Electronic Supplementary Information (ESI)

MSC-Derived Osteogenic Cell Sheets on Stiffness-Tuned Hyaluronic Acid-Gelatin Hydrogels

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Phenolated HA (HA-Ph) synthesis:

For the low-substitution phenolated hyaluronic acid (HA-Ph 3.7), sodium hyaluronate (Na-HA, Mw ≈ 800 kDa, 1.87 g) was dissolved in 500 mL of 0.1 M MES buffer (pH 6.0) and stirred overnight, after which tyramine hydrochloride (6.95 g) was added, followed by WSCD·HCl (1.38 g) and NHS (0.785 g). For the medium-substitution phenolated HA (HA-Ph 4.3), the same Na-HA and tyramine quantities were used, but with WSCD·HCl (2.76 g) and NHS (1.57 g). For the high-substitution variant (HA-Ph 5.2), double the coupling reagent amounts were applied WSCD·HCl (5.52 g) and NHS (3.14 g) while keeping all other reaction conditions identical. Each reaction mixture was stirred at room temperature for 20 h, transferred to dialysis tubing (MWCO 12–14 kDa), and dialyzed against deionized water for 3 days until no absorbance at 275 nm was detected in the diffusate, after which the purified solutions were lyophilized to obtain each HA-Ph variant. The degree of phenol substitution for each polymer was quantified by UV–Vis spectroscopy at 275 nm using tyramine calibration standards and confirmed by ^{1}H NMR in D_2O (Figure S1 and Figure S2).

Phenolated gelatin (Gelatin-Ph) synthesis:

Phenolated gelatin (Gelatin-Ph) was prepared by coupling 3-(4-hydroxyphenyl)propionic acid to gelatin through an NHS/EDC-mediated reaction. Gelatin type B (20 g) was dissolved in 500 mL of dimethylformamide (DMF, pH 4.2), after which 3-(4-hydroxyphenyl)propionic acid (6.64 g), WSCD·HCl (7.6 g), and NHS (6.4 g) were added. The reaction mixture was stirred for 20 h at room temperature. Then, unreacted 3-(4-hydroxyphenyl)propionic acid was removed by dialyzing the reaction mixture against deionized water until no absorbance at 275 nm was detected in the

diffusate. The resulting solution was then lyophilized to obtain Gelatin-Ph. The phenolation was confirmed by ^{1}H NMR analysis (signals at 6–8 ppm) (Figure S3).

