Nitric Oxide-Releasing Hyaluronic Acid as an Antibacterial and Immunomodulatory Acne Therapeutic

Supporting Information

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CONTENTS

Scheme S1. (A) Modification of HA with Secondary Amine-Containing Alkylamines and (B) *N*-Diazeniumdiolate Formation on Secondary Amines and Release of NO.

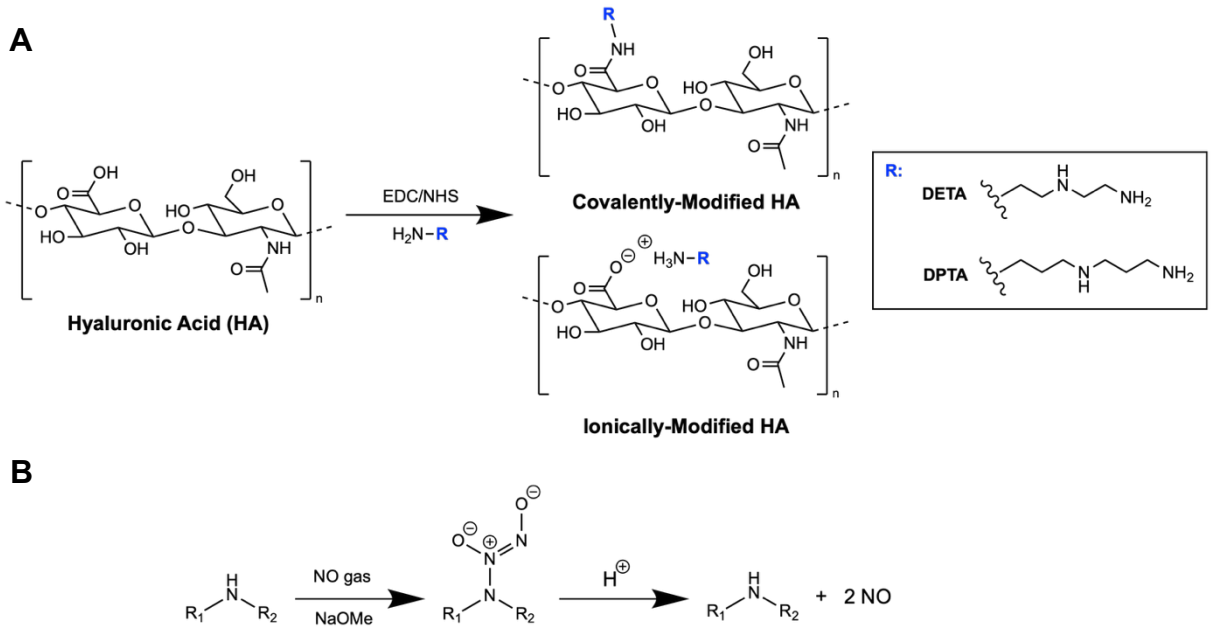

Table S1. Weight-Average Molecular Weight (M_w) and Dispersity(Đ) of Unmodified and Amine-Modified HA Derivatives.[a,b]

Material	M_w (kDa)	Đ
HA6	9.0 ± 2.3	1.7 ± 0.1
HA90	89.3 ± 8.0	1.8 ± 0.1
HA1000	1150.8 ± 161.0	1.2 ± 0.1
HA6-DETA	9.3 ± 1.5	1.7 ± 0.2
HA90-DETA	92.9 ± 15.1	1.6 ± 0.1
HA1000-DETA	1031.8 ± 122.7	1.3 ± 0.1
HA6-DPTA	9.4 ± 2.1	1.7 ± 0.2
HA90-DPTA	95.6 ± 11.5	1.7 ± 0.1
HA1000-DPTA	1225.0 ± 266.6	1.3 ± 0.1

[a]Determined by GPC-MALS in 0.1 M pH 7.0 phosphate buffer supplemented with 0.1 M sodium nitrate and 0.02 wt% sodium azide. [b]Error represents standard deivation from $n \geq 3$ separate syntheses.

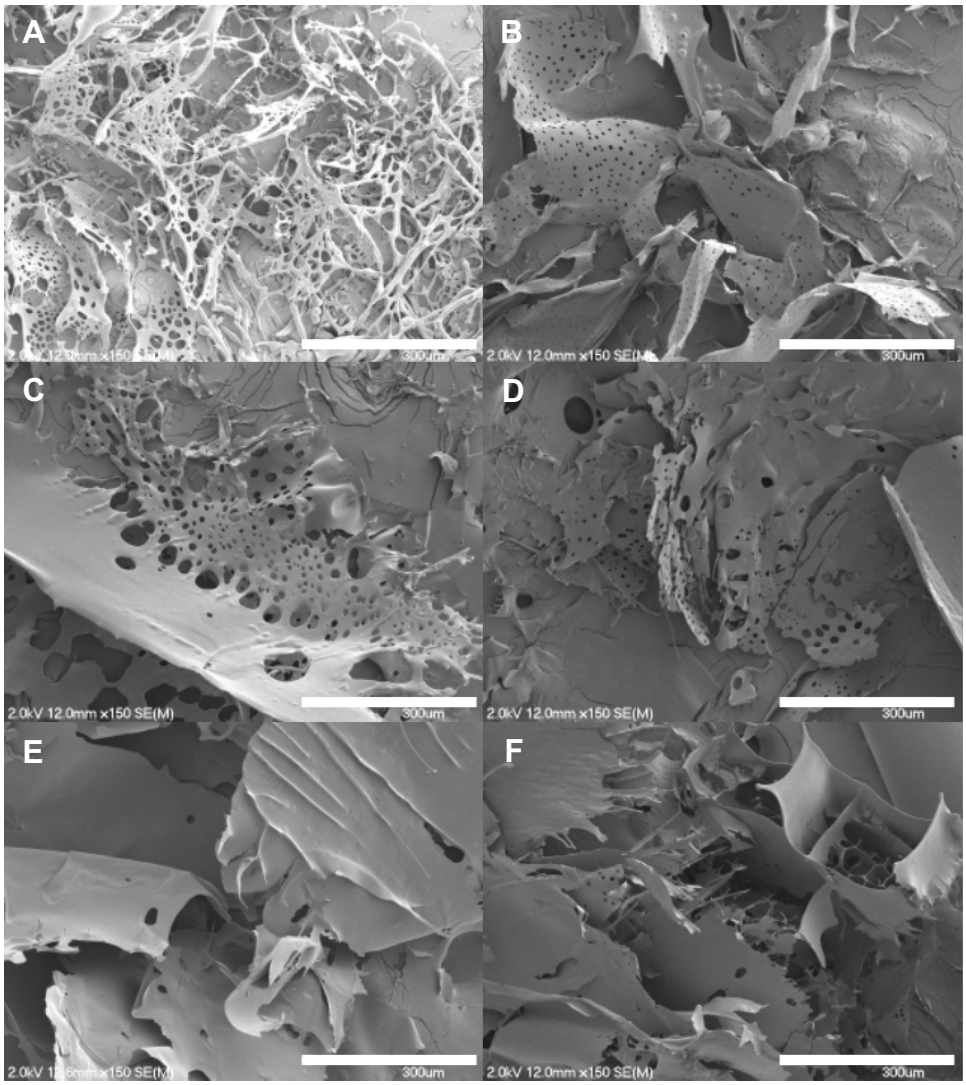

Figure S1. Representative scanning electron micrographs of (A) HA6, (B) HA6-DETA, (C) HA90, (D) HA90-DETA, (E) HA1000, and (F) HA1000-DETA. Scale bars represent 300 μm.

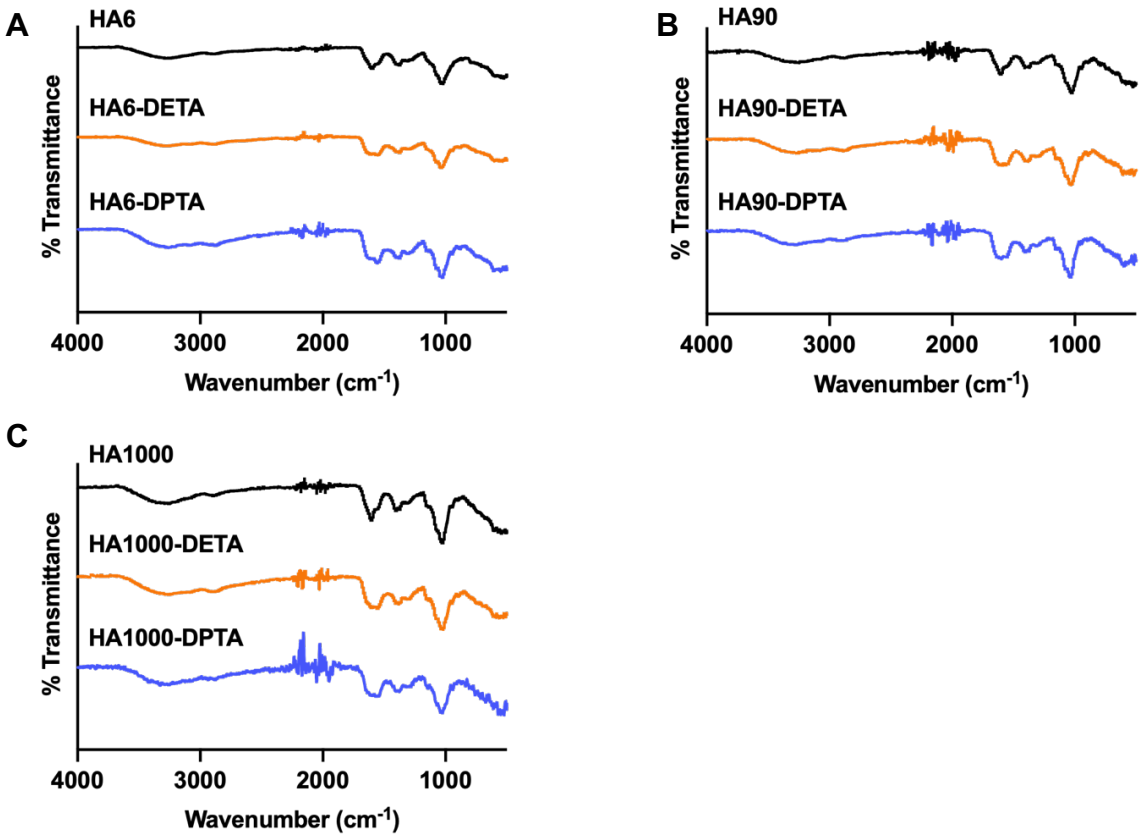

Figure S2. Representative FTIR spectra of unmodified and amine-modified (A) HA6, (B) HA90, and (C) HA1000 derivatives.

