SUPPLEMENTARY INFORMATION

Evidence supporting the safety of pegylated diethylaminoethyl-chitosan polymer as a nanovector for gene therapy applications

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This work was done in Montréal (QC, Canada) and Sao Jose do Rio Preto (SP, Brazil).

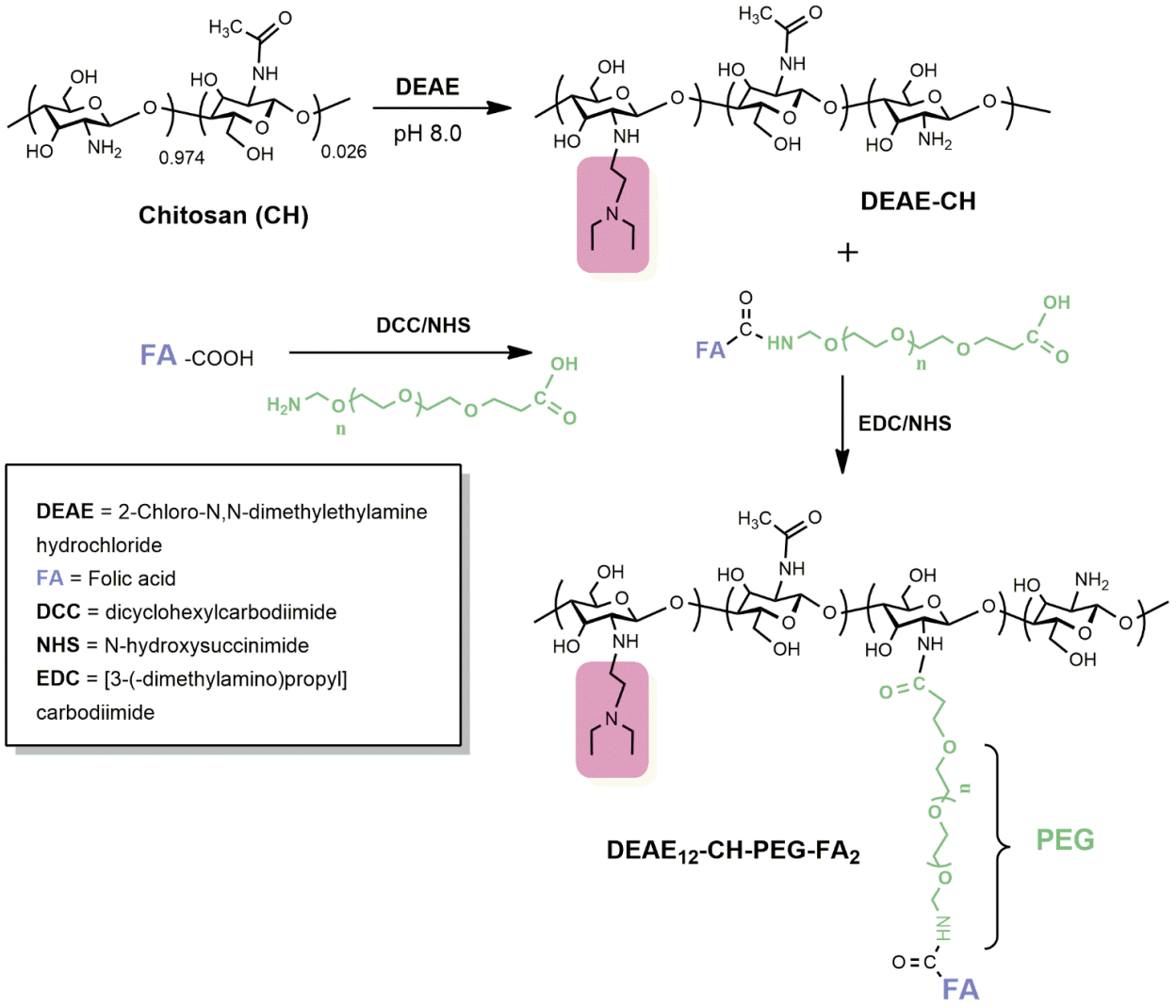
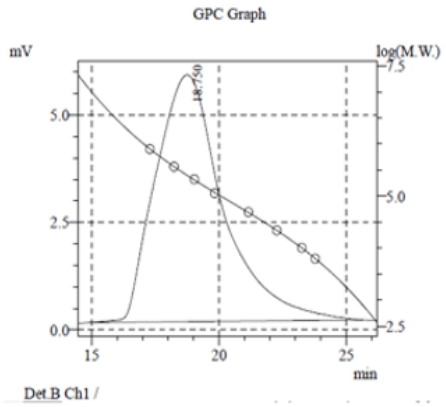


