Supporting Information for

Thiol-Mediated Synthesis of Hyaluronic Acid-Epigallocatechin-3-*O*-Gallate Conjugates for the Formation of Injectable Hydrogels with ROS Scavenging Property and Degradation Resistance

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Supplementary Methods

Preparation of DyLight 488-labeled HA and HA-EGCG. DyLight 488 maleimide was dissolved in 300 μL of DMF at a concentration of 10 mg mL^{-1}. Then, 30 μL of DyLight 488 maleimide was added dropwise into 10 mL of PBS (pH 7.4) containing 20 mg of thiolated HA. The pH of the mixture was adjusted to 7.5 by adding 0.1 M NaOH. The resulting solution was stirred at 25ºC for 24 h in a dark place under nitrogen atmosphere. After the pH of the mixture was brought to 6, the solution was transferred to dialysis tubes with a molecular weight cutoff of 10 kDa. The tubes were dialyzed against deionized water for 3 days at 4ºC. The purified solution was lyophilized to obtain thiolated HA labeled with DyLight 488. The degree of labeling was determined to be 0.2 by measuring the fluorescence of DyLight 488 dyes on a Horiba FluoroMax-4 spectrofluorometer with an excitation wavelength at 400 nm and an emission wavelength at 518 nm. For conjugation of EGCG, 40 mg of thiolated HA labeled with DyLight 488 was dissolved in 7 mL of PBS (pH 7.4) under nitrogen atmosphere. This solution was added dropwise to 3 mL of PBS (pH 7.4) containing excess of EGCG with continuous stirring. The pH of the mixture was adjusted to 7.4 by dropwise addition of 1 M NaOH. After reaction for 3 h at 25ºC, the pH of the mixture was adjusted to 6. The resultant solution was transferred to dialysis tubes with a molecular weight cutoff of 3,500 Da. The tubes were dialyzed against 25% ethanol for 1 day and deionized water for 2 days under nitrogen atmosphere. The purified solution was lyophilized to obtain DyLight 488-labeled HA-EGCG. The degree of EGCG conjugation was determined by measuring the absorbance of EGCG at 274 nm on a Hitachi U-2810 spectrophotometer. The degree of EGCG conjugation of DyLight 488-labeled HA-EGCG (*ca.* 6.26) was comparable to that of unlabeled HA-EGCG (6.55 ± 1.17).

Analysis of the binding activity of DyLight 488-labeled HA and HA-EGCG to cell surface CD44. CD44-positive human ovarian carcinoma SKOV-3 cells were cultured in McCoy's 5A medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. These cells were seeded on a black-walled 96-well plate at a density of 5×10^4 cells per well and then allowed to attach

for 3 h at 37°C. Subsequently, the cells were treated with 200 μL of serum-free medium containing either DyLight 488-labeled HA or HA-EGCG at final HA concentrations of 50 and 100 μg mL^{-1}, and then incubated for 30 min at 25°C. After rinsing with serum-free media twice, the cellular fluorescence was measured using a Tecan M200 Infinite microplate reader with an excitation wavelength at 490 nm and an emission wavelength at 520 nm. The amount of HA bound to the cells was quantified by comparing the observed fluorescence intensity with the standard solution containing various concentrations of either DyLight 488-labeled HA or HA-EGCG.

Rheological measurement. A stock solution of HA-EGCG conjugate was prepared by dissolving the conjugate in deionized water at a concentration of 11.5 mg mL^{-1} at 25ºC. The resultant solution was sterilized by filtration through a 0.22 μm syringe filter. To form HA-EGCG hydrogels, 780 μL of the filtered stock solution was mixed with 15 μL of HRP solution, 15 μL of H_2O_2 solution and 90 μL of 100 mM PBS (final ionic strength: 0.16 M, pH 7.4) in LoBind tubes (Eppendorf, Germany). The final concentrations of HA-EGCG, HRP and H_2O_2 were 10 mg mL^{-1}, 0.12-0.36 units mL^{-1} and 0.76 mM, respectively. The mixture was immediately vortexed and 210 μL of which was applied to the bottom plate of a HAAKE Rheoscope 1 rheometer (Karlsruhe, Germany). For comparison, HA-Tyr hydrogels were prepared using HA-Tyr solution (11.5 mg mL^{-1}) instead of HA-EGCG solution. The final concentrations of HRP and H_2O_2 used to form HA-Tyr hydrogels were 0.15 units mL^{-1} and 0.27-0.41 mM, respectively. Rheological measurement was carried out at 37ºC in the dynamic oscillatory mode with a constant deformation of 1% and a frequency of 1 Hz, using a cone and plate geometry of 3.5 cm diameter and 0.949º cone angle. The evolution of storage modulus (G') and loss modulus (G'') was monitored as a function of time. The gel point, an indicator of gelation rate, was determined as the time at which crossover of the two moduli occurred. For frequency sweep experiments, HA-EGCG hydrogels formed at the optimized concentrations of HRP (0.24 units mL^{-1}) and H_2O_2 (0.76 mM) were subjected to frequency scanning from 0.2 to 20 Hz under a constant shear stress (1 Pa) and temperature (25°C). The G' and strain were recorded as a function of frequency.