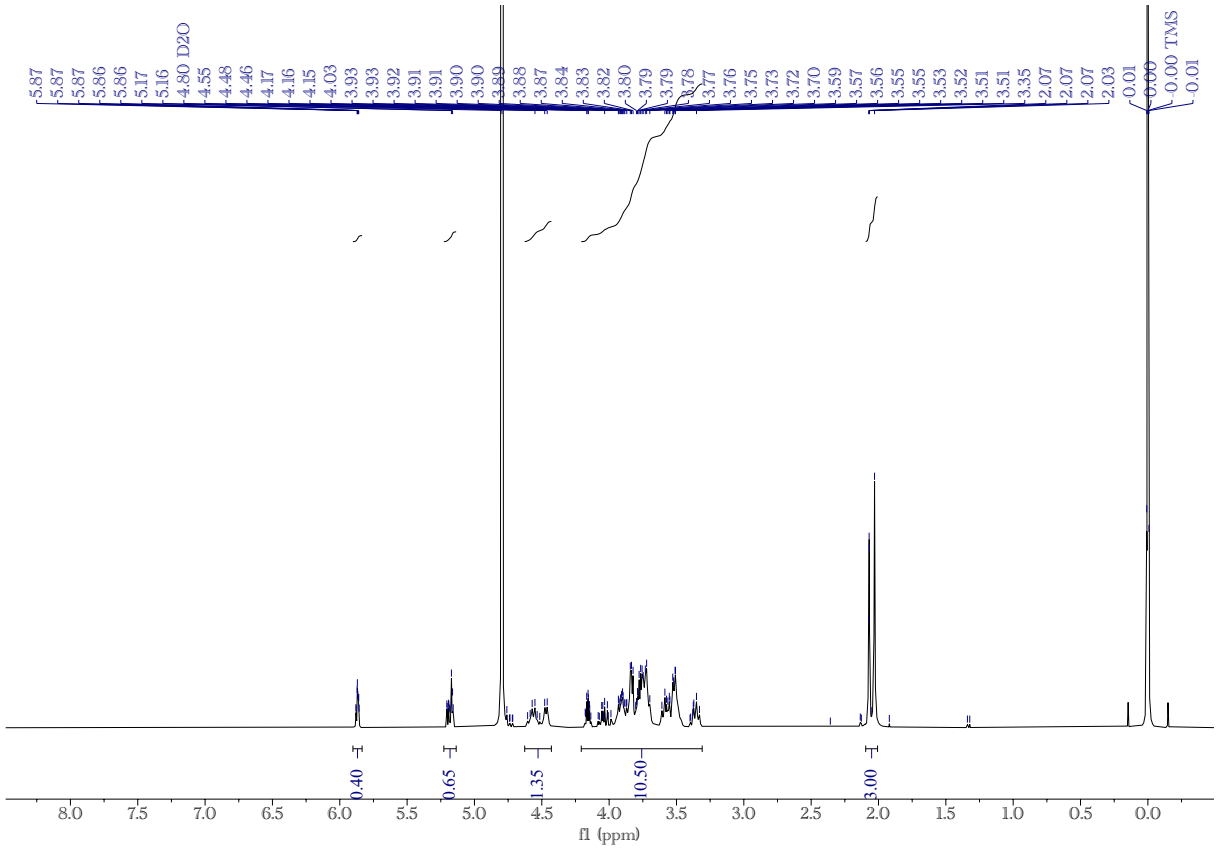

Figure S3. ^{1}H NMR (400 MHz, D_2O) spectrum of HA6 (starting material).

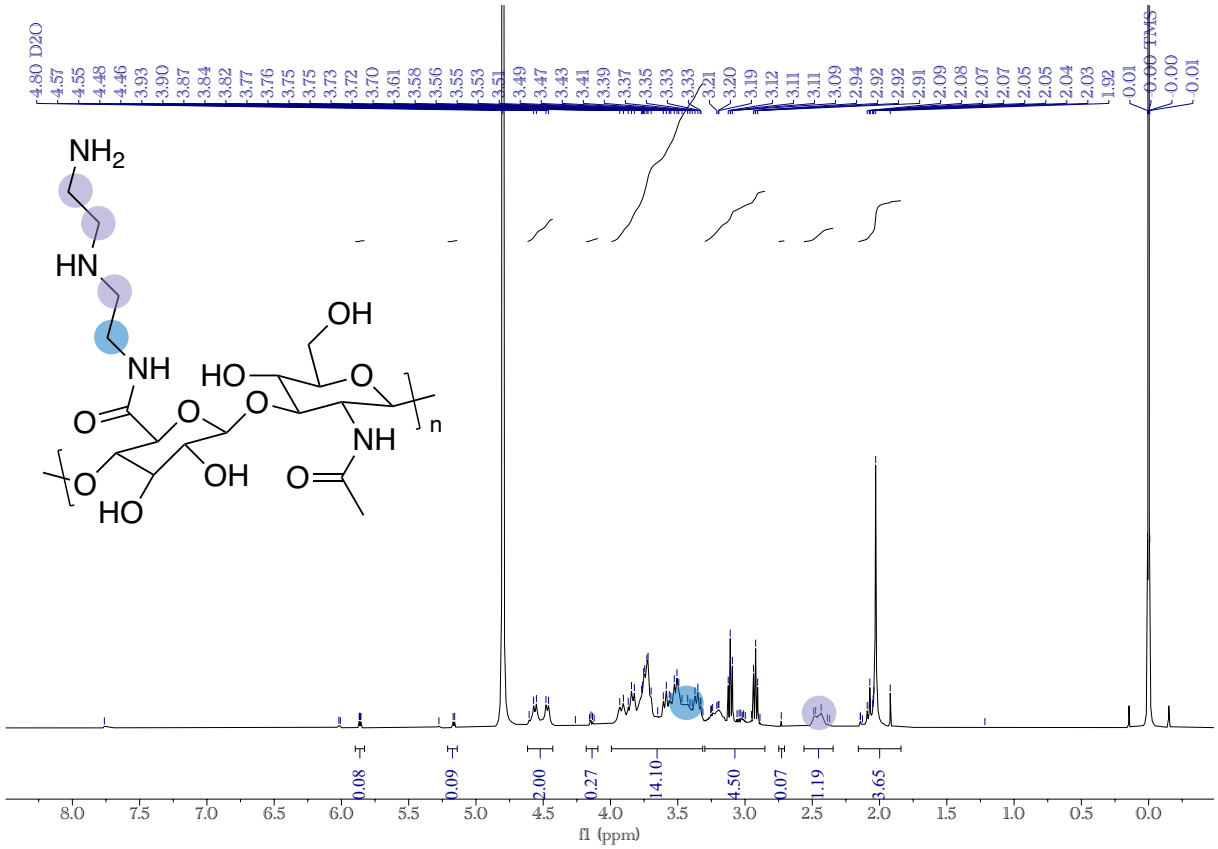

Figure S4. ^{1}H NMR (400 MHz, D_2O) spectrum of HA6-DETA. The highlighted peaks suggest covalent conjugation of DETA to HA.

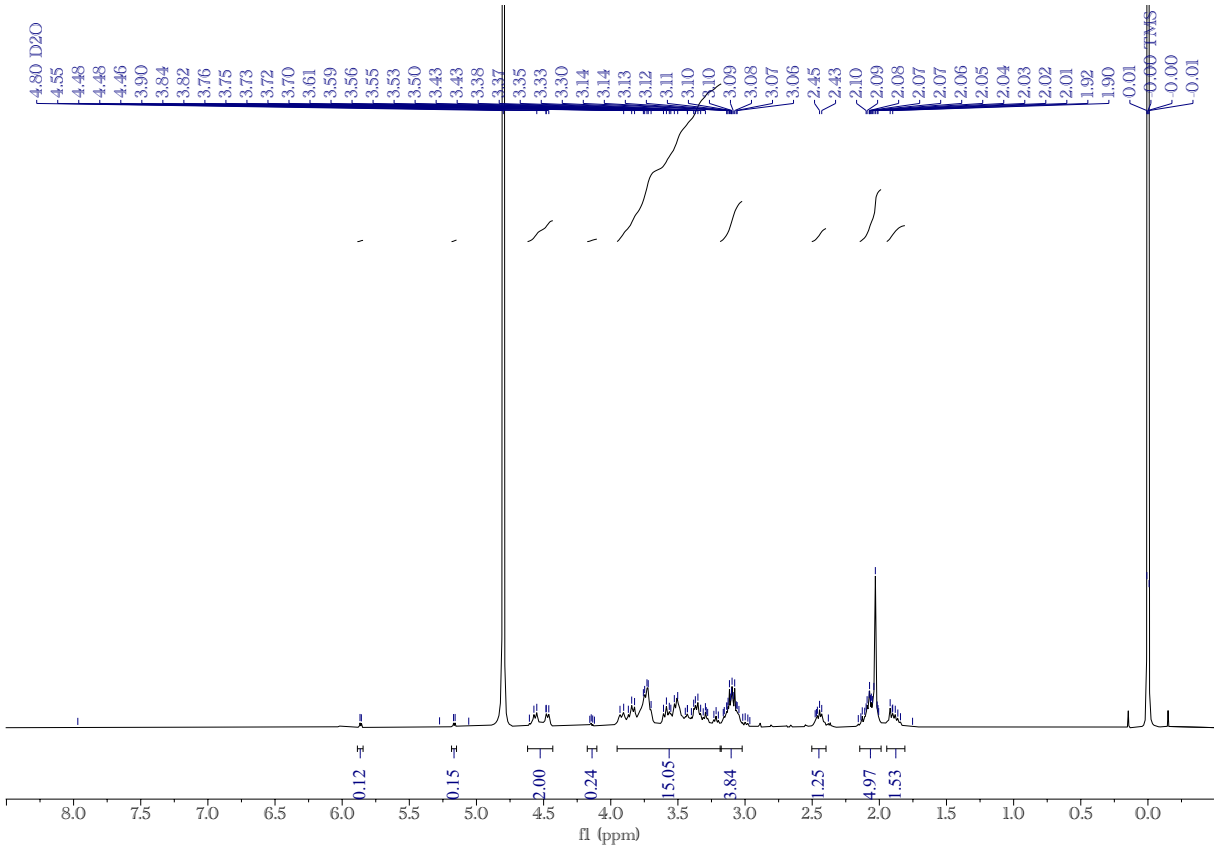

Figure S5. ^{1}H NMR (400 MHz, D_2O) spectrum of HA6-DPTA.

