Enhancing Efficacy and Stability of an Anti-Heroin Vaccine: Examination of Antinociception, Opioid Binding Profile, and Lethality

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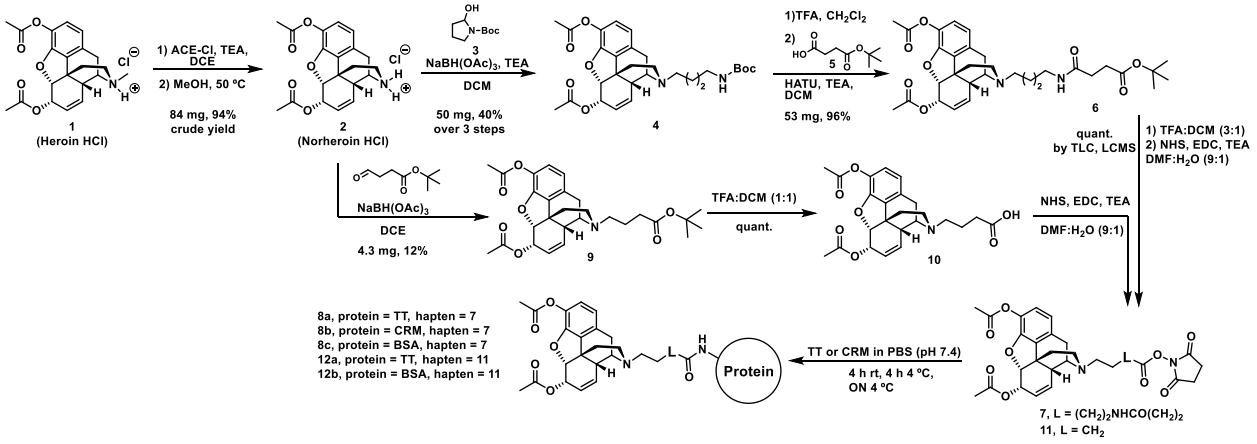
Supplementary Information

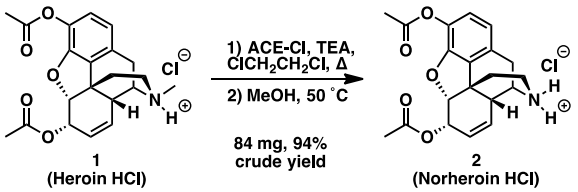
Materials and methods

^{1}H and ^{13}C NMR spectra were obtained on Bruker spectrometers. Multiplicities are quoted as singlet (s), doublet (d), triplet (t), unresolved multiplet (m), or broad signal (br). All chemical shifts are given on the δ–scale in parts per million ((ppm) relative to internal $CDCl_3$ (δ 7.26, ^{1}H NMR; δ 77.0, ^{13}C NMR). ^{1}H coupling constants (*J* values) are given in Hz. The concentration of the NMR samples was in the range of 2-5 mg/mL. Analytical LCMS was performed on an Agilent ESI-ToF (LC/MSD ToF) with an Agilent Zorbax 300SB-C_8 (4.6 x 50 mm) 5 µm column using a flow rate of 0.5 mL/min. The LCMS was run using the following solvents: Solvent A: 0.1% formic acid, Solvent B: 0.1% formic acid in acetonitrile (MeCN) and each run was ten minutes (0-7 min: 5-95% Solvent B, 7-10 min: 95% Solvent B) with detection at wavelength 254 nm. Matrix-assisted laser desorption/ionization (MALDI) mass spectra were obtained using an Applied Biosystems Voyager DE. All chemicals were purchased from commercial sources, with the exception of heroin, which was obtained from NIDA, and used without further purification. Sodium triacetoxyborohydride ($NaBH(OAc)_3$), 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU), α-chloroethylchloroformate (ACE-Cl), *N*-hydroxysuccinimide (NHS), and bovine serum albumin (BSA) were purchased from Sigma. Mono-*t*-butyl succinate was purchased from Combi-Blocks, Inc. Tetanus toxoid (TT) was purchased from UMass Biologics and mutant nontoxic form of diphtheria toxin (CRM197) was purchased from Fina Biosolutions. *N*-Boc-δ-aminobutanal **3** was synthesized according to literature procedure.[1] All reactions were run under inert gas and with dry, distilled solvents unless otherwise noted. The previously mentioned, LCMS as well as TLC visualized with UV light and ninhydrin staining were routinely used to monitor reactions. All exact masses were computed for the following isotopic compositions: ^{1}H, ^{12}C, ^{14}N, and ^{16}O.