Figure S1. Diagram representing the synthesis procedure of $DEAE_{12}$-CH-PEG-FA_2.

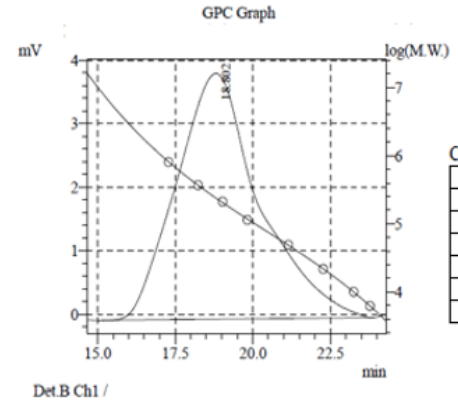


CH DDA 97.4%

GPC Summary

Chromatogram Det.B Ch1

#	Title	Mn	Mw	Mz	Mw/Mn
1	CH DDA	91327	270048	460946	2.95693
	Average	91327	270048	460946	2.95693
	%RSD	0.000	0.000	0.000	0.000
	Maximum	91327	270048	460946	2.95693
	Minimum	91327	270048	460946	2.95693
	SD	0	0	0	0.00000



$DEAE_{12}$-CH-PEG-FA_2

GPC Summary

Chromatogram Det.B Ch1

#	Title	Mn	Mw	Mz	Mw/Mn
1	DEAE-CH-PEG-FA.lcd	124288	259410	452577	2.08717
	Average	124288	259410	452577	2.08717
	%RSD	0.000	0.000	0.000	0.000
	Maximum	124288	259410	452577	2.08717
	Minimum	124288	259410	452577	2.08717
	SD	0	0	0	0.00000

Figure S2. GPC traces for the original CH and $DEAE_{12}$-CH-PEG-FA_2.