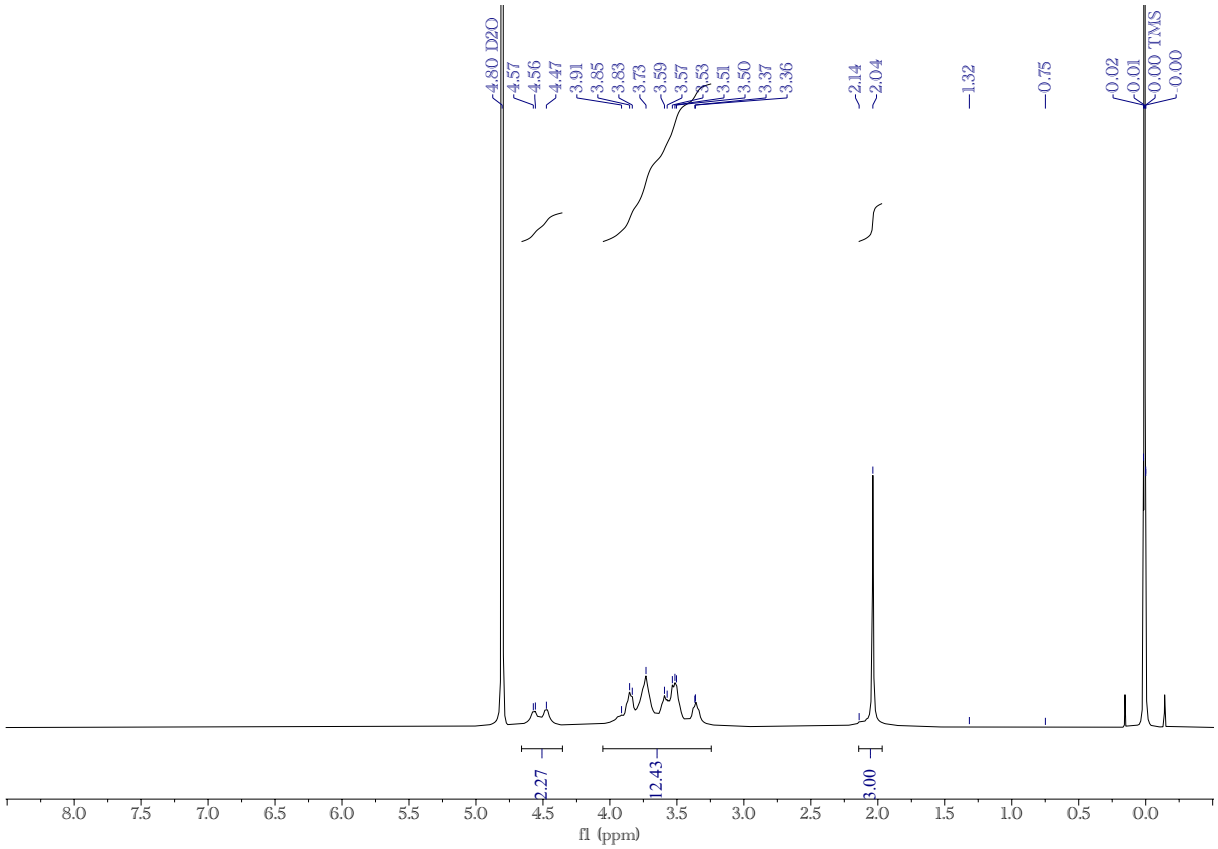

Figure S6. ^{1}H NMR (400 MHz, D_2O) spectrum of HA90 (starting material).

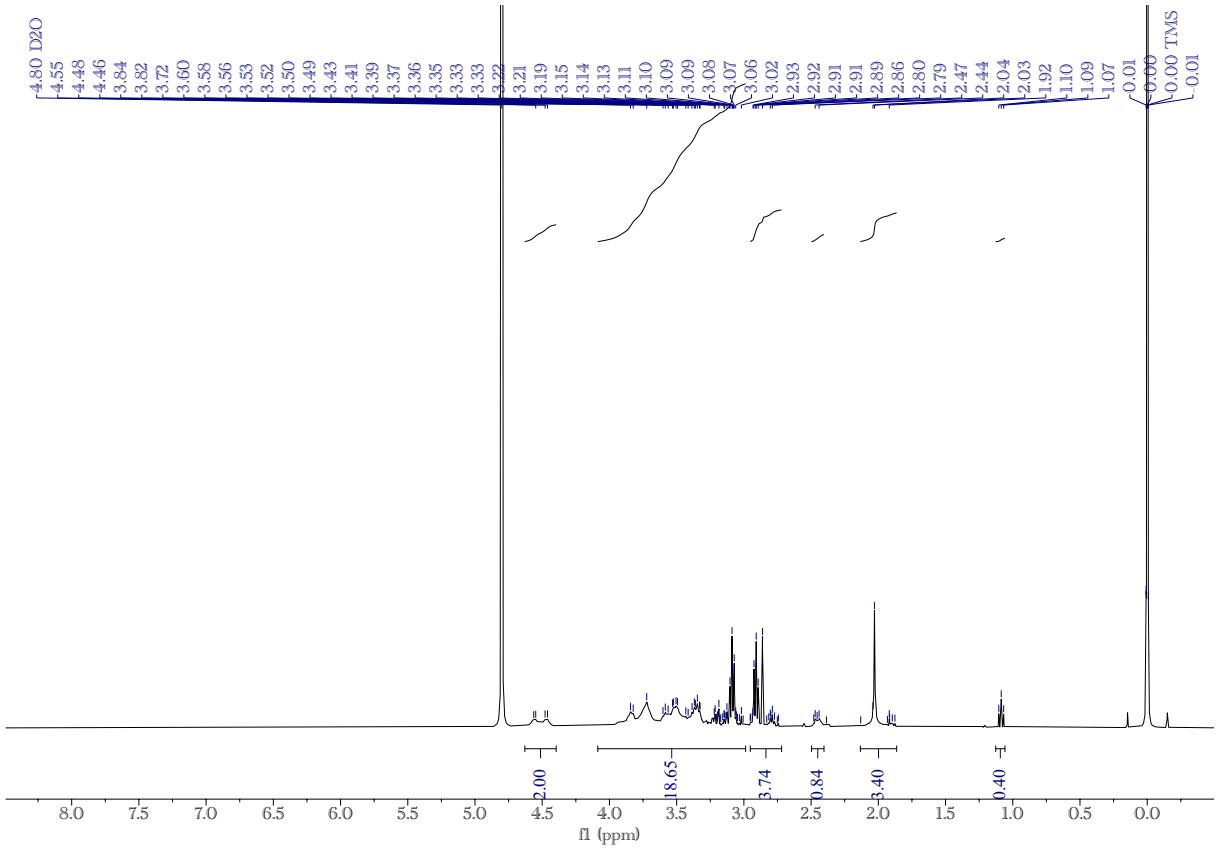

Figure S7. ^{1}H NMR (400 MHz, D_2O) spectrum of HA90-DETA.

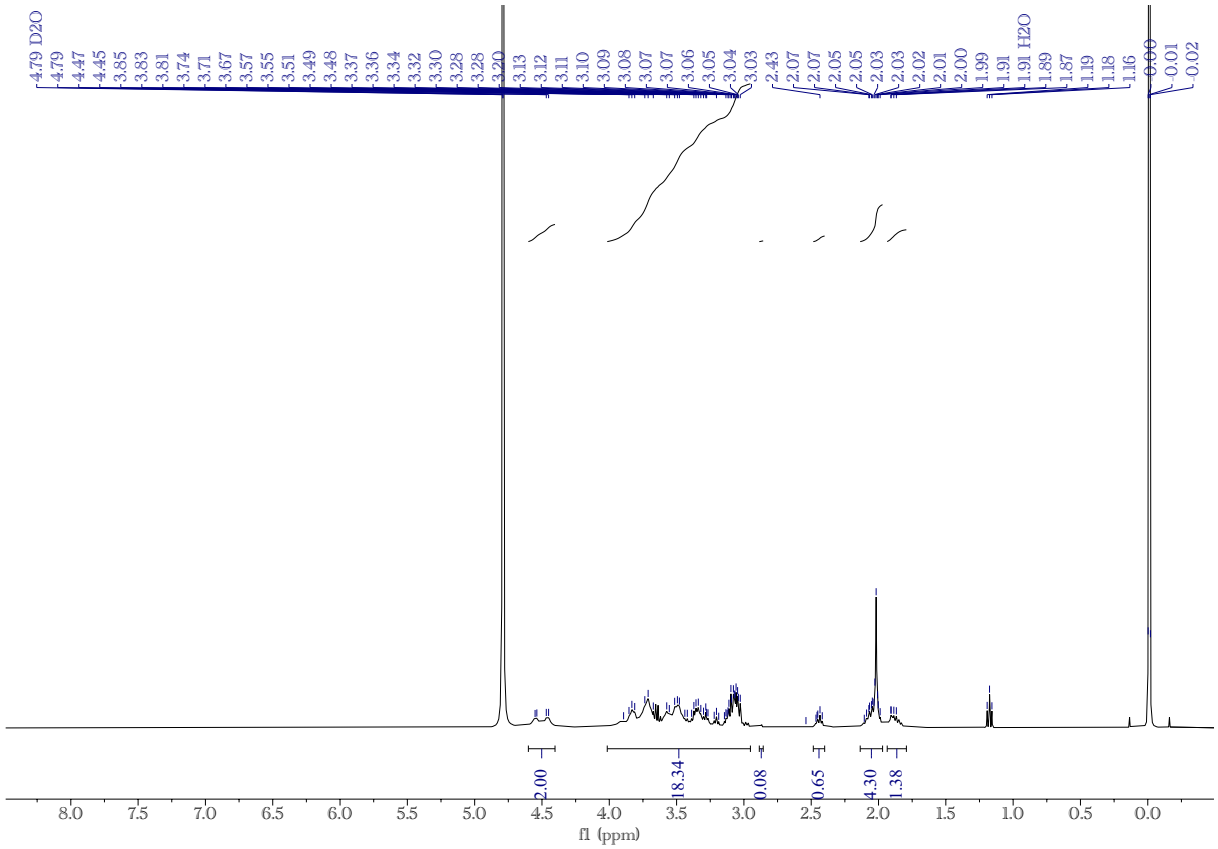

Figure S8. ^{1}H NMR (400 MHz, D_2O) spectrum of HA90-DPTA.