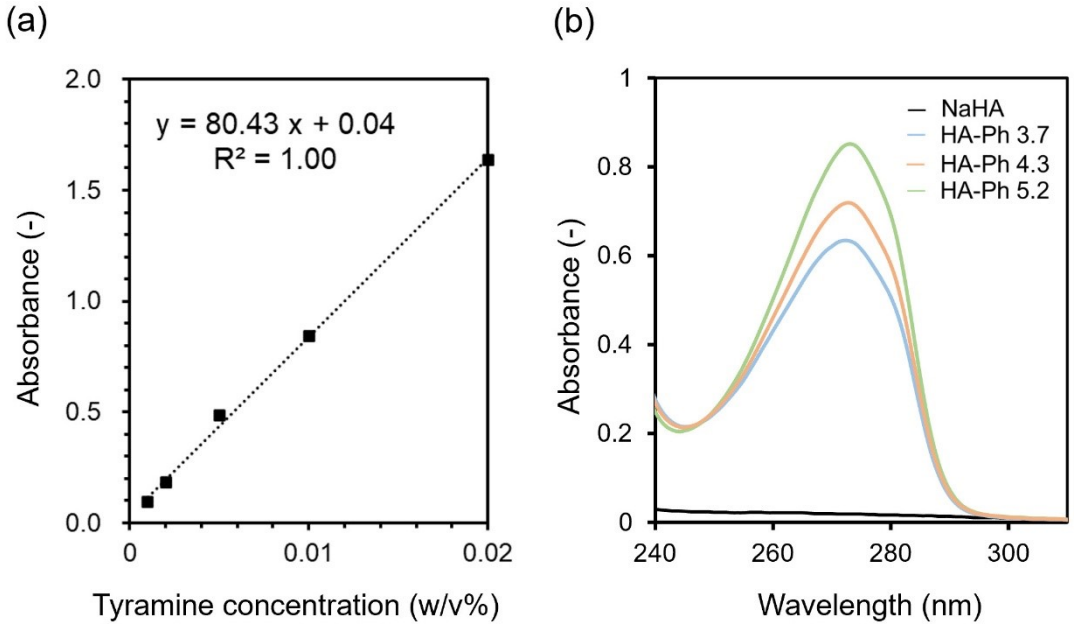

Figure S1. UV-Vis absorbance spectrum of (a) tyramine hydrochloride standard curve and (b) NaHA, HA-Ph 3.7, HA-Ph 4.3, and HA-Ph 5.2.

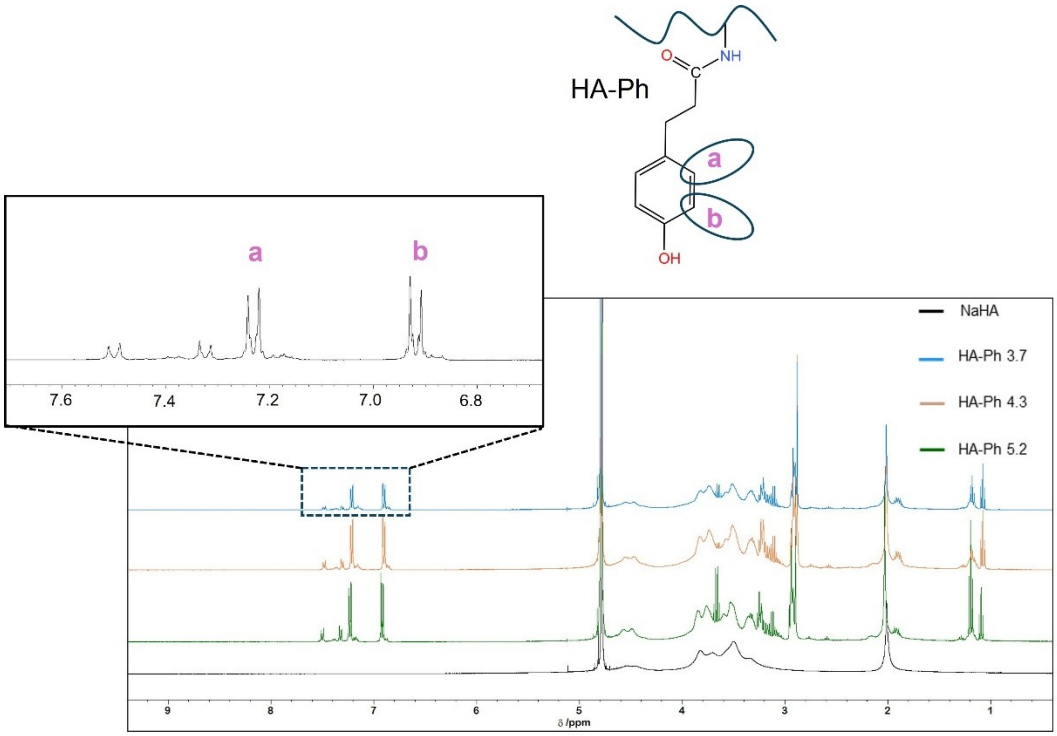

Figure S2. ^{1}H NMR spectroscopy of the (a) Na-HA, HA-Ph 3.7, HA-Ph 4.3, and HA-Ph 5.2.

The successful phenolation of HA was confirmed by the appearance of new aromatic proton signals in the ^{1}H NMR spectra. Native sodium hyaluronate (NaHA) showed no resonances in the aromatic region, whereas all HA-Ph samples showed two distinct sets of peaks between 6.8–7.4 ppm. These signals correspond to the phenyl ring protons of the tyramine moiety, specifically the protons labeled a and b in the HA-Ph structure. The emergence and gradual increase in the intensity of these aromatic peaks from HA-Ph 3.7 to HA-Ph 5.2 directly reflect the increasing incorporation of phenolic groups into the HA backbone. This clear difference between NaHA and HA-Ph confirms the successful covalent conjugation of tyramine onto HA, consistent with previous reports by wang et al. on phenol-modified polysaccharides (DOI: 10.1039/C9RA09531D).

