MMP-8 responsive poly(ethylene glycol) hydrogel for intraoral drug delivery

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APPENDIX

Materials and methods

Reagents

Trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), methanol and dichloromethane (DCM) were acquired from MilliporeSigma (St. Louis, MO) and distilled prior to use. Quadridentate-PEG-acrylate (4-arm-PEG-DA, M_W 20 kDa) was purchased from Ponsure Biotechnology (Shanghai, China). Matrix metalloproteinase-8 was purchased from Sino Biological Inc. (Beijing, China).

Peptide synthesis

Peptide chains were suspended on 2-chlorotriyl chloride resin. Coupling of the first residue used 4 equivalents of Fmoc-protected amino acid and 6 equivalents of diisopropylethylamine in DMF solution for 2 hours. Other amino acid couplings were performed with 4 equivalents of Fmoc-protecting amino acid, 4 equivalents of o-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluorophosphate and 6 equivalents of diisopropylethylamine in DMF for 4 hours. During synthesis, the Fmoc-protected groups were deprotected twice with 20% piperidine/DMF (v/v) for 15 min each. At the end of synthesis, acetic acid was conjugated to the peptide segments. The resin was then rinsed with DMF (four times) and DCM (four times) and dried under vacuum for 24 hours. Cleavage of the anticipated peptide from dried resin was performed by suspending the resin in a cleavage cocktail containing TFA (0.5%) and DCM (99.5%) for 15 min. The peptides were subsequently precipitated in ice cold diethyl ether, centrifuged and rinsed four times in diethyl ether.

Peptide molecular weights were identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS, AB Sciex LLC, Farmingham, MA). Peptide purity was evaluated by high performance liquid chromatography (HPLC, Shimadzu Prominence, Shimadzu Scientific Instruments, Columbia, MD) with a C18 column using gradient elution, with the mobile phase consisting of acetonitrile, water and 1% TFA. Peptide concentrations were measured via absorbance at 205 nm. Peptide purity was evaluated by comparing target peak area to total area. Solid peptide was stored at -20 ºC until use.

Hydrogel characterization

Gelation time*:* The vial-tilting method was employed for measuring glelation time (Lih *et al*. 2012). Precursor solutions were added to vials and the time when there was no flow upon inverting the vial was recorded as the gelation time.

Fourier-transform infrared spectroscopy (FTIR): Spectra of freeze-dried hydrogels were collected using a FTIR 5700 spectrometer (ThermoFisher Scientific, Waltham, MA) using the potassium bromide tablet technique. Freeze-dried 4-arm-PEG-DA powder was used as control.

Scanning electron microscopy: Freeze-dried hydrogels were affixed to aluminum stubs and sputter-coated with gold for examination by field emission scanning electron microscopy (FESEM, Zeiss SIGMA, Germany).

Rheology: Rheological characteristics were monitored with a DHR-2 rheometer (TA Instruments, New Castle, DE) at 37 ºC. Hydrogel discs were examined at 1 Hz at a gap distance of 1 mm. The elapsed time for mixing and injecting specimens was 30 seconds.

Swelling ratio: Measurements were conducted in phosphate buffer saline (PBS). For each group, five gel discs with the same weight and volume were dried, weighed (W_0) and immersed in PBS at 37 ºC. At designated time intervals, the specimens were retrieved and re-weighed (Wt). Mass swelling ratio was defined as Wt/W_0 (Lin *et al*. 2014).

Hydrogel degradation: Hydrogels were immersed in PBS, 10 nM or 20 nM of activated recombinant human MMP-8 at 37 ºC. Because MMP-8 inactivates over time, all buffer solutions were replaced every 48 hours. The hydrogels were examined every 12 hours until complete degradation. Degradation was assumed to be complete when no remaining material could be detected on disentanglement.