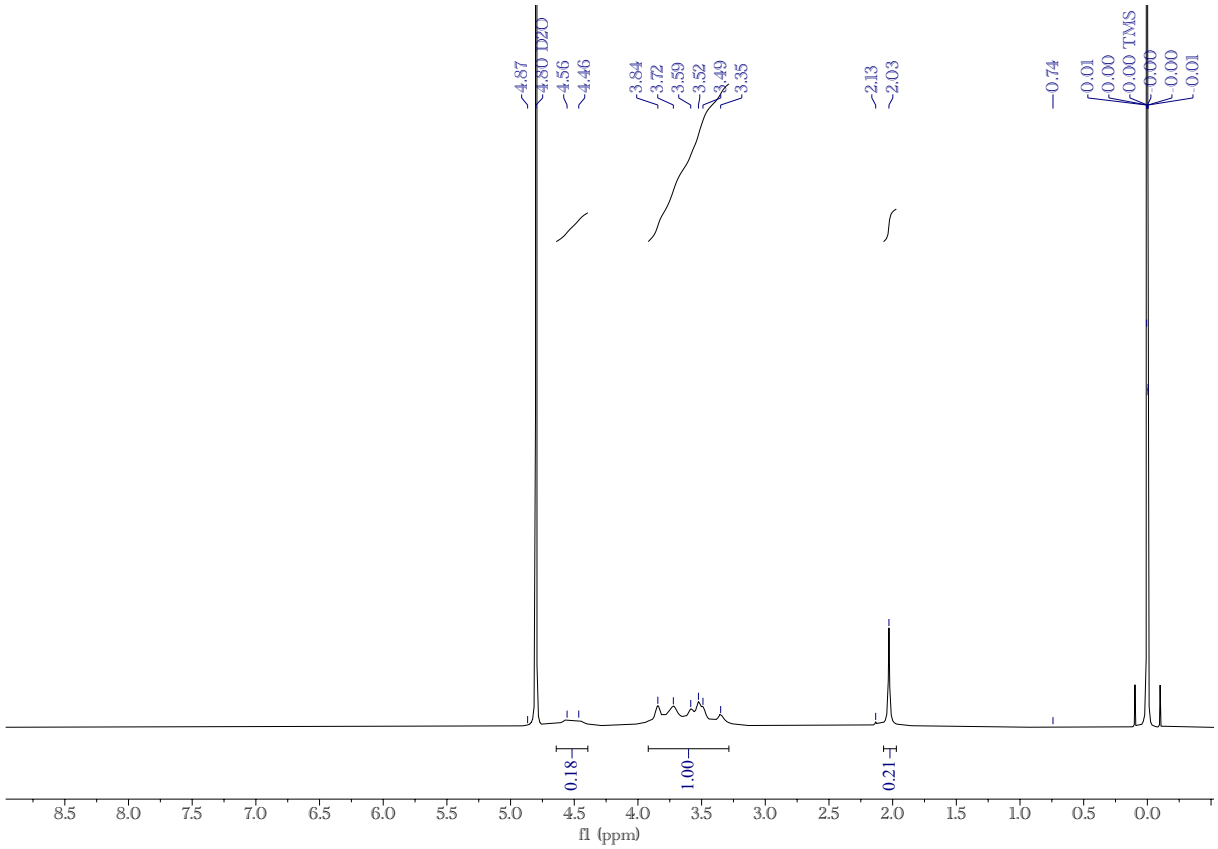

Figure S9. ^{1}H NMR (400 MHz, D_2O) spectrum of HA1000 (starting material).

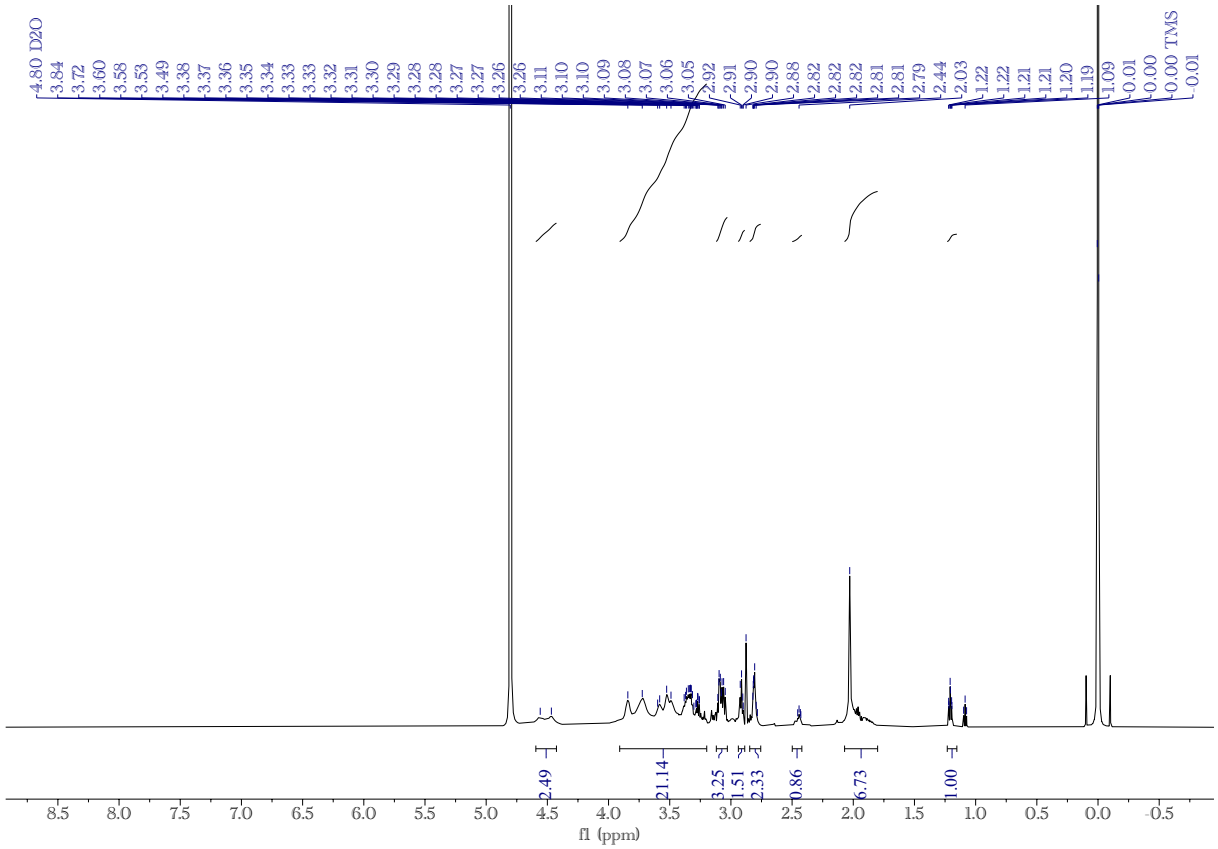

Figure S10. ^{1}H NMR (400 MHz, D_2O) spectrum of HA1000-DETA.

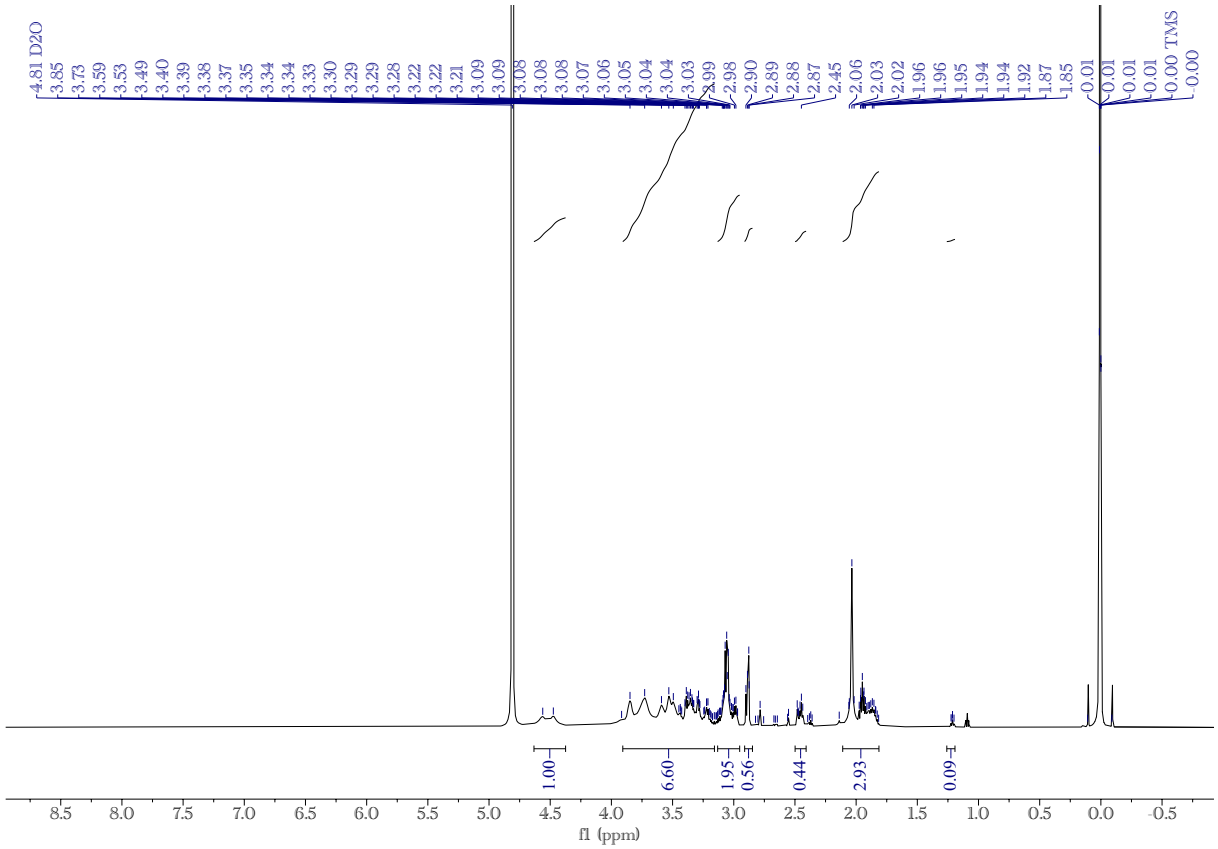

Figure S11. ^{1}H NMR (400 MHz, D_2O) spectrum of HA1000-DPTA.