Formation of HA-EGCG hydrogels through autoxidation. A stock solution of HA-EGCG was prepared by dissolving the conjugate in deionized water at a concentration of 11.5 mg mL^{-1} at 25ºC. To form HA-EGCG hydrogels, 780 μL of the filtered stock solution was mixed with 30 μL of water and 90 μL of 100 mM PBS (final ionic strength: 0.16 M, final pH ranging from 7.5 to 9.0) in Eppendorf tubes. The final concentration of HA-EGCG was 10 mg mL^{-1}. The mixture was immediately vortexed and 210 μL of which was applied to the bottom plate of a HAAKE Rheoscope 1 rheometer (Karlsruhe, Germany). Rheological measurement was carried out according to the methods described above.

Examination of cytocompatibility of HA-EGCG and HA-Tyr conjugates. NIH 3T3 mouse fibroblasts were purchased from the American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. NIH 3T3 cells were seeded on 96-well plates at a density of 2×10^4 cells per well and then allowed to adhere for 18 h at 37°C. The cells were incubated for 24 h with 200 μL of DMEM/FBS media containing either HA-EGCG or HA-Tyr conjugate at various concentrations. Cell viability was evaluated using the alamarBlue® cell viability assay reagent which measures cellular metabolic reduction. Briefly, 100 μL of serum-free DMEM containing 10% (v/v) AlamarBlue® reagent was added to each well of the 96-well plates. After incubation for 4 h at 37°C, the cellular fluorescence was measured using a Tecan Infinite microplate reader (Tecan Group, Switzerland) with an excitation wavelength at 530 nm and an emission wavelength at 590 nm. The cell viability was expressed as percentages derived from the fluorescence intensity from the treated cells relative to untreated cells.

Hematological examination. Female athymic NCR nude mice (CrTac:NCr-Fixn1^{nu}, 5-6 weeks old, 20-25 g) were subcutaneously injected with 100 μL of either HA-EGCG or HA-Tyr hydrogel under anesthesia. Three weeks after hydrogel injection, blood (150 μL) was collected into a Microvette 200 tube containing potassium-EDTA (Sarstedt, Germany). As a control, blood samples were withdrawn from age-matched mice without hydrogel treatment. The blood cell counts were performed with a Hemavet 950FS hematology analyzer (Drew Scientific Inc., Oxford, USA).

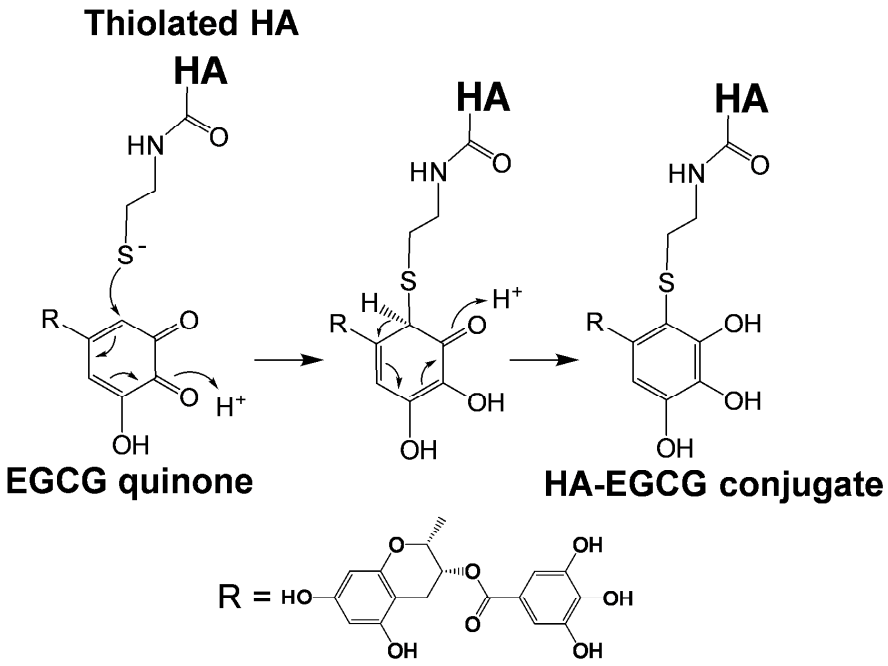

Figure S1. Proposed mechanism of the formation of HA-EGCG conjugate through nucleophilic addition reaction and rearrangement.

