Antibiotic-loaded Chitosan Hydrogel with Superior Dual Functions: Antibacterial Efficacy and Osteoblastic Cell Responses

Fang Wu[a], Guolong Meng[a], Jing He[a], Yao Wu[a], Fang Wu[a,]*, Zhongwei Gu[a]

[a] National Engineering Research Center for Biomaterials, Sichuan University, Chengdu, 610064, P.R China

[*]Corresponding author: Prof. Fang Wu, *E-mail address*: <u>fangwu0808@yahoo.com</u>

Supporting Information

Supplemental Data 1: UV-Vis spectra of the synthesized chitosan-GS hydrogels

The CM-chitosan-GS hydrogel were prepared by adding GS aqueous solution (20 mg/mL) and 1 mL of genipin solution (with various concentrations) to 20 mL of 50 mg/mL CM-chitosan aqueous solution aseptically. After the addition of the genipin, both the chitosan and GS solutions changed their color, likely due to the reaction between genipin and the amino groups of the GS and chitosan, yielding a heterocyclic cross-linking structure [1]. The likely conjugations of the hydrogels were further examined by the UV-vis spectroscopy (UV-2100, Shimadzu, Japan) and the spectra were shown in Figure S1. New absorption at 596 nm, 586 nm, 595 nm appeared in the UV-vis spectra of chitosan-genipin, GS-genipin and chitosan-GS-genipin complexes, respectively (Figure S1), indicating the formation of the corresponsive new compounds and likely conjugation between the genipin and chitosan/antibiotic.
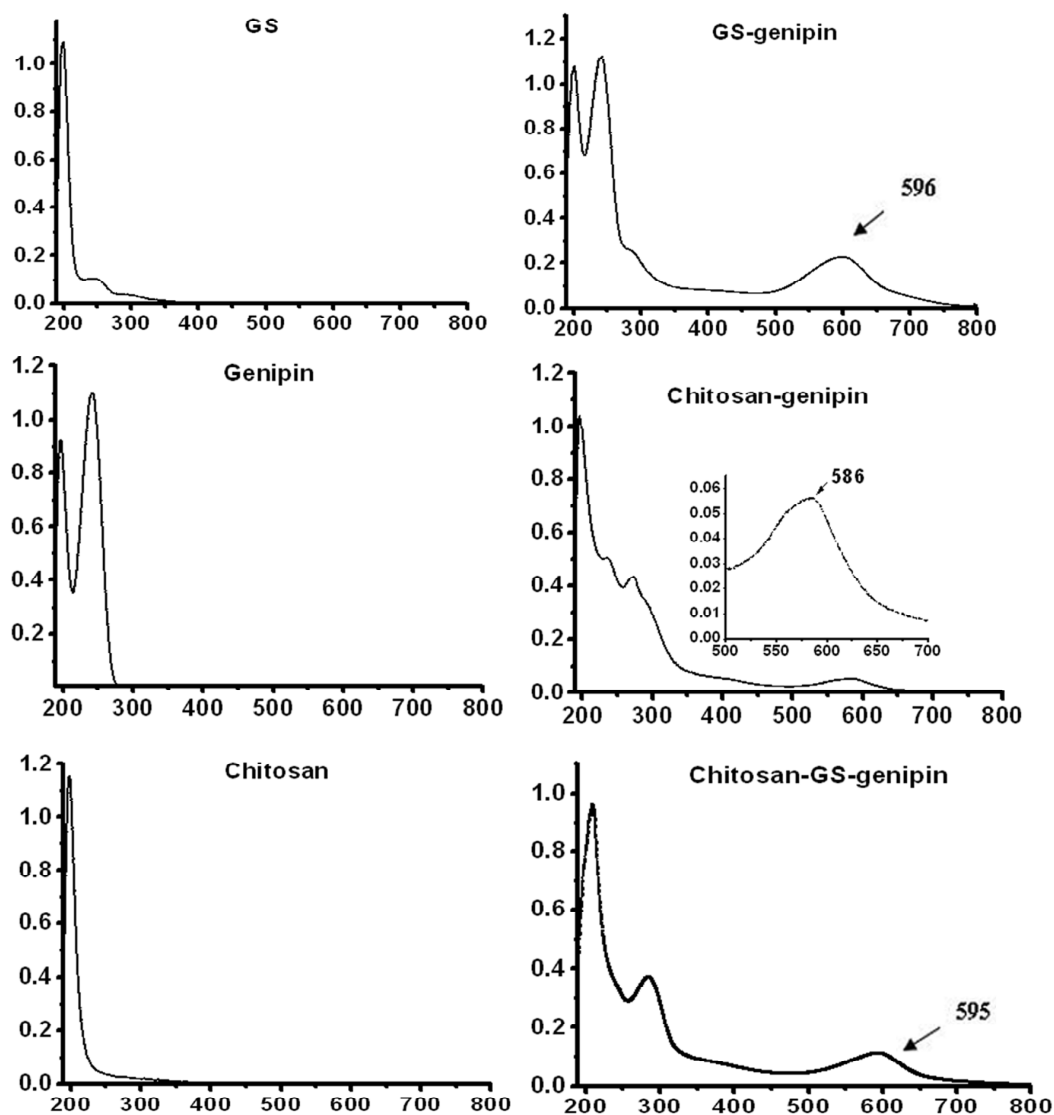
Figure S1: UV-Vis spectra of GS, genipin, chitosan, GS-genipin, chitosan-genipin, chitosan-GS-genipin, indicating the reactions and formation of new compounds between chitosan and genipin and between GS and genipin, respectively.