Table S2. Elemental Analysis (CHN) of Unmodified and Amine-Modified HA Derivatives.[a]

Material	% C	% H	% N
HA6	36.9 ± 0.3	5.2 ± 0.2	3.1 ± 0.0
HA90	35.3 ± 1.5	6.1 ± 0.4	3.3 ± 0.7
HA1000	34.0 ± 0.2	6.3 ± 0.8	2.8 ± 0.0
HA6-DETA	39.5 ± 1.1	7.1 ± 0.8	9.3 ± 1.0
HA90-DETA	40.0 ± 0.5	7.1 ± 0.4	9.9 ± 0.2
HA1000-DETA	38.6 ± 1.1	7.5 ± 0.3	8.0 ± 0.7
HA6-DPTA	41.6 ± 1.5	6.8 ± 0.2	8.3 ± 0.6
HA90-DPTA	41.9 ± 0.7	8.1 ± 0.3	8.4 ± 0.1
HA1000-DPTA	39.8 ± 1.1	7.5 ± 0.9	8.7 ± 0.7

[a]Error represents standard deivation from $n = 3$ separate syntheses.

Table S3. Efficiency of Covalent Amine Modification and Total Amine Content of Amine-Modified HA Derivatives.

Material	Covalently Bound Amine (%)[a]	Total Amine Content (%)[b]
HA6-DETA	19.8	62 ± 12
HA90-DETA	14.0	70 ± 3
HA1000-DETA	14.3	47 ± 9
HA6-DPTA	20.8	54 ± 8
HA90-DPTA	10.8	55 ± 2
HA1000-DPTA	7.3	59 ± 10

[a]Determined by ^{1}H NMR analysis. [b]Determined by CHN elemental analysis, where error represents standard deviation from $n = 3$ separate syntheses.

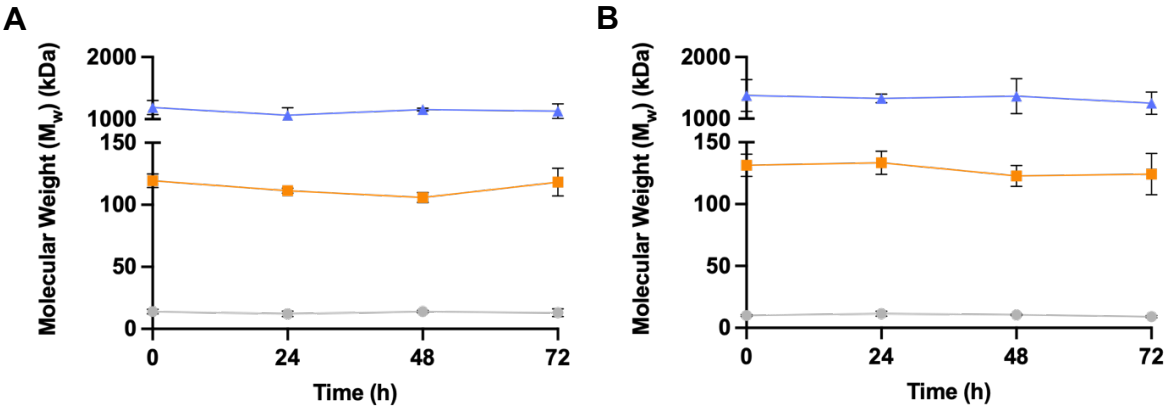

Figure S12. Short-term stability of (A) unmodified and (B) DETA-modified HA6 (light gray circle), HA90 (orange square), and HA1000 (blue triangle) derivatives in 0.22 µm-filtered 0.1 M pH 7.0 phosphate buffer supplemented with 0.1 M sodium nitrate and 0.02 wt% sodium azide over 72 h. Error bars represent the standard deviation from $n = 3$ separate samples.

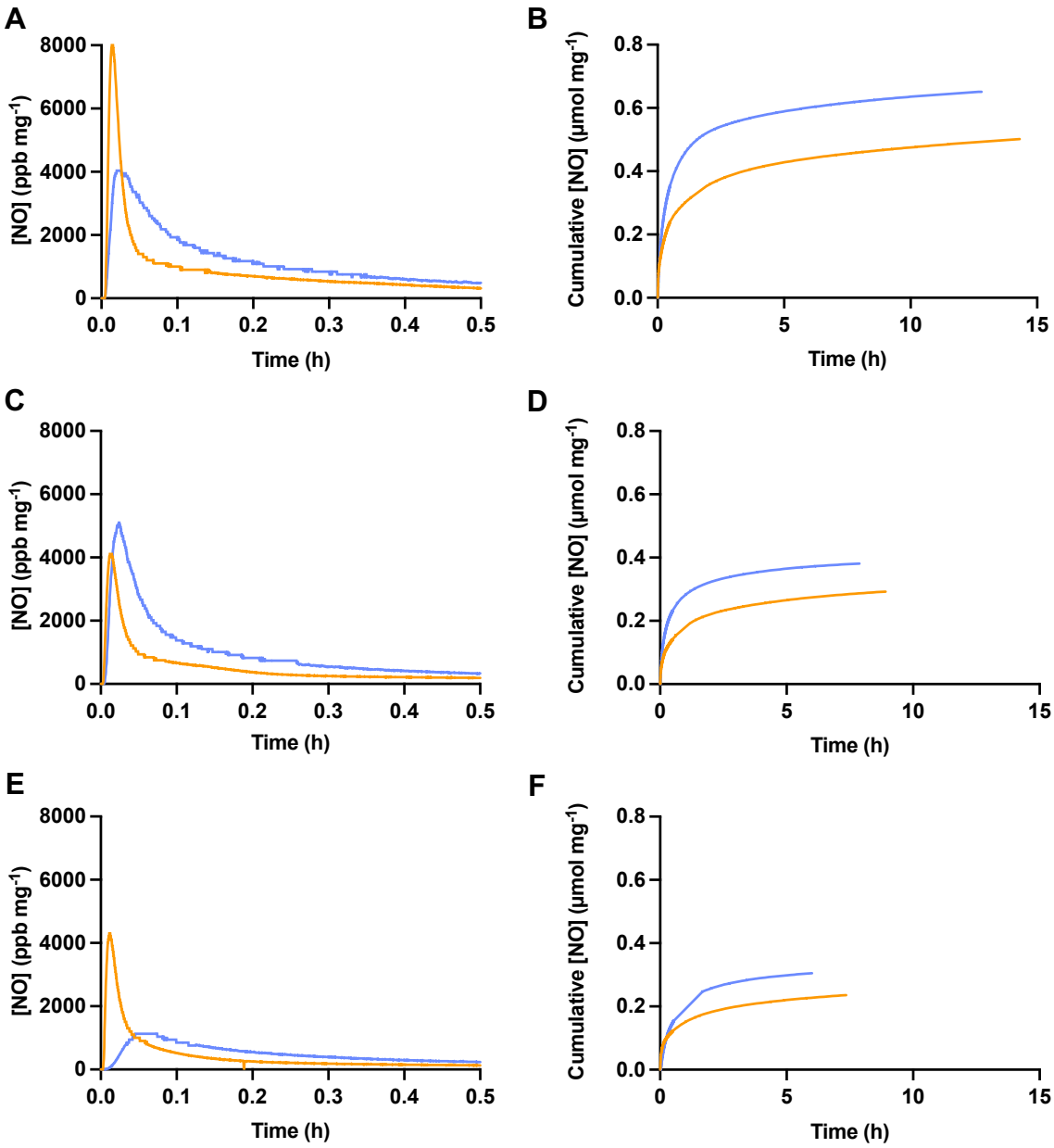

Figure S13. Representative real-time NO-release profiles over the first 0.5 h for (A) HA6, (C) HA90, and (E) HA1000 with DETA (orange) or DPTA (blue) modifications. Cumulative NO release over the entire release duration for (B) HA6, (D) HA90, and (F) HA1000 with DETA (orange) or DPTA (blue) modifications.

Table S4. Efficiency of NO Donor Functionalization.[a,b]

Material	NO Donor Functionalization (%)
HA6-DETA	20 ± 3
HA90-DETA	12 ± 3
HA1000-DETA	15 ± 3
HA6-DPTA	32 ± 5
HA90-DPTA	19 ± 2
HA1000-DPTA	17 ± 5

[a]Determined by experimental NO totals and CHN elemental analysis. [b]Error represents standard deivation from $n \geq 3$ separate syntheses.

Table S5. Minimum Bactericidal Concentrations (MBCs) of Unmodified and Amine-Modified HA Against *C. acnes* and *S. aureus* Following 24-h Exposure.[a]

	***C. acnes*[b]**	***S. aureus*[c]**
Material	**MBC_{24h} (mg mL^{-1})**	**MBC_{24h} (mg mL^{-1})**
HA6	>16	>16
HA90	>16	>16
HA1000	>8	>8
HA6-DETA	>16	>16
HA90-DETA	>16	>16
HA1000-DETA	>8	>8
HA6-DPTA	>16	>16
HA90-DPTA	>16	>16
HA1000-DPTA	>8	>8

[a]MBC_{24h} determined from $n \geq 3$ separate experiments. [b]*C. acnes* treated in 2% v/v WCB in pH 7.4 PBS. [c]*S. aureus* treated in 2% v/v MHB in pH 7.4 PBS.