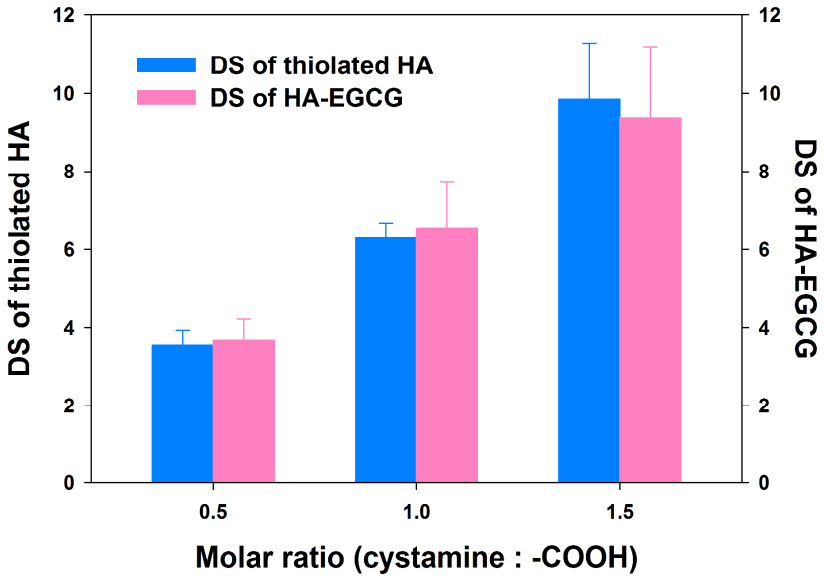

Figure S2. Comparison of the degree of substitution (DS) between thiolated HA derivatives and HA-EGCG conjugates.

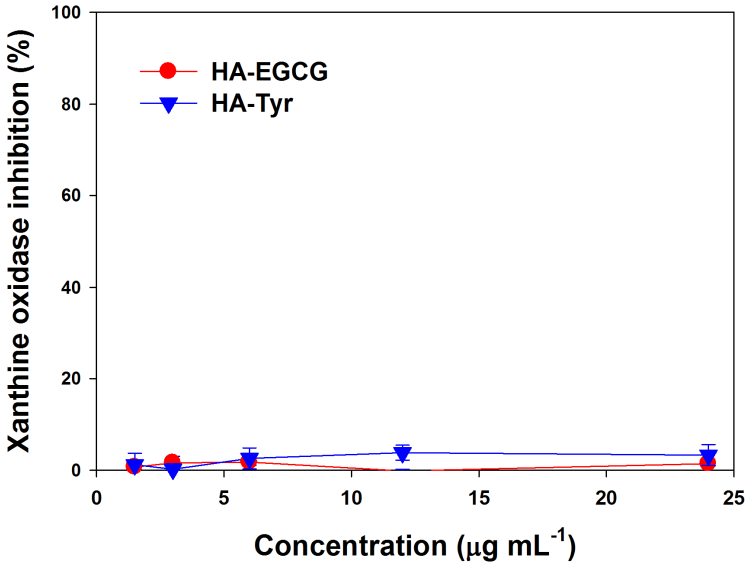

Figure S3. Xanthine oxidase-inhibitory activity of HA-EGCG and HA-Tyr conjugates as a function of concentration. Mean ± SD (n = 3). Neither HA-EGCG nor HA-Tyr conjugate had any significant influence on the activity of xanthine oxidase in the range of concentrations tested in the superoxide scavenging assays (1.5-24 μg mL^{-1}).