Supplemental Data 2: *In vitro* **drug release kinetics of GS from CM-chitosan hydrogels**

To understand the *in vitro* release kinetics of gentamycin sulfate from CM-chitosan hydrogel with various genipin concentrations, four most applied mathematical models were used [2-5] i.e. the zero order model, first order model, Higuchi square root model and Korsmeyer-Peppas model.

Zero order model: $\% Q_t/\% Q_\infty = Kt$;

First order model: $\% Q_t/\% Q_\infty = 1-e^{-Kt}$;

Higuchi square root model: $\% Q_t/\% Q_\infty = Kt^{1/2\,t}$;

Korsmeyer-Peppas model: $\% Q_t/\% Q_\infty = Kt^n$.

where $\% Q_t$ represents the percentage of drug released at time t, and $\% Q_\infty$ represents the total percentage of drug released; $\% Q_t/\% Q_\infty$ is the fractional release of drug at time t. K is the release rate constant and n is the diffusional exponent which could indicate the drug release mechanism and t is the time. When $n \leq 0.5$, it is Fickian diffusion (diffusion-controlled release); when $0.5 < n < 1$, it is defined as non-Fickian release (anomalous transport); and n=1 (zero order release), it is Case-II transport; when n>1, it is defined as Super Case-II transport [4-5]. The correlation coefficients (R^2) of these four models were calculated and values nearer to 1 considered as the best fitting model.

Table S1 showed the result of the curve fitting into different mathematical models. All these hydrogels followed the Korsmeyer-Peppas model (R^2=0.8755, 0.9699, 0.9855 at chitosan-0.5%, chitosan-1%, chitosan-2%, respectively). Fitting the first 60% of the total amount release to the Korsmeyer-Peppas equation proposed that the antibiotic release from chitosan-0.5% (n=1.2545) was through Super Case-II transport which was likely through polymer relaxation. On the other hand, the release behaviors of chitosan-1% (n=0.9002) and chitosan-2% hydrogel (n=0.7764) were non-Fickian diffusion which might involve in the combination of diffusion and erosion.

Table S1: Correlation coefficient (R^2) and release exponent (n) calculated after curve fitting of the in vitro release profile of chitosan-0.5% genipin, chitosan-1% genipin and chitosan-2% genipin.