Cytotoxicity

MTT assay: Human gingival fibroblasts (Creative Bioarray, Shirley, NY) were suspended in cell culture medium and seeded in 96-well plates at 1×10^4 cells/100 μL/well. Seeded plates were incubated at 37 °C in 5% CO_2 for 24 hours. The original culture medium was subsequently replaced with media with or without hydrogel extracts and further incubated for 24 or 72 hours. Specimens were then processed for MTT assay (Bu *et al*. 2016). Relative cell viability was defined as the ratio between the mean absorbance value of the sample and that of cells cultured in the respective replacement medium (N=3).

***Cell attachment*:** Cell attachment on the hydrogel was used to evaluate the biocompatibility of the hydrogels using rhodamine-phalloidin staining. The hydrogels were placed in 24-well plates and sterilized with ultraviolet light. After aspiration of PBS, the specimens were incubated overnight in α-Minimum Essential Medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution in 5% CO_2 at 37 °C. After removal of the media, 2×10^5 3T3 mouse fibroblasts were seeded on the hydrogels. After culturing for 24 hours at 37 °C in 5% CO_2, the cells were fixed, stained with rhodamine-phalloidin (Cytoskeleton, Inc., Denver, CO) and 4′,6-diamidino-2-phenyl-indole (DAPI; Invitrogen, Carlsbad, CA) and examined with a florescence microscope.

Drug loading and release

Each drug/protein was added to the hydrogel precursor (1 mg/100 μL) prior to adding the peptide crosslinker. The MMP-8 sensitive hydrogel was prepared as described, with exception the

CGPQG↓IWGQC peptide was replaced with CGPQG↓IWGQ-KKVVFKVKFK-GPQG↓IWGQC. After freeze-drying, the drug/protein-loaded hydrogels were incubated in PBS at 37 °C for 24 hours. The solutions were replaced with fresh PBS, 10 nM or 20 nM of activated recombinant human MMP-8. At designated time-periods, 1 mL of the medium was collected and replaced with an equivalent volume of fresh medium. The amount of MH released was determined by HPLC at 350 nm (Yao *et al*. 2014), using a pre-determined standard curve that correlated absorbance with known MH concentrations. The amount of BSA released was determined using BSA assay (Yang *et al*. 2013). The concentrations of released peptide fragment (IWGQ-KKVVFKVKFK-GPQG) were determined using HPLC as described previously.

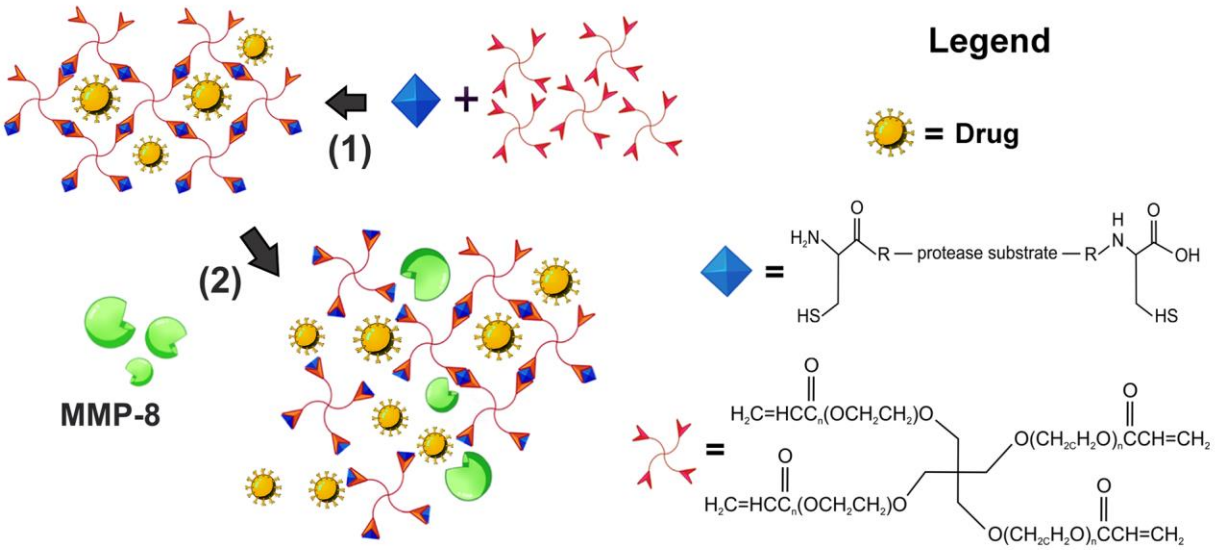
Drug release kinetics were analyzed by fitting the release data to zero-order kinetics, first-order kinetics, Korsmeyer-Peppas, Higuchi and Hixson-Crowell release models using the equations employed by Dipankar (Das *et al.* 2015). The relative correlation coefficient values were calculated.