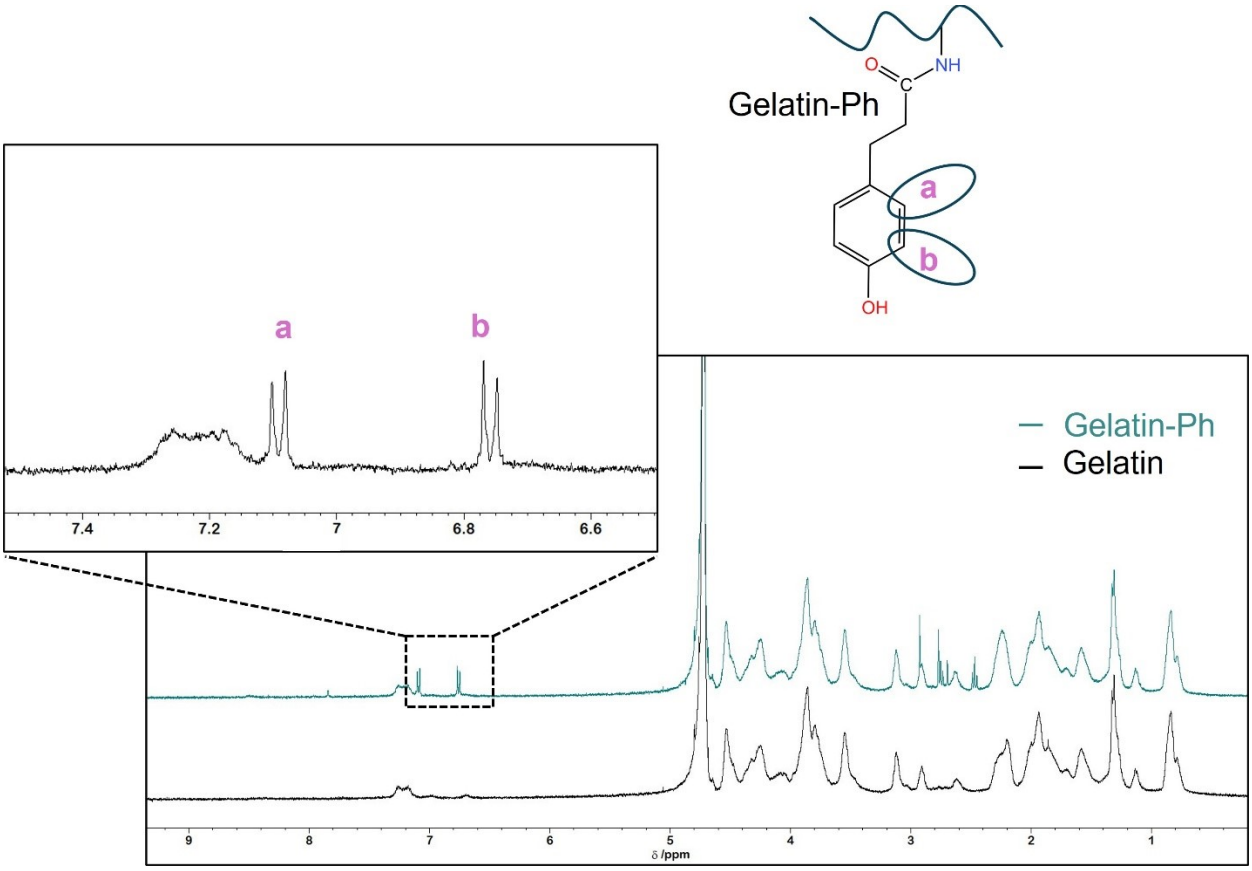

Figure S3. ^{1}H NMR spectroscopy of the (a) Gelatin-Type A, and Gelatin-Ph.