Materials	Zero order	Frist order	Higuchi	Korsmeyer-Peppas	
	R^2	R^2	R^2	R^2	Release exponent (n)
Chitosan-0.5% genipin	0.0962	0.4524	0.6790	0.8755	1.2545
Chitosan-1% genipin	0.5856	0.6813	0.8767	0.9699	0.9002
Chitosan-2% genipin	0.9213	0.9548	0.9613	0.9855	0.7764

Supplemental Data 3: SEM micrograph showing osteoblastic cell adhesion

After 5-day culture, SEM was used to analyze the morphology and adherence of the MC3T3-E1 cells (Figure S2). The result was in agreement with the MTT observations, showing increased cell adhesion on chitosan hydrogel samples compared with the control. Cell on the chitosan-2% genipin sample had the best spreading, indicating that the increase of genipin concentration was beneficial for the osteoblastic cell adhesion. Overall, the results suggested that positively charged chitosan might attract negatively charged cells and enhance cell adhesion.
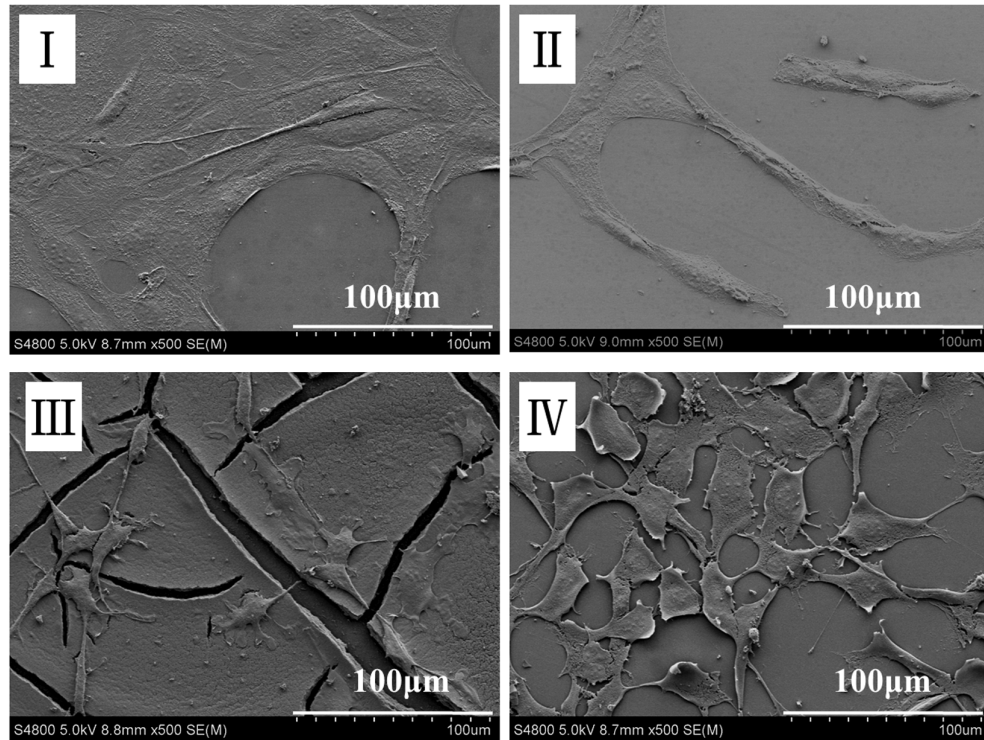

Figure S2: SEM images of MC3T3-E1 cells on the different material surfaces after 5 days, indicating the beneficial effect of increasing genipin concentration on the osteoblastic cell adhesion: I: Control; II: Chitosan-0.5% genipin; III: Chitosan-1% genipin; IV: Chitosan-2% genipin.

Supplemental Data 4: Osteoblastic cell differentiation under the osteogenic induction medium