Table S6. Minimum Bactericidal Concentrations (MBCs) for *C. acnes* and *S. aureus* Normalized to NO Dose Following 24-h Exposure.[a,b]

	***C. acnes*[c]**	***S. aureus*[d]**
Material	**MBC_{24h} (μg NO mL^{-1})**	**MBC_{24h} (μg NO mL^{-1})**
HA6-DETA/NO	7	14
HA90-DETA/NO	19	38
HA1000-DETA/NO	17	34
HA6-DPTA/NO	9	19
HA90-DPTA/NO	24	24
HA1000-DPTA/NO	34	34

[a]MBC_{24h} determined from $n \geq 3$ separate experiments. [b]MBCs are reported as the NO dose over 24 h. [c]*C. acnes* treated in 2% v/v WCB in pH 7.4 PBS. [d]*S. aureus* treated in 2% v/v MHB in pH 7.4 PBS.

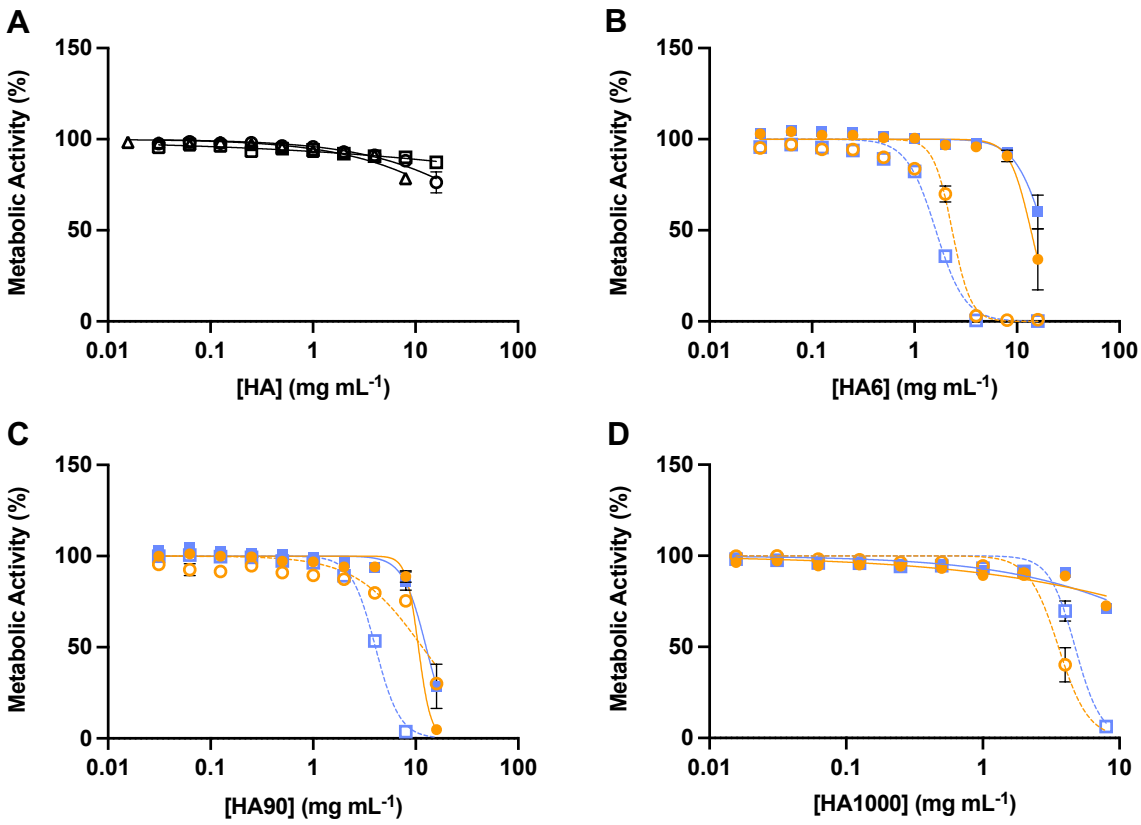

Figure S14. (A) Dose-response curves after 24-h treatment of keratinocytes with unmodified HA6 (black circle), HA90 (black square), and HA1000 (black triangle). Dose-response curves after 24-h treatment of keratinocytes with amine-modified (solid) and NO-releasing (dashed) (B) HA6, (C) HA90, and (D) HA1000 with DETA (orange circle) or DPTA (blue square) modifications. Error bars represent the standard error of the mean from $n \geq 3$ separate experiments.

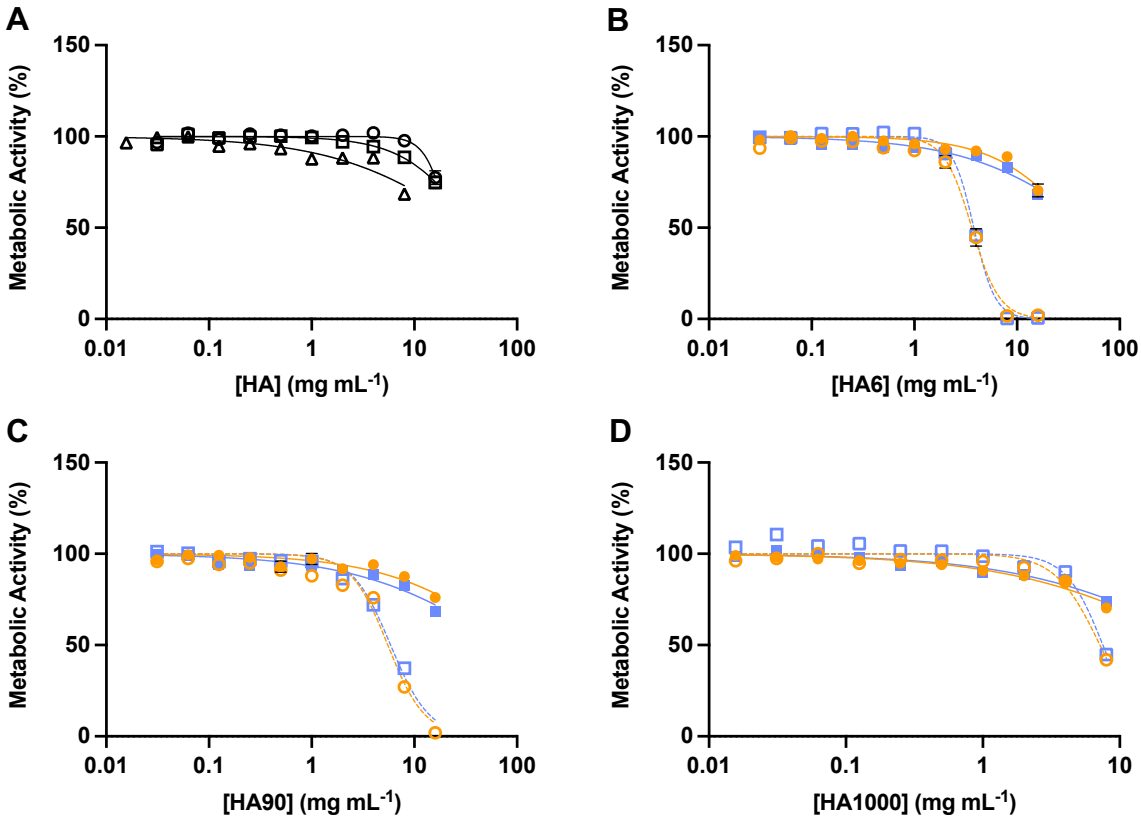

Figure S15. (A) Dose-response curves after 24-h treatment of sebocytes with unmodified HA6 (black circle), HA90 (black square), and HA1000 (black triangle). Dose-response curves after 24-h treatment of sebocytes with amine-modified (solid) and NO-releasing (dashed) (B) HA6, (C) HA90, and (D) HA1000 with DETA (orange circle) or DPTA (blue square) modifications. Error bars represent the standard error of the mean from $n \geq 3$ separate experiments.

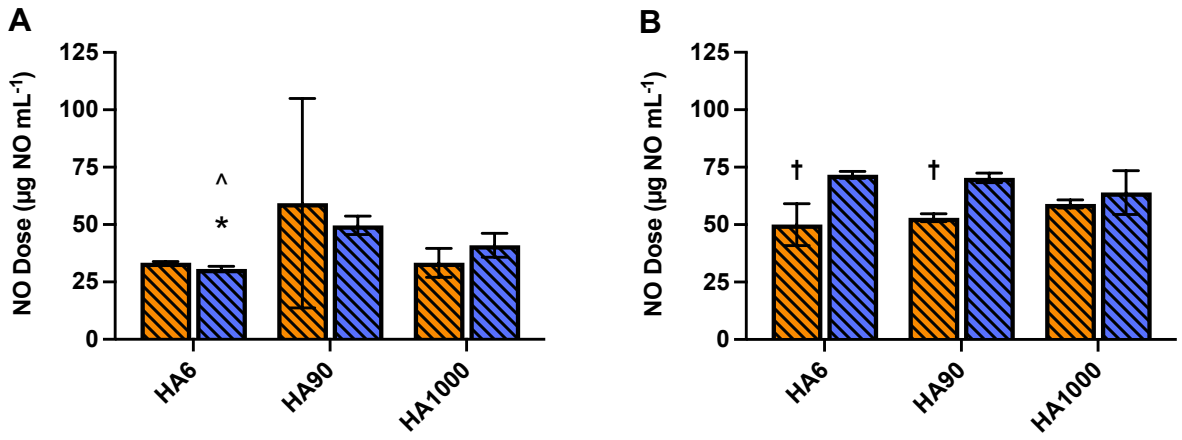

Figure S16. IC_{50} normalized to NO dose (μg NO mL^{-1}) of DETA-modified (striped, orange) and DPTA-modified (striped, blue) NO-releasing HA for (A) keratinocytes and (B) sebocytes. Error bars represent the standard deviation from $n \geq 3$ separate experiments. $^{*}p < 0.05$ for HA6 compared to HA90. $^{\wedge}p < 0.05$ for HA6 compared to HA1000. $^{\dagger}p < 0.05$ for DETA modifications compared to DPTA modifications.