Evaluation of drug bioactivity after their release from the M8SH hydrogels

After preparation of the activated MMP-8 solutions, M8SH+MH and ABPM8SH were synthesized and immersed in 0 (PBS only), 10 nM (PBS+10 nM MMP-8) and 20 nM MMP-8 (PBS+20 nM MMP-8) solutions with continuous agitation at 37 °C. Solutions were replaced every 48 hours because MMP-8 inactivates over time. A 100 mL aliquot of the solution was collected daily and replaced with the same volume of fresh solution. This procedure was continued until the hydrogels were completely disintegrated. The aliquots were used to investigate the antibacterial activity of the released drugs (Xiao *et al*. 2016). Briefly, 75 μL of liquid broth was added to a 96-well plate and a 75 μL aliquot of each sample was transferred to the first well. After mixing, 75 μL of the solution from the previous well was transferred to the next well to decrease its concentration by 50%. In control groups, the collected samples were replaced with PBS. Seventy-five microliter of *Porphyromonas gingivalis* (ATCC® 33277™; American Type Culture Collection, Manassas, VA) suspension was added to each well. The plate was placed in an aerobic incubator at 37 °C for 48 hours. Antibacterial activity was evaluated by recording the optical density of those samples at 600 nm (N = 3).

Statistical analyses

Analyses of the various parameters were performed using one-factor analysis of variance after verification of the normality and homoscedasticity assumptions of the corresponding data sets. If either of those assumptions was violated, the corresponding data set was non-linearly transformed to satisfy those assumptions prior to the adoption of parametric statistical methods. Statistical significance was pre-set at $\alpha = 0.05$.



Appendix Figure. Schematic illustrating the mechanism of MMP-8-responsive drug delivery system. (1) The MMP-8-responsive hydrogel system is produced by Michael-type addition reaction between the quadridentate-PEG-DA and bi-cysteine MMP-8 substrate peptides. (2) Drug-loaded network is responsive to local increase in MMP-8 activity.

Appendix Table. Different models fitted values of *in-vitro* drugs release

Models		Minocycline			BSA			ABP		
		PBS	10 nM MMP-8	20 nM MMP-8	PBS	10 nM MMP-8	20 nM MMP-8	PBS	10 nM MMP-8	20 nM MMP-8
Zero-order	R^2	0.865	0.865	0.923	0.914	0.965	0.948	0.934	0.972	0.885
First-order	R^2	0.976	0.958	0.961	0.671	0.983	0.984	0.871	0.984	0.785
Korsmeyer -Peppas	R^2	0.953	0.955	0.957	0.871	0.981	0.988	0.883	0.989	0.931
	n	0.470	0.597	0.485	0.828	0.465	0.510	0.575	0.635	0.726
Higuchi	R^2	0.948	0.866	0.945	0.816	0.985	0.968	0.927	0.981	0.845
Hixson-Crowell	R^2	0.959	0.969	0.975	0.953	0.989	0.979	0.934	0.984	0.903

PBS, phosphate buffered saline; BSA, bovine serum albumin; ABP, antimicrobial peptide.

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