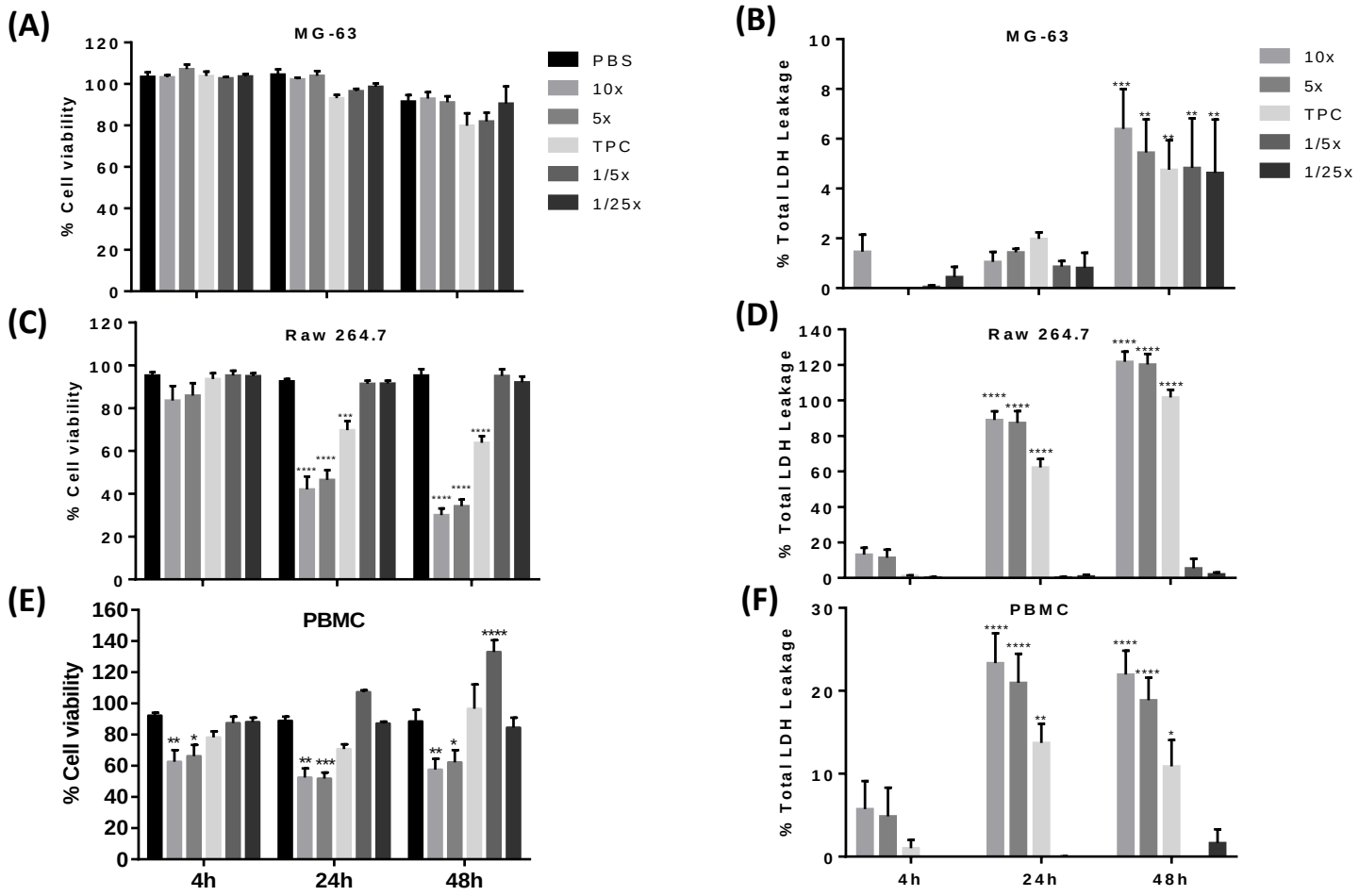


Figure S3. Cell viability and toxicity of $DEAE_{12}$-CH-PEG-FA_2. The cytotoxic effect of free $DEAE_{12}$-CH-PEG-FA_2 was evaluated using the MTS (A, C, E) and LDH assays (B, D, F) in MG-63 cells, Raw 264.7 macrophage cells and PBMC. Cells were incubated with samples for 4, 24 and 48 h at 37°C. DPBS was used as a negative control. 500 μM H_2O_2 and 1% v/v triton X-100 were used as positive controls for MTS and LDH assays,

respectively. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 are significantly different from the negative control. Data are expressed as the mean ± SEM of three independent experiments and were analyzed by Two-way ANOVA (post hoc Dunnett's test). Blood samples to obtain PBMC were collected from three different healthy human donors. See Table 1 for $DEAE_{12}$-CH-PEG-FA_2 concentrations.