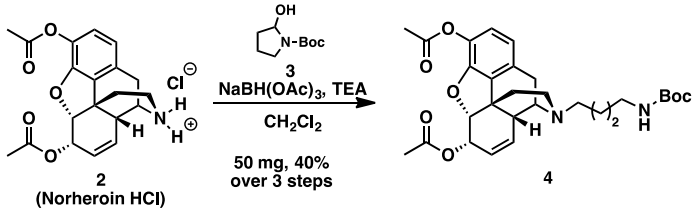
Scheme 1. Synthesis of heroin haptens and conjugation to carrier proteins



(4a*R*,7*S*,7a*R*,12b*S*)-3-(4-((*tert*-butoxycarbonyl)amino)butyl)-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinoline-7,9-diyl diacetate (**4**)



To a solution of heroin hydrochloride (100 mg, 0.25 mmol) in 4 mL of dry, 1,2-dichloroethane was added *N*,*N*-diisopropylethylamine (343 µL, 2.0 mmol, 8 equiv) and ACE-Cl (216 µL, 2.0 mmol, 8 equiv) at rt. The solution was then heated to reflux for 4 h under argon with monitoring by TLC (9:1 CH_2Cl_2:MeOH). The reaction solution was then cooled and the solvent was removed under reduced pressure. The residue was then dissolved in 10 mL CH_2Cl_2 and washed with saturated bicarbonate (2 x 10 mL). The aqueous layers were combined and washed with EtOAc (1 x 10 mL). The organic layers were combined and dried with sodium sulfate (Na_2SO_4) and the solution was filtered and solvents were removed. The residue was then dissolved in a portion of MeOH, stirred at 50 ˚C for 12 minutes and monitored by TLC. Hydrolysis of the carbamate with MeOH must be carefully monitored, as deacetylation of norheroin may occur with proloned heating. The solvents were evaporated and the product **2** (84 mg, 94% crude yield) was used in the next step as obtained. ESI-MS: MS (*m/z*): *calcd* for $C_{20}H_{22}NO_5^+$: 356.2, *found*: 356.2 $[M + H]^+$.



Crude norheroin **2** (84 mg, 0.24 mmol) was dissolved in 4 mL of dry 1,2-dichloroethane, followed by addition of **3** (88 mg, 0.48 mmol, 2 equiv)[1], triethylamine (66 µL, 0.48 mmol, 2 equiv), and $NaBH(OAc)_3$ (150 mg, 0.72 mmol, 3 equiv). The reaction solution was allowed to stir for 4 h and was monitored by TLC. The reaction was quenched with water and washed with saturated sodium bicarbonate (2 x 10 mL). The organic layer was dried with Na_2SO_4 and solvents were evaporated. The residue was purified by flash chromatography using 5% MeOH in EtOAc. The fractions were collected and the solvents were evaporated to give 50 mg of **4** (40% yield over three steps). NMR spectra were consistent with literature values.[2] ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.75 (d, *J* = 8.1 Hz, 1H), 6.56 (d, *J* = 8.2 Hz, 1H), 5.61 (dt, *J* = 10.0, 2.5 Hz, 1H), 5.41 (dt, *J* = 10.1, 2.5 Hz, 1H), 5.24 (s, 1H), 5.14 (d, *J* = 2.7 Hz, 1H), 5.10 (d, *J* = 6.6 Hz, 1H), 3.43 (dd, *J* = 6.0, 3.2 Hz, 1H), 3.17 – 3.11 (m, 2H), 2.98 (d, *J* = 18.7 Hz, 1H), 2.77 (s, 1H), 2.67 (dd, *J* = 12.6, 4.6 Hz, 1H), 2.53 (q, *J* = 6.1 Hz, 2H), 2.35 (d, *J* = 6.0 Hz, 1H), 2.32 (s, 1H), 2.26 (s, 3H), 2.12 (s, 3H), 2.03 (s, 1H), 1.87 (d, *J* = 13.0 Hz, 1H), 1.56 (q, *J* = 6.9 Hz, 4H), 1.43 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.40, 168.40, 156.04, 149.32, 132.12, 131.74, 131.50, 129.52, 128.41, 121.88, 119.28, 88.67, 78.94, 68.06, 56.98, 54.33, 44.59, 43.31, 40.45, 40.35, 34.94, 28.46, 27.81, 24.88, 21.57, 20.63, 20.60. ESI-MS: MS (*m/z*): *calcd* for $C_{29}H_{39}N_2O_7^+$: 527.3, *found*: 527.3 $[M + H]^+$.