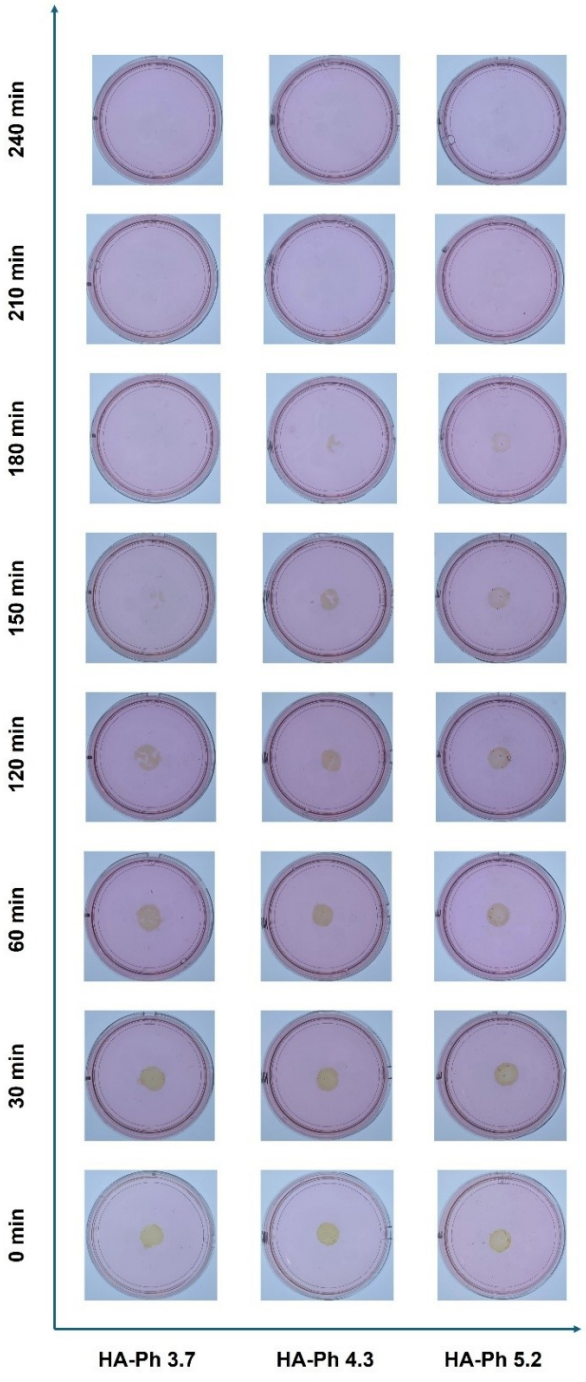

Figure S4. Time-lapse images of enzymatic degradation of HA-Ph/Gelatin-Ph hydrogels prepared with three phenol substitution degrees (HA-Ph 3.7, HA-Ph 4.3, and HA-Ph 5.2). Hydrogels were incubated in DMEM containing 0.1% w/v hyaluronidase at 37 °C, and their morphological changes were recorded from 0 to 240 min.