To examine the osteoblastic differentiation course, 1 mL of MC3T3-E1 cells suspension were cultured on three prepared hydrogels or tissue culture plates at 1, 3, 5, 7 days by using the osteogenic induction medium (the induction medium consisted of α-MEM supplemented 10% FBS, 0.1 μmol/L of dexamethasone, 10 mmol/L of β-glycerophosphate and 50 μmol/L of ascorbic acid) [6]. As shown in Figure S3b, chitosan hydrogels showed higher OD values and the chitosan-2% genipin sample had the highest OD values. All chitosan-based hydrogels showed significant higher N-cadherin, Runx2 and ALP expressions compared with the control (Figure S3), indicating enhanced cell adhesion and differentiation of MC3T3-E1 cells. Similar trend has been observed for the cell adhesion, proliferation and differentiation of osteoblastic cells in comparison with that of the normal culture medium. But a clearer upward trend was observed with the increase of the OD values and protein expressions with the increase of the genipin concentration (degree of cross-linking) and significantly enhanced osteoblastic cell response over the control for all three genipin concentrations in the conditional medium. The results also demonstrated a significantly stronger dependence of N-cad expressions on genipin concentration under osteogenic induction media, compared with the normal culture media used above.
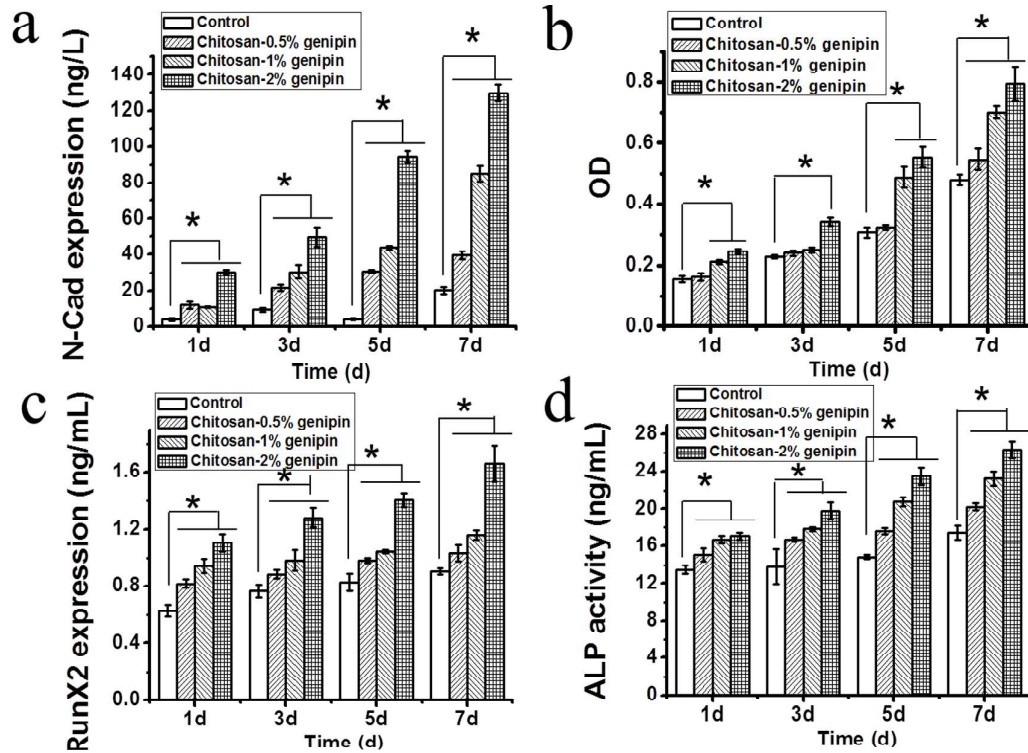
Figure S3: The effect of the chitosan hydrogels (with the tissue culture dishes as control) on the osteoblastic cell response with the introduction medium. (a): Adhesion-related protein (N-cadherin) expressions of MC3T3-E1 cells on control, chitosan-0.5% genipin, chitosan-1% genipin and chitosan-2% genipin for 1, 3, 5, and 7 days. (b): Proliferations of MC3T3-E1 cells cultured on control, chitosan-0.5% genipin, chitosan-1% genipin and chitosan-2% genipin for 1, 3, 5, and 7 days. Differentiation-related expressions of MC3T3-E1 cells on control, chitosan-0.5% genipin, chitosan-1% genipin and chitosan-2% genipin for 1, 3, 5, and 7 days. (e): RunX2; (f): ALP. Error bars represented standard deviation of the mean for n=3 (*p <0.05 vs control as statistically significant).

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