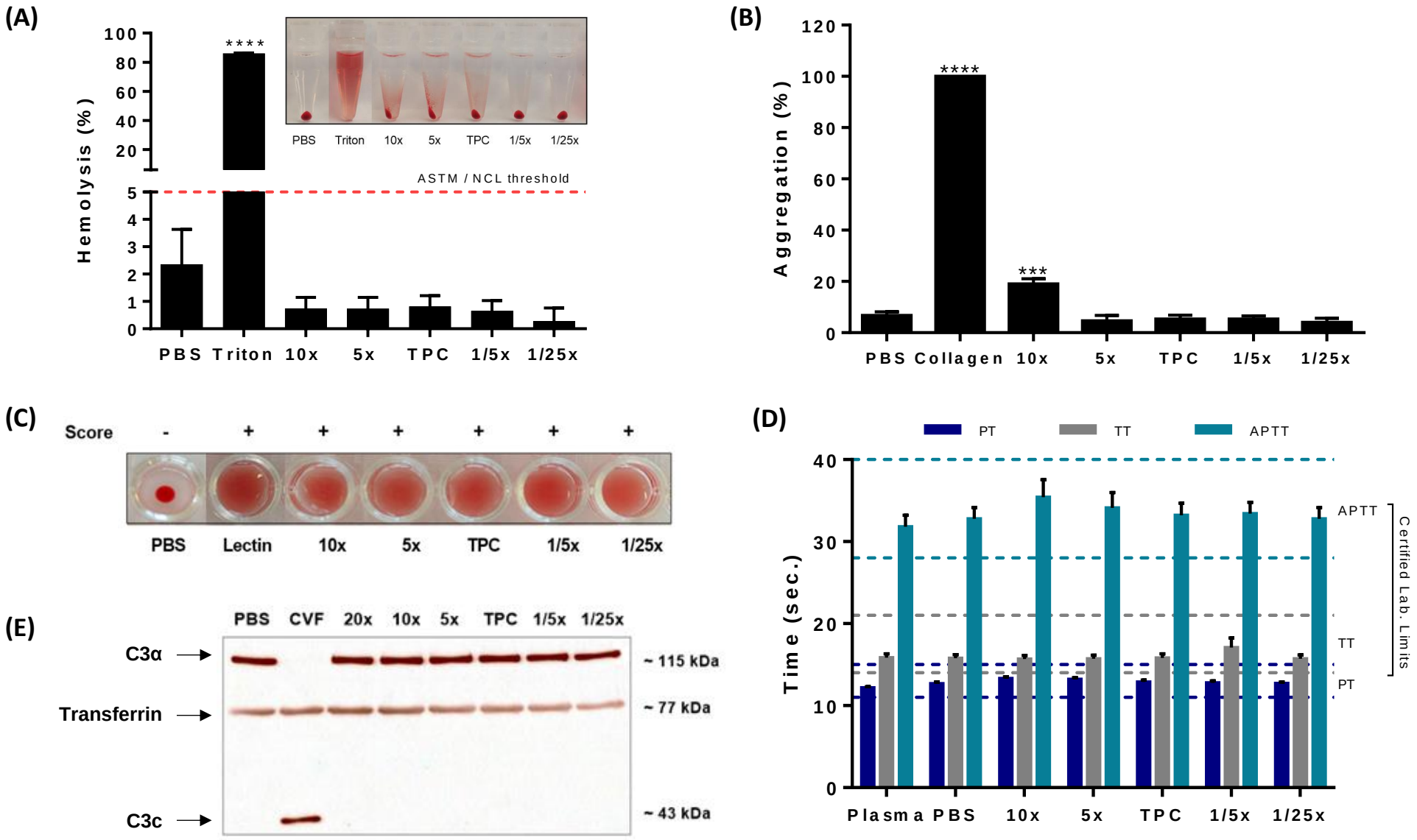

(A)
Hemolysis (%)
100
80
60
40
20
5
4
3
2
1
0
****
PBS
Triton
10x
5x
TPC
1/5x
1/25x
ASTM / NCL threshold
(B)
Aggregation (%)
120
100
80
60
40
20
0
****
***
PBS
Collagen
10x
5x
TPC
1/5x
1/25x
(C)
Score
-
+
+
+
+
+
+
PBS
Lectin
10x
5x
TPC
1/5x
1/25x
(D)
PT
TT
APTT
Time (sec.)
40
30
20
10
0
APTT
TT
PT
Certified Lab. Limits
Plasma
PBS
10x
5x
TPC
1/5x
1/25x
(E)
PBS
CVF
20x
10x
5x
TPC
1/5x
1/25x
C3α
Transferrin
C3c
~ 115 kDa
~ 77 kDa
~ 43 kDa