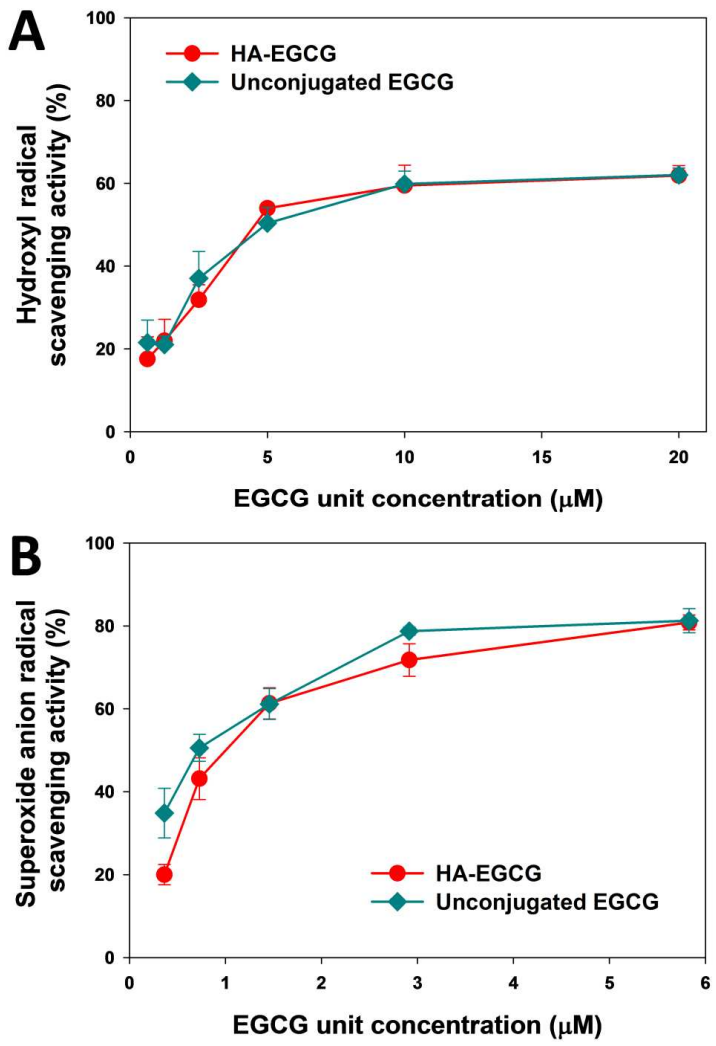

Figure S4. (A) Hydroxyl radical (•OH) and (B) superoxide anion radical (O_2•¯) scavenging activities of HA-EGCG conjugate and unconjugated EGCG as a function of EGCG unit concentration. All the results are expressed as mean ± SD (n = 3).

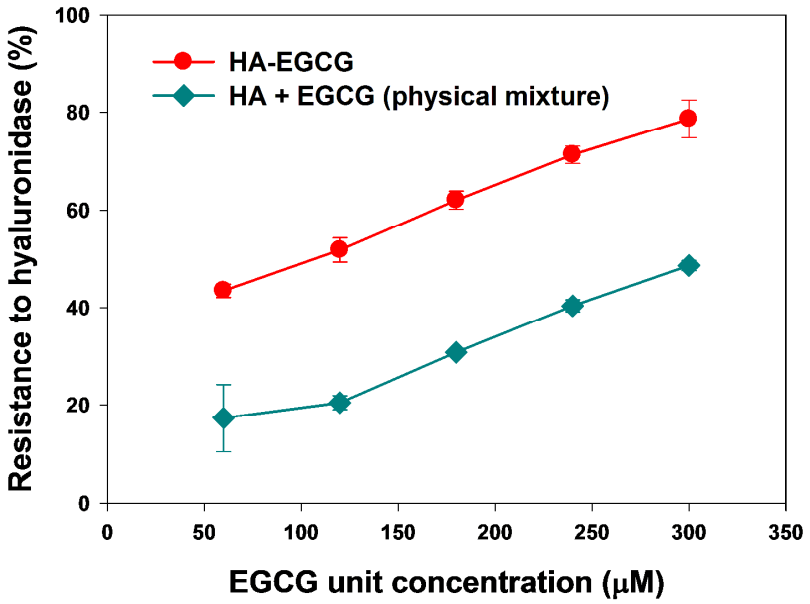

Figure S5. Hyaluronidase resistance of HA-EGCG conjugates in comparison with a physical mixture containing equivalent amounts of HA and EGCG. Mean ± SD (n = 3).

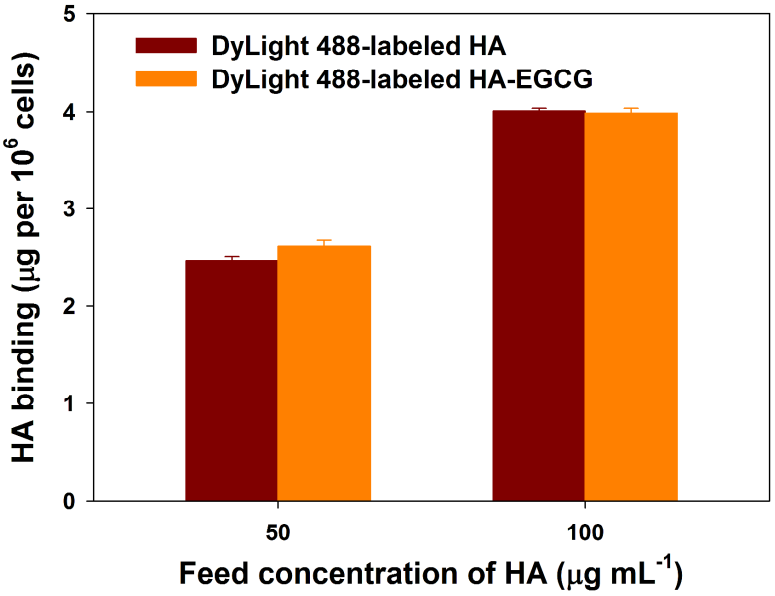

Figure S6. Amount of HA bound to CD44-expressing SKOV-3 cells following incubation with either DyLight 488-labeled HA or HA-EGCG for 30 min at 25°C. Mean ± SD (n = 3).