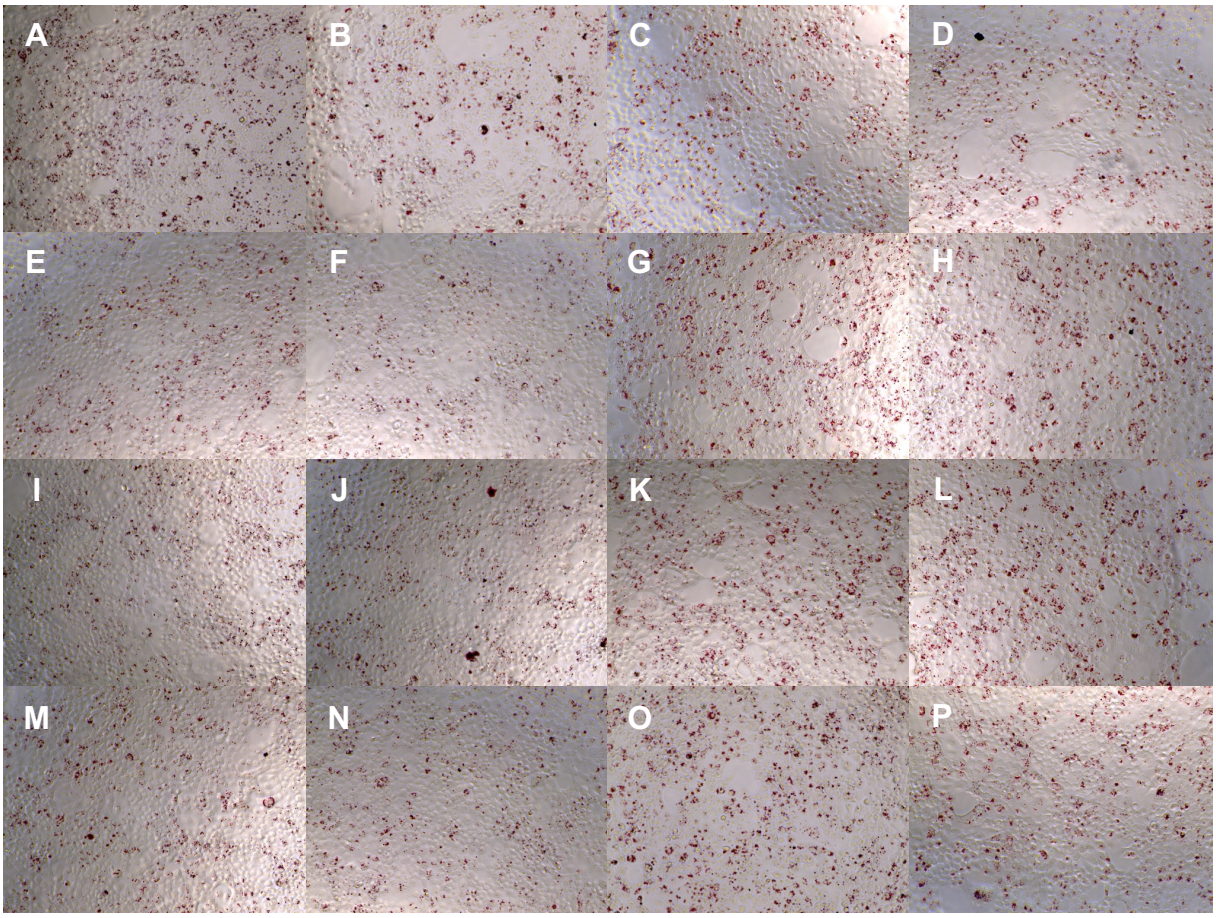

Figure S17. Representative images of sebocytes stained with Oil Red O for lipid quantification after 24-h treatment with 1000 μg mL^{-1} of unmodified, amine-modified, and NO-releasing HA modified with DETA or DPTA. Cells were stimulated with 1 μg mL^{-1} of insulin and either (A) left untreated, or treated with (B) HA6, (C) HA90, (D) HA1000, (E) HA6-DETA, (F) HA6-DPTA, (G) HA6-DETA/NO, (H) HA6-DPTA/NO, (I) HA90-DETA, (J) HA90-DPTA, (K) HA90-DETA/NO, (L) HA90-DPTA/NO, (M) HA1000-DETA, (N) HA1000-DPTA, (O) HA1000-DETA/NO, and (P) HA1000-DPTA/NO.

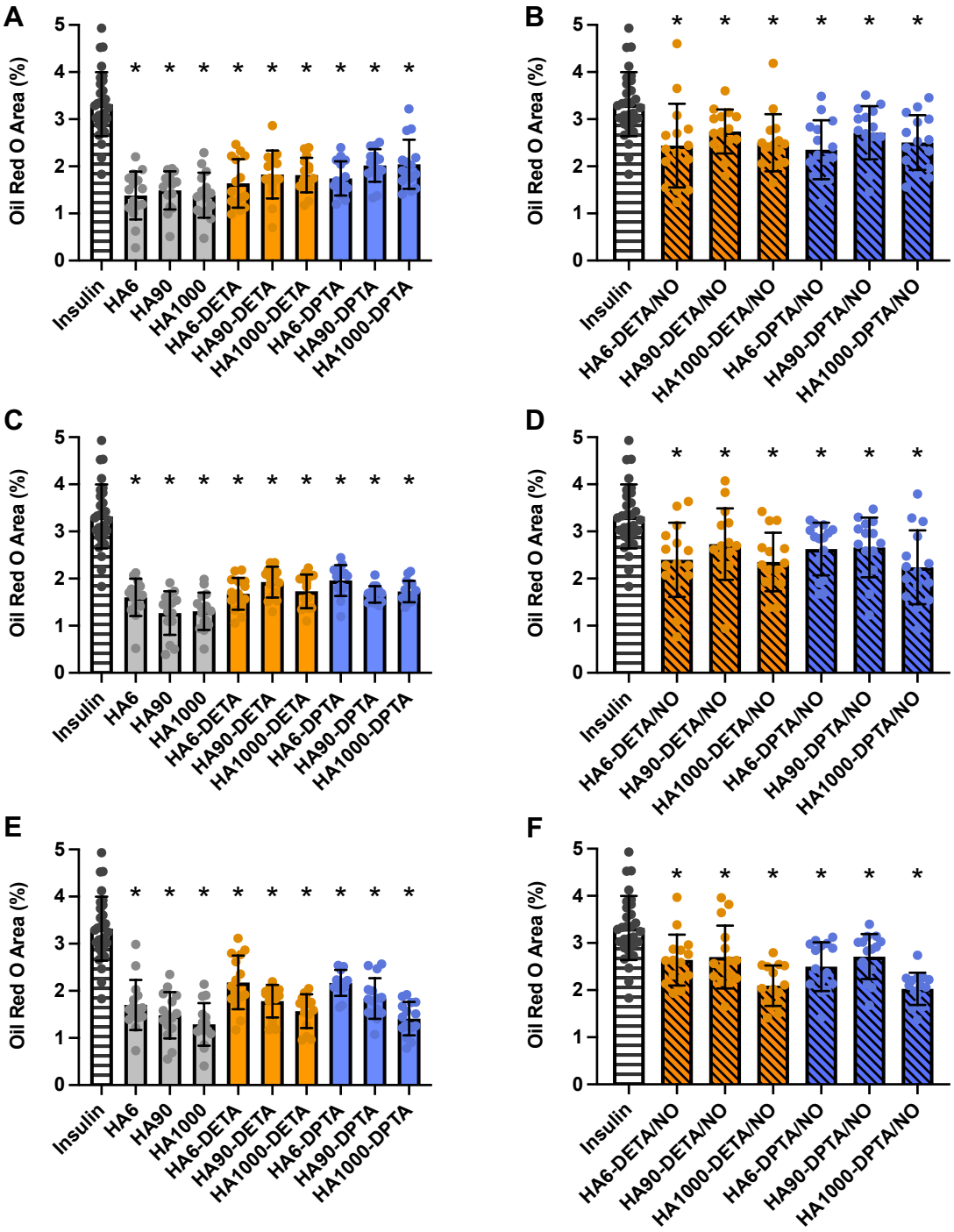

Figure S18. Influence of (A, B) 100, (C, D) 10, and (E, F) 1 μg mL^{-1} of (A, C, E) unmodified (light gray) and amine-modified (solid) HA, and (B, D, F) NO-releasing (striped) HA with DETA (orange) or DPTA (blue) modifications on lipid production of insulin-stimulated (1 μg mL^{-1}) sebocytes. Insulin-stimulated cells without treatment (hollow, striped) were included as a control. Error bars represent the standard deviation from $n \geq 15$ processed images. $^{*}p < 0.05$ for HA derivatives compared to the control.