Figure S4. Hemocompatibility of $DEAE_{12}$-CH-PEG-FA_2. (A) Hemolysis percentage induced by free $DEAE_{12}$-CH-PEG-FA_2 and visual inspection of tubes after the centrifugation step (inset). Human blood was incubated with samples for 3 h at 37°C. DPBS and triton represent the negative and positive controls, respectively. Pink dash line shows the 5% threshold of ASTM from which a sample is considered to have hemolytic properties. Data are expressed as the mean ± SEM of three independent experiments and were analyzed by One-way ANOVA (post hoc Dunnett's test). (B) The platelet aggregation profile of free $DEAE_{12}$-CH-PEG-FA_2, after platelet-rich plasma was incubated with samples for a 6 min run at 37°C. PBS and collagen represent the negative and positive controls, respectively. Data are expressed as the mean ± SEM of three independent experiments and were analyzed by One-way ANOVA (post hoc Dunnett's test). (C) Hemagglutination activity produced by $DEAE_{12}$-CH-PEG-FA_2 in an erythrocyte suspension after 1 h incubation at 37°C. DPBS and lectin represent the negative and positive controls, respectively. Pictures represent one of three independent experiments with similar results. The agglutination analysis was performed as described in the methods. (D) Effect of free $DEAE_{12}$-CH-PEG-FA_2 on plasma coagulation times: prothrombin time (PT), thrombin time (TT) and activated partial thromboplastin time (APTT). Measures were taken after 30 min incubation of human plasma with samples at 37°C. The normal coagulation time limits are indicated with a colored dash line (PT 11 ≤ 15s (dark blue), TT 14 ≤ 21s (gray) and APTT 28 ≤ 40s (light green)). Non-treated plasma and DPBS were used as internal controls for the test. Data are expressed as the mean ± SEM of four independent experiments and were analyzed by Two-way ANOVA (post hoc Dunnett's test). (E) Complement activation assay showing the expression levels of a native C3α chain (~115 kDa) and its cleavage product C3c (~43 kDa), after human plasma exposure to $DEAE_{12}$-CH-PEG-FA_2 for 30 min at 37°C. DPBS and CVF represent the negative and positive controls, respectively. Transferrin (~77 kDa) was used as a serum loading control. Blots represent one of three independent experiments with similar results. For all experiments, blood samples were collected from at least three healthy human donors. ***$p<0.001$, ****$p<0.0001$ are significantly different from the negative control. See Table 1 for $DEAE_{12}$-CH-PEG-FA_2 concentrations.