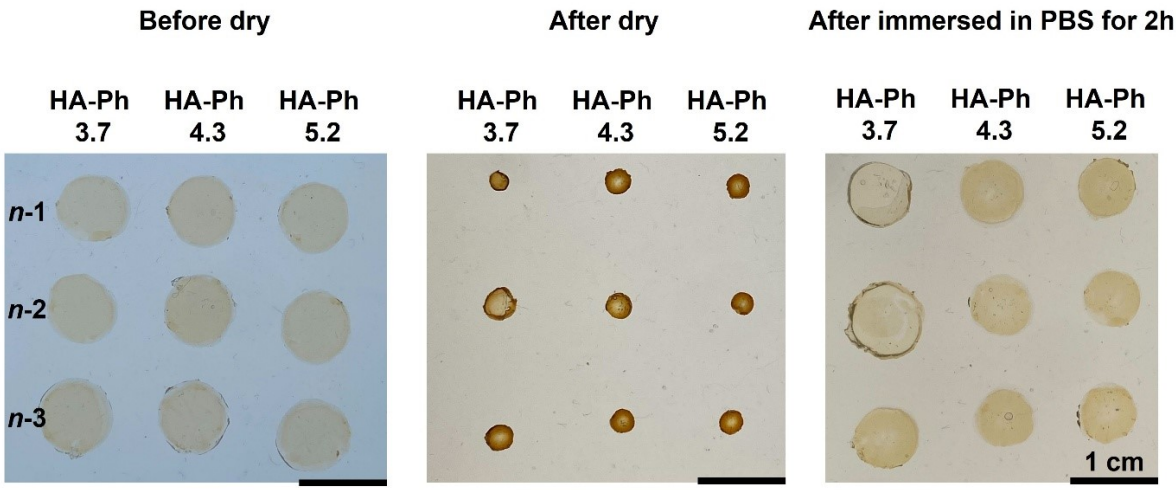

Figure S5. Macroscopic appearance of HA-Ph/Gelatin-Ph hydrogels prepared with three phenol substitution degrees (HA-Ph 3.7, HA-Ph 4.3, and HA-Ph 5.2) before drying, after drying, and after rehydration in PBS. Each hydrogel was fabricated using 2% w/v HA-Ph, 0.5% w/v Gelatin-Ph, and 50 U/mL HRP, followed by exposure to 16 ppm H_2O_2 for 30 min. Left: Hydrogels immediately after gelation (n = 3 per condition). Middle: Corresponding dried hydrogels demonstrating shrinkage due to water removal. Right: Hydrogels after immersion in PBS at 37 °C for 2 h, showing rehydration and swelling behavior characteristic of each HA-Ph variant. Scale bar:1 cm.

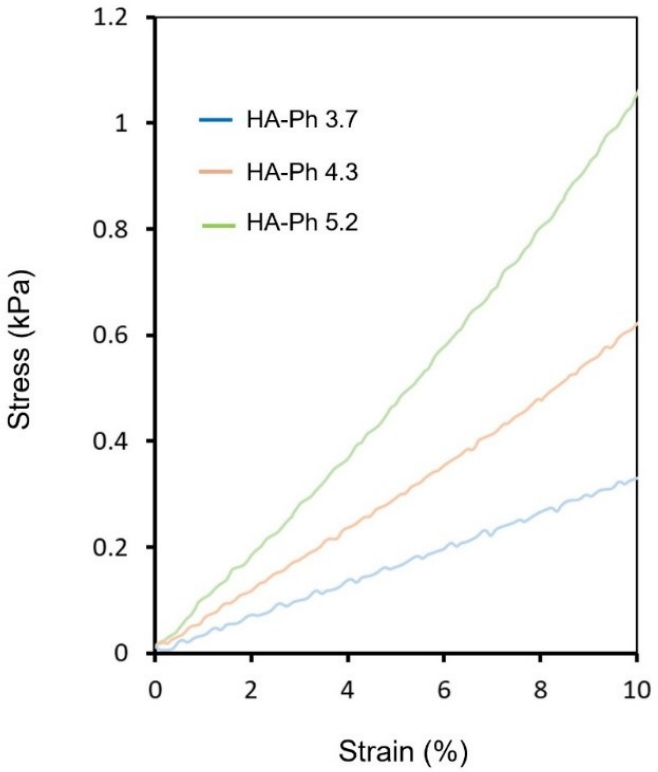

Figure S6. Stress-strain curve of HA-Ph/Gelatin-Ph hydrogels obtained by exposing 16 ppm H_2O_2 to PBS containing 2 w/v% HA-Ph (HA-Ph 3.7, HA-Ph 4.3 or HA-Ph 5.2), 0.5 w/v% Gelatin-Ph, and 50 U/mL HRP for 30 min. Hydrogels were compressed at 0.6 mm/s using an 8 mm probe.

Table S1: Primers used for qPCR.

Gene	Forward primer	Reverse primer
RUNX2	CCCAGTATGAGAGTACGTGTCC	GGGTAAGACTGGTCATAGGACC
COL1A1	GATTCCCTGGACCTAAAGGTGC	AGCCTCTCCATCTTTGCCAGCA
ALP1	GCTGTAAGGACATCGCCTACCA	CCTGGCTTTCTCGTCACTCTCA
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG