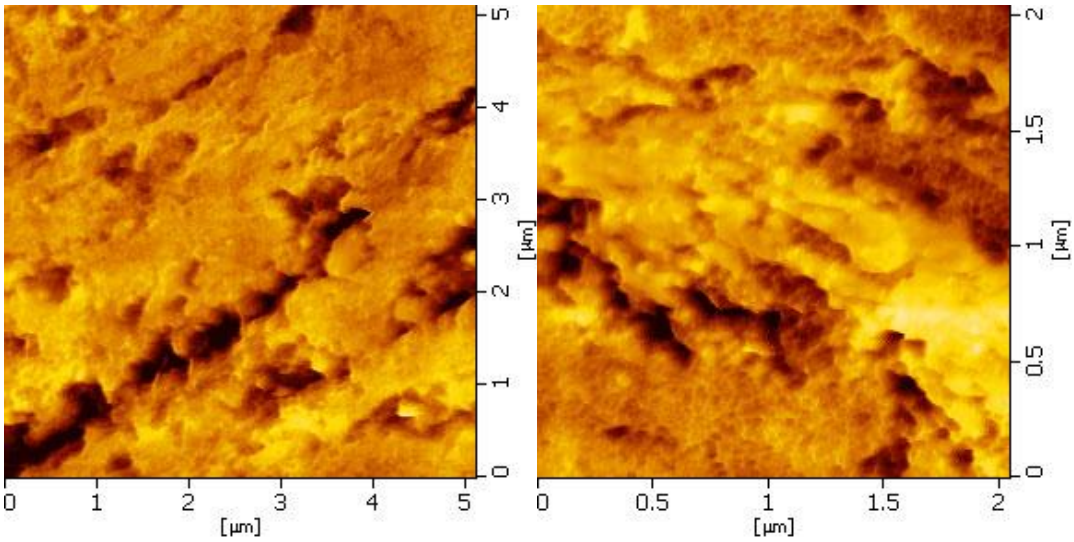
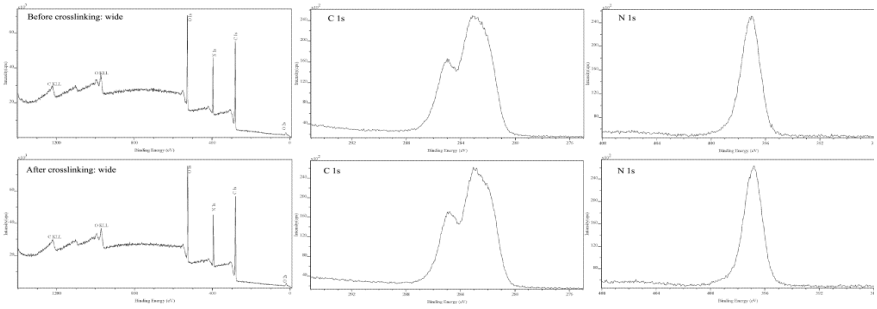


Figure S2. The representative SFM images of AE-Ti group. The images in the right panel are the magnified images of the left panel.

5. *The detection of C_{1s} and N_{1s} spectra of the coating and the C and N contents of the coating in the BEC-CL-Ti group before and after crosslinking via XPS*



A

	Peak			
	N 1S		C 1S	
	Before crosslinking	After crosslinking	Before crosslinking	After crosslinking
Atomic Mass	14.007	14.007	12.011	12.011
Atomic Concentration	21.11	21.6	78.39	78.4
Mass Concentration	23.78	24.32	76.22	75.68

B

Figure S3. A) C_{1s} and N_{1s} spectra of the coating in the BEC-CL-Ti group before and after crosslinking. B) N and C contents in the coating of the BEC-CL-Ti group before and after crosslinking, which not only showed the success of the assembly strategy, but also showed tiny difference in the N and C contents in the coating before and after crosslinking (n= 5).

6. *The surgical procedures of animal experiment*

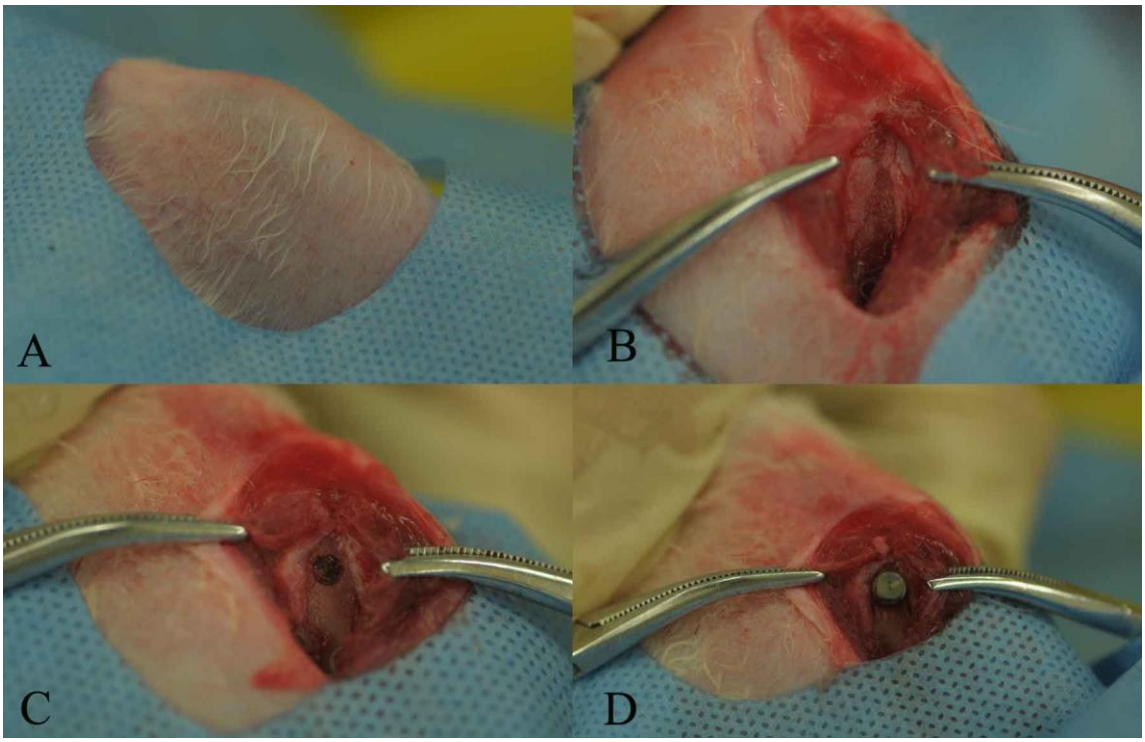


Figure S4. (A, B, C, D) simply introduced the surgical procedure of in vivo test. (A) The rabbit was tied to the operation table after general anesthetized. The surgical sites were shaved and the skin was disinfected. (B) The surgical site was incised in layers. (C) The implant site osteotomic was prepared in usual manner at the flat surface on the medial condyle of the femur and a final drill diameter of 3.0 mm was used. (D) Screw implant with a covering abutment (used for biomechanical test) was inserted, the inferior margin of which was level with the bone surface.