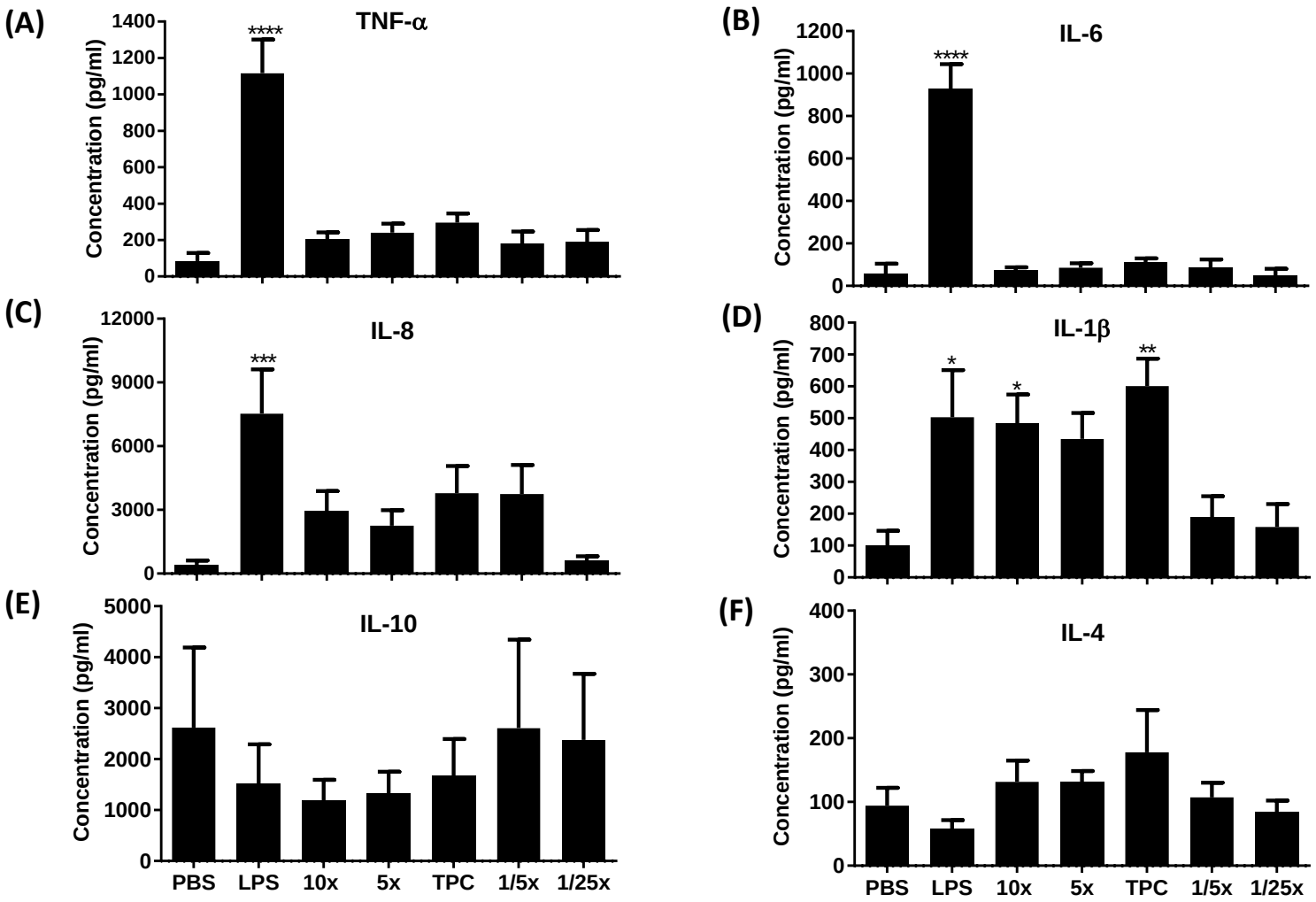

(A)
TNF-α
Concentration (pg/ml)
1400
1200
1000
800
600
400
200
0
****
(B)
IL-6
Concentration (pg/ml)
1200
1000
800
600
400
200
0
****
(C)
IL-8
Concentration (pg/ml)
12000
9000
6000
3000
0
***
(D)
IL-1β
Concentration (pg/ml)
800
700
600
500
400
300
200
100
0
*
*
**
(E)
IL-10
Concentration (pg/ml)
5000
4000
3000
2000
1000
0
(F)
IL-4
Concentration (pg/ml)
400
300
200
100
0
PBS
LPS
10x
5x
TPC
1/5x
1/25x

Figure S5. $DEAE_{12}$-CH-PEG-FA_2 potential to induce cytokines. (A) TNF-α, (B) IL-6, (C) IL-8, (D) IL-1β, (E) IL-10, and (F) IL-4 cytokines were detected by ELISA, after PBMC incubation with free $DEAE_{12}$-CH-PEG-FA_2 for 24 h at 37°C. DPBS and LPS (20 ng/mL) were used as negative and positive controls, respectively. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 are significantly different from the negative control. Data are expressed as the mean ± SEM of five independent experiments, each one from a different healthy human donor, except for IL-4 where N=4. Data were analyzed by One-way ANOVA (post hoc Dunnett's test). See Table 1 for $DEAE_{12}$-CH-PEG-FA_2 concentrations.