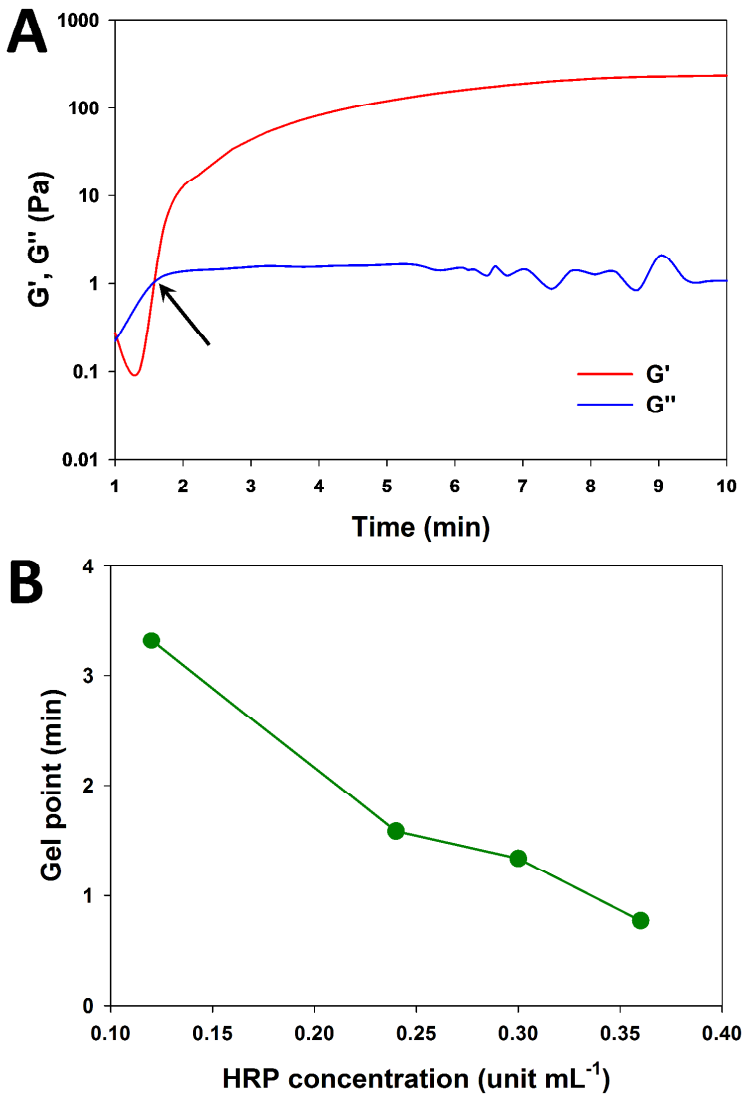

Figure S7. (A) Time course of changes in the storage modulus (G', red curve) and loss modulus (G'', blue curve) of HA-EGCG hydrogel formed at the optimized concentrations of HRP (0.24 units mL^{-1}) and H_2O_2 (0.76 mM). The arrow indicates the gel point at which the crossover of G′ and G″ occurs. (B) Gel points of HA-EGCG hydrogels formed at various concentrations of HRP. The final concentration of H_2O_2 was fixed at 0.76 mM.

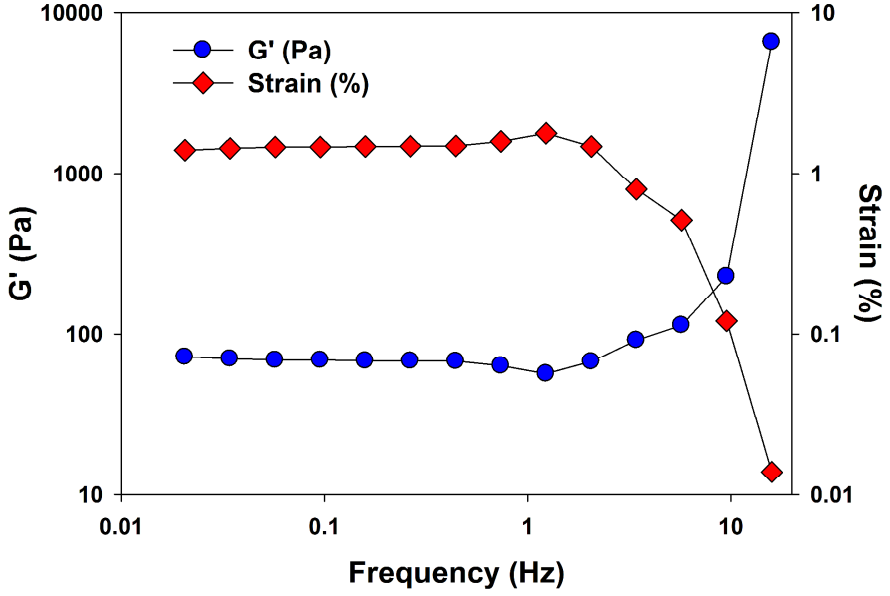

Figure S8. Evolution of the storage modulus (G') and strain of HA-EGCG hydrogel as a function of applied frequency of oscillatory shear stress. HA-EGCG hydrogel was formed at the optimized concentrations of HRP (0.24 units mL^{-1}) and H_2O_2 (0.76 mM).