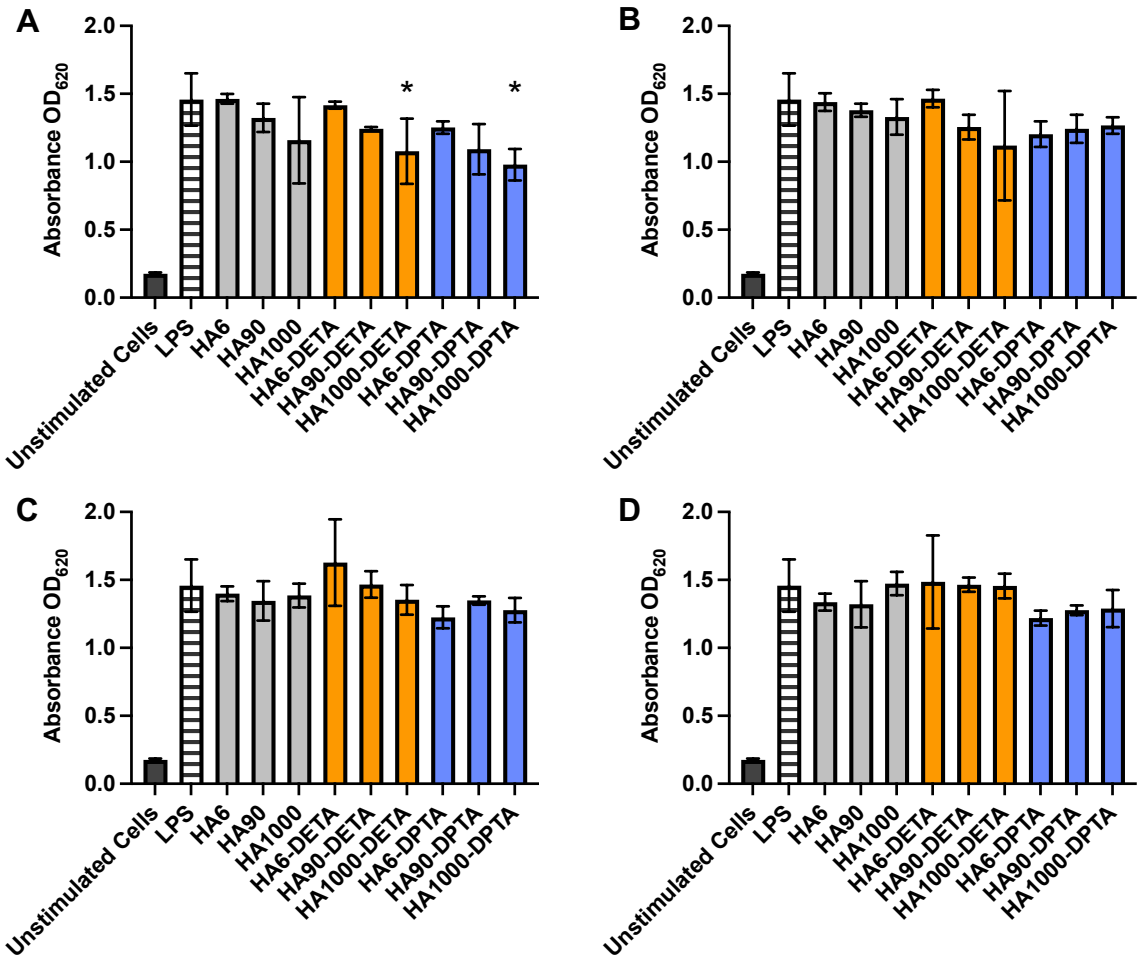

Figure S19. Influence of (A) 1000, (B) 100, (C) 10, and (D) 1 μg mL^{-1} of unmodified HA (light gray) and amine-modified HA with DETA (orange) or DPTA (blue) modifications on modulation of TLR2-mediated NF-κB activation. Unstimulated cells (dark gray) and LPS-stimulated cells (100 ng mL^{-1}) without treatment (hollow, striped) were included as negative and positive controls, respectively. Error bars represent the standard deviation from $n = 3$ separate samples. $^{*}p < 0.05$ for HA derivatives compared to the positive control.

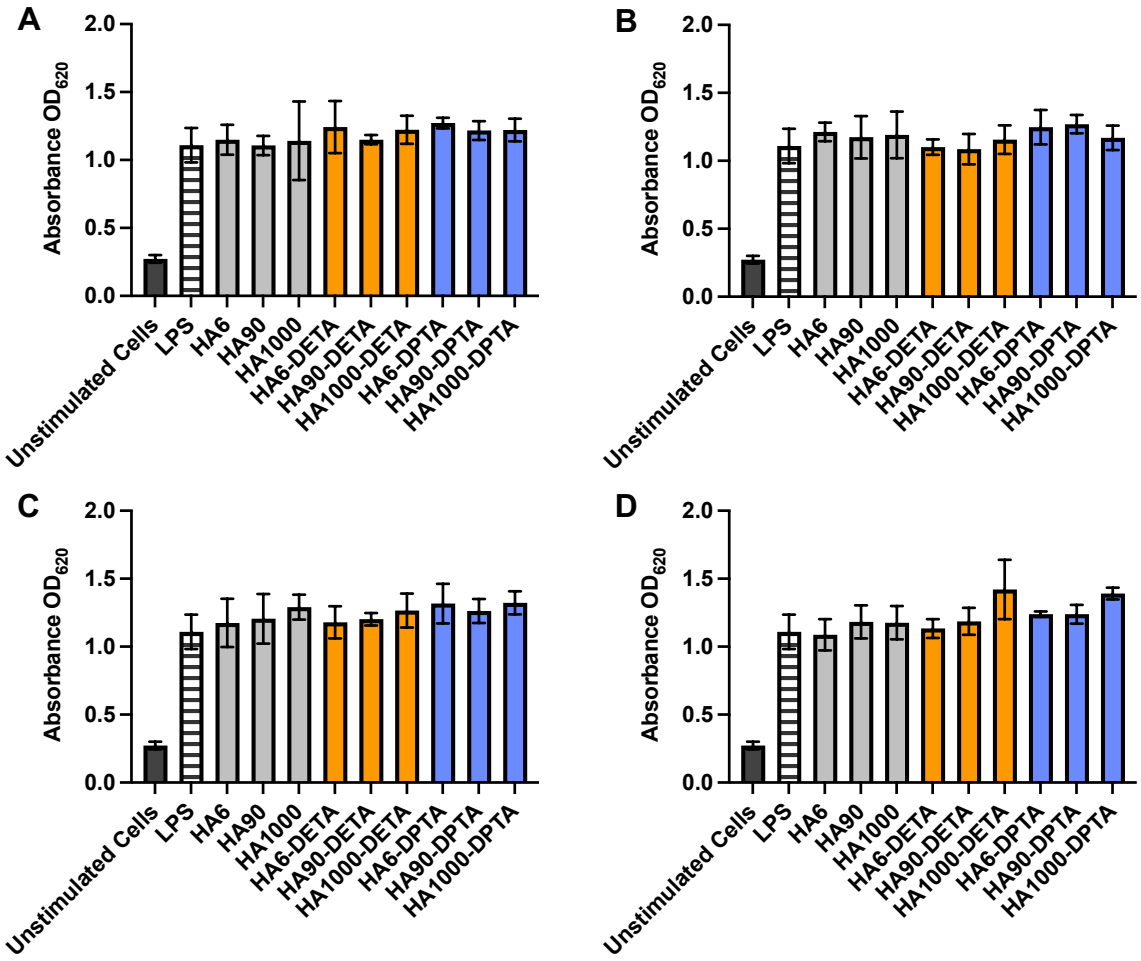

Figure S20. Influence of (A) 1000, (B) 100, (C) 10, and (D) 1 μg mL^{-1} of unmodified HA (light gray) and amine-modified HA with DETA (orange) or DPTA (blue) modifications on modulation of TLR4-mediated NF-κB activation. Unstimulated cells (dark gray) and LPS-stimulated cells (100 ng mL^{-1}) without treatment (hollow, striped) were included as negative and positive controls, respectively. Error bars represent the standard deviation from $n = 3$ separate samples.

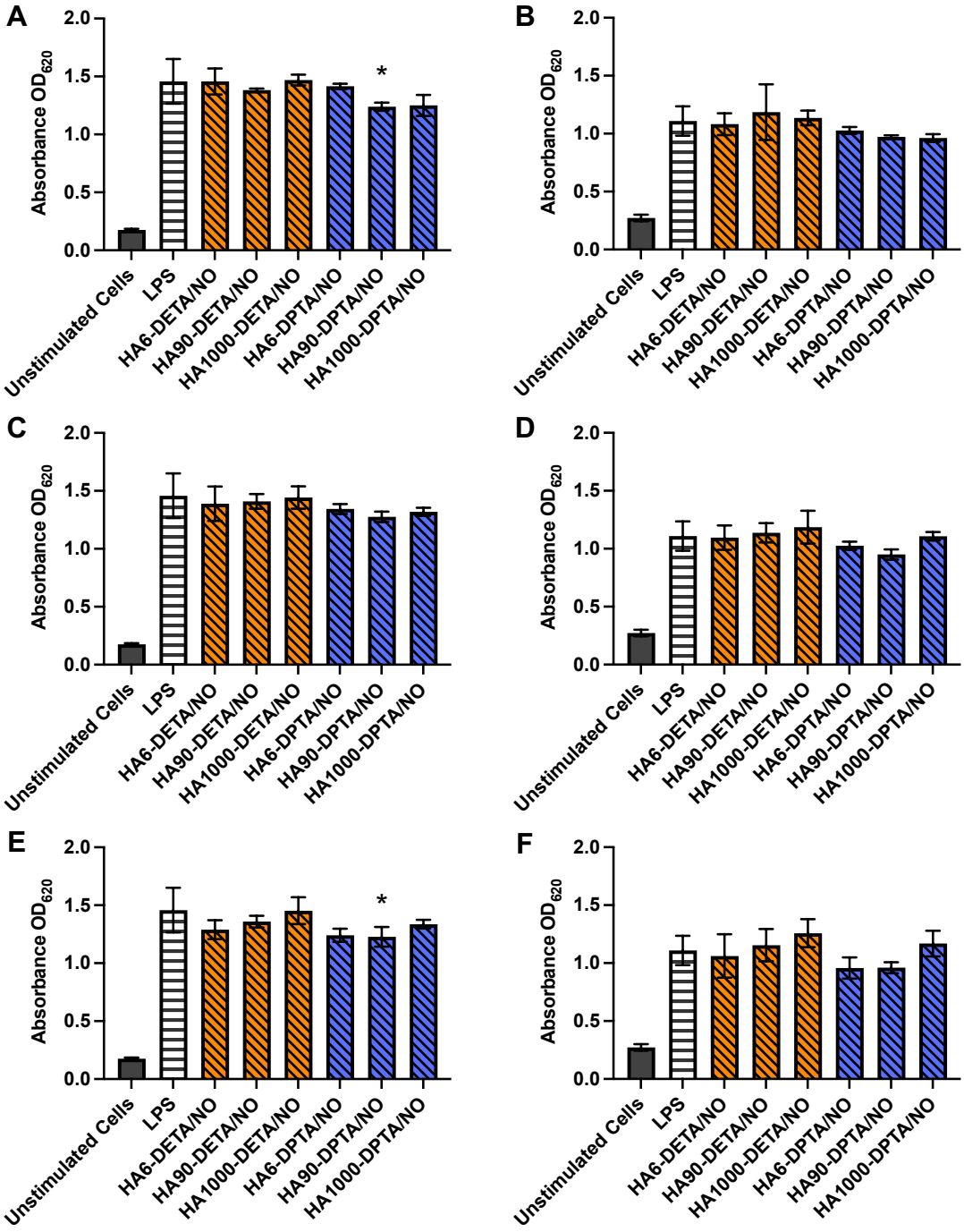

Figure S21. Influence of (A, B) 100, (C, D) 10, and (E, F) 1 μg mL^{-1} of NO-releasing HA with DETA (striped, orange) or DPTA (striped, blue) modifications on modulation of (A, C, E) TLR2- and (B, D, F) TLR4-mediated NF-κB activation. Unstimulated cells (dark gray) and LPS-stimulated cells (100 ng mL^{-1}) without treatment (hollow, striped) were included as negative and positive controls, respectively. Error bars represent the standard deviation from $n = 3$ separate samples. $^{*}p < 0.05$ for NO-releasing HA derivatives compared to the positive control.