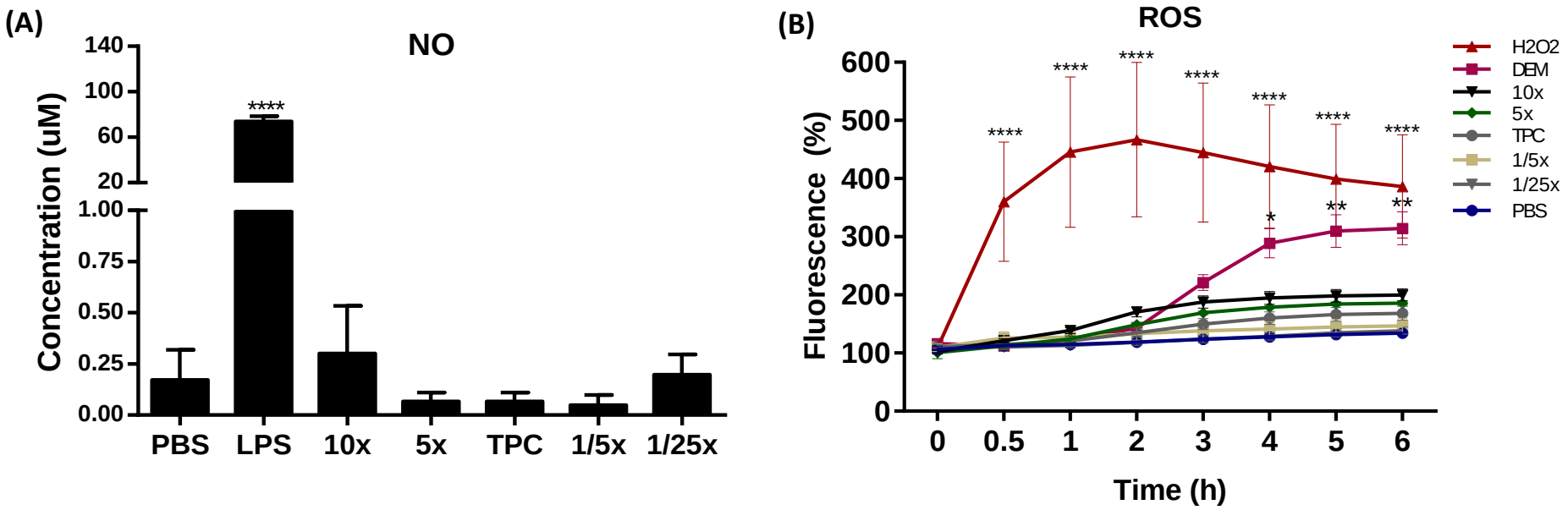


Figure S6. Nitric oxide (NO) and reactive oxygen species (ROS) production in response to free $DEAE_{12}$-CH-PEG-FA_2. (A) NO concentration produced by Raw 264.7 macrophage cells in response to $DEAE_{12}$-CH-PEG-FA_2. Cells were incubated with samples for 48 h at 37°C. NO_2^- concentration was measured in cell supernatants using the Greiss reagent. DPBS and LPS (100 ng/mL) were used as negative and positive controls, respectively. Data were analyzed by One-way ANOVA (post hoc Dunnett's test). (B) ROS induction by $DEAE_{12}$-CH-PEG-FA_2 in Raw 264.7 cells. Cells were exposed to samples for 6 h, after pre-incubation with 20 μM of DCFH-DA probe. Fluorescence readings (ex. 485 nm and em. 530 nm) were performed at the indicated time points. 500 μM H_2O_2 and 5 mM DEM were used as positive controls. DPBS in blue represents the negative control. Data were analyzed by Two-way ANOVA (post hoc Dunnett's test). Data from NO and ROS are expressed as the mean ± SEM of three independent experiments. *p<0.05, **p<0.01, ****p<0.0001 are significantly different from the negative control. See Table 1 for $DEAE_{12}$-CH-PEG-FA_2 concentrations.