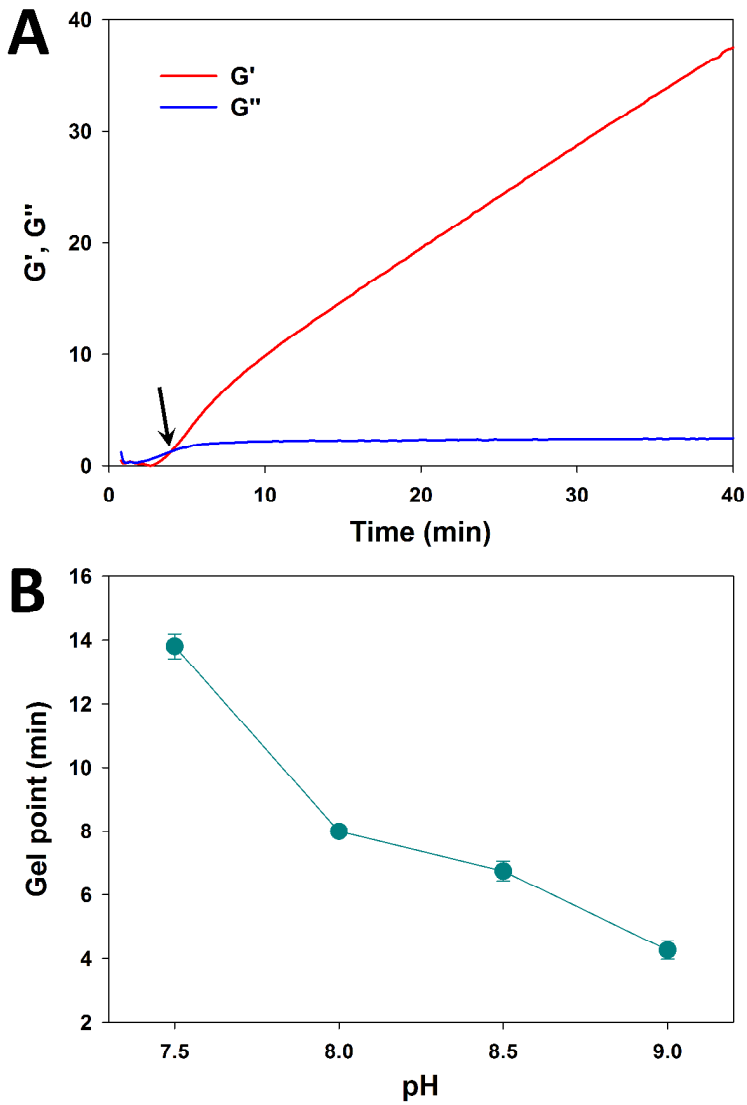

Figure S9. (A) Time course of changes in the storage modulus (G') and loss modulus (G'') of HA-EGCG hydrogel formed by autoxidation at pH 9.0. The arrow indicates the gel point at which the crossover of G′ and G″ occurs. (B) Gel points of HA-EGCG hydrogels formed by autoxidation at various pH.