Figure S1. ^{1}H NMR (500 MHz, $CDCl_3$) of **4**

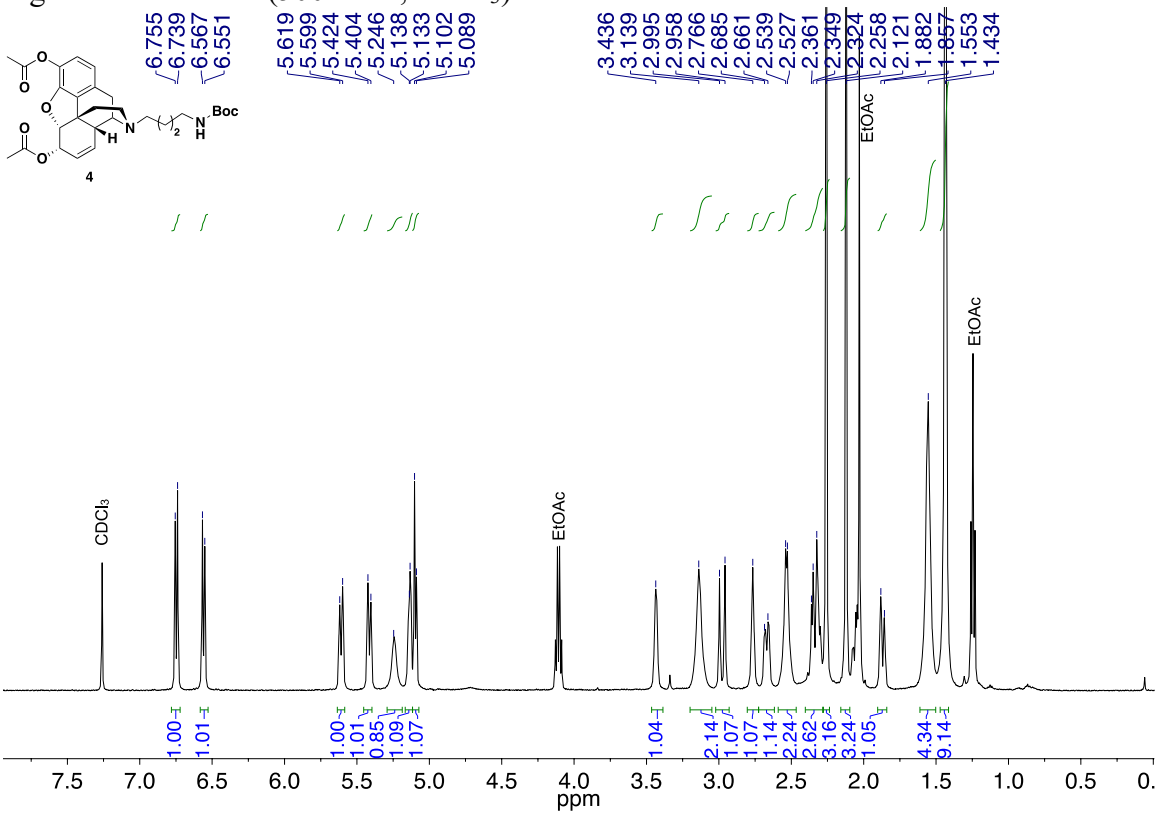