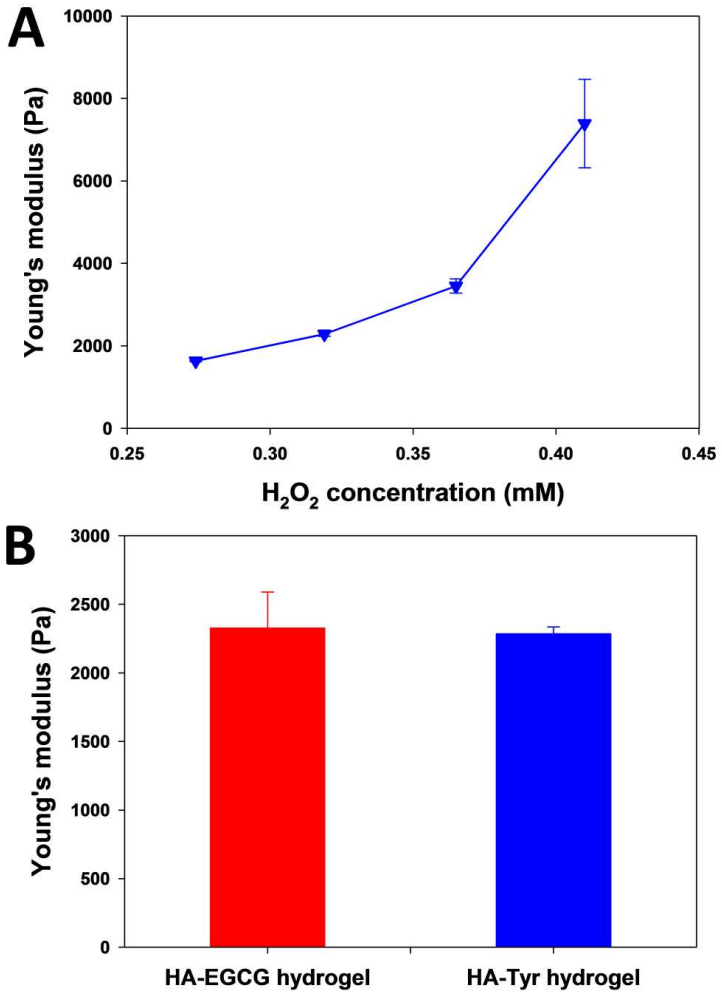

Figure S10. (A) Young's moduli of HA-Tyr hydrogels as a function of H_2O_2 concentration. The final concentration of HRP was fixed at 0.15 units mL^{-1}. (B) Comparison of Young's moduli between HA-EGCG and HA-Tyr hydrogels tested in this study. HA-EGCG hydrogel was formed at the optimized concentrations of HRP (0.24 units mL^{-1}) and H_2O_2 (0.76 mM). The final concentrations of HRP and H_2O_2 used to form HA-Tyr hydrogels were 0.15 units mL^{-1} and 0.32 mM, respectively. All the results are expressed as mean ± SD (n = 3).

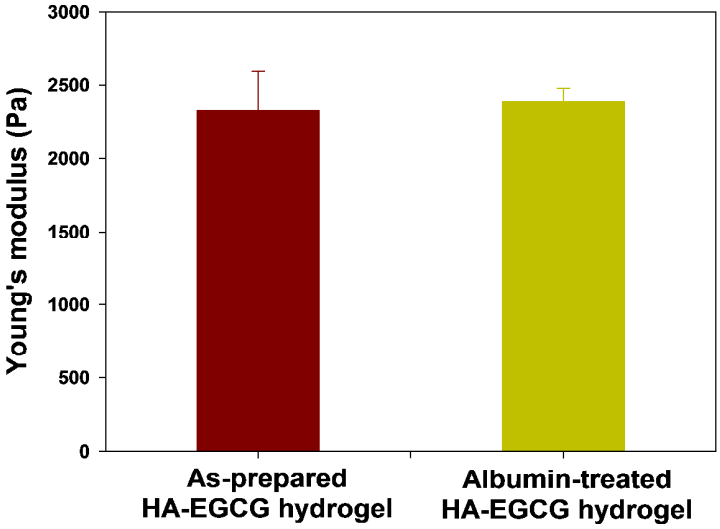

Figure S11. Comparison of Young's moduli between as-prepared and albumin-treated HA-EGCG hydrogels tested in this study. HA-EGCG hydrogels were formed at the optimized concentrations of HRP (0.24 units mL^{-1}) and H_2O_2 (0.76 mM). For albumin treatment, hydrogel disks were immersed in 2 mL of PBS (pH 7.4) containing bovine serum albumin (100 mg mL^{-1}) for 72 h at 37°C. All the results are expressed as mean ± SD (n = 3).

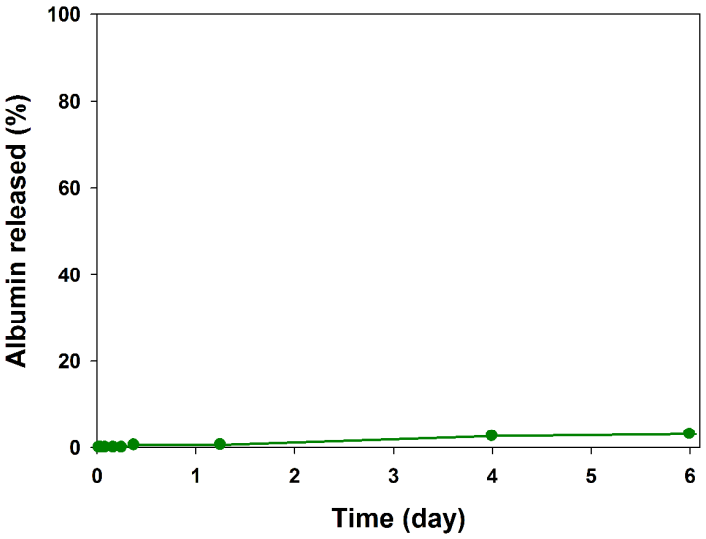

Figure S12. Percent of albumin released over time from albumin-treated HA-EGCG hydrogel in 20 mL of PBS (pH 7.4) at 37ºC. Mean ± SD (n = 4).

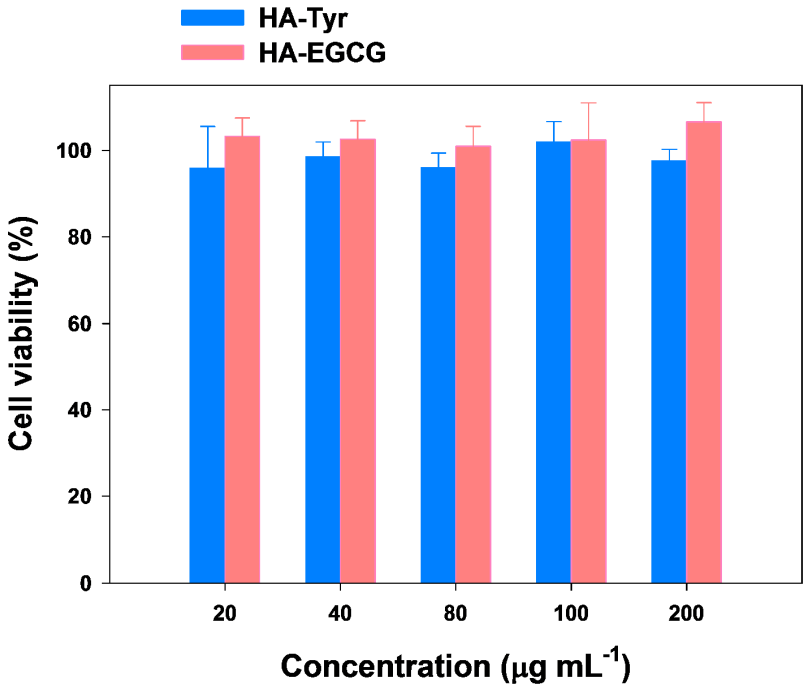

Figure S13. Viability of NIH 3T3 mouse fibroblasts treated for 24 h with either HA-EGCG or HA-Tyr conjugate at various concentrations. Mean ± SD (n = 5).

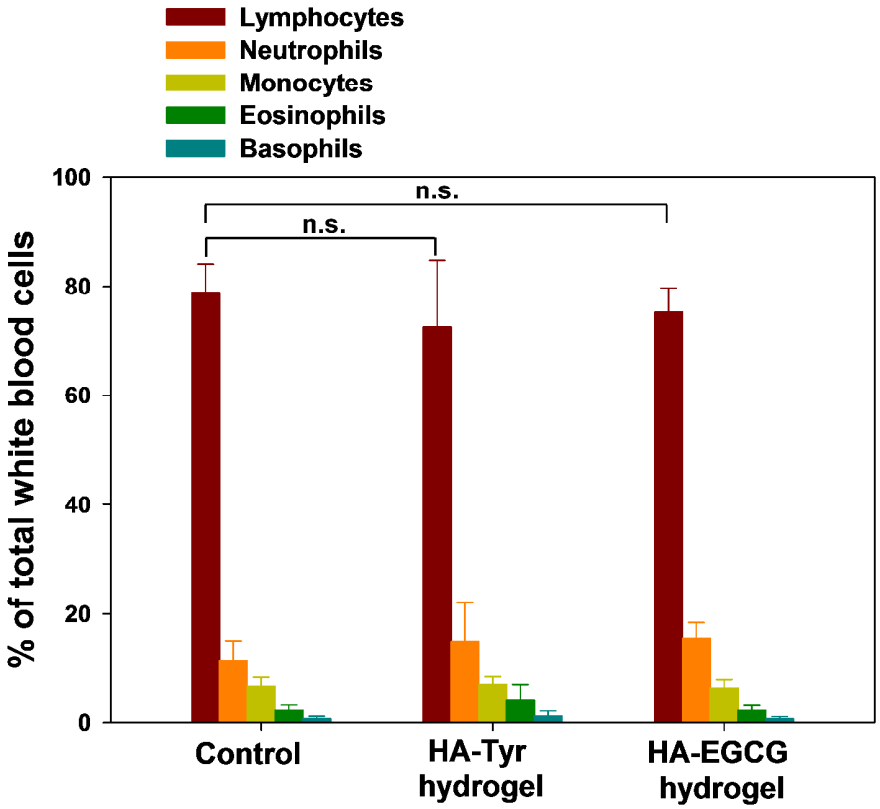

Figure S14. Quantitative analysis of leukocytes in the blood retrieved from the mice injected with either HA-Tyr or HA-EGCG hydrogel at 3 weeks post-injection. The control blood samples were withdrawn from age-matched mice without hydrogel treatment. There was no statistically significant difference in the leukocyte population between groups; n.s.: nonsignificant. Mean ± SD (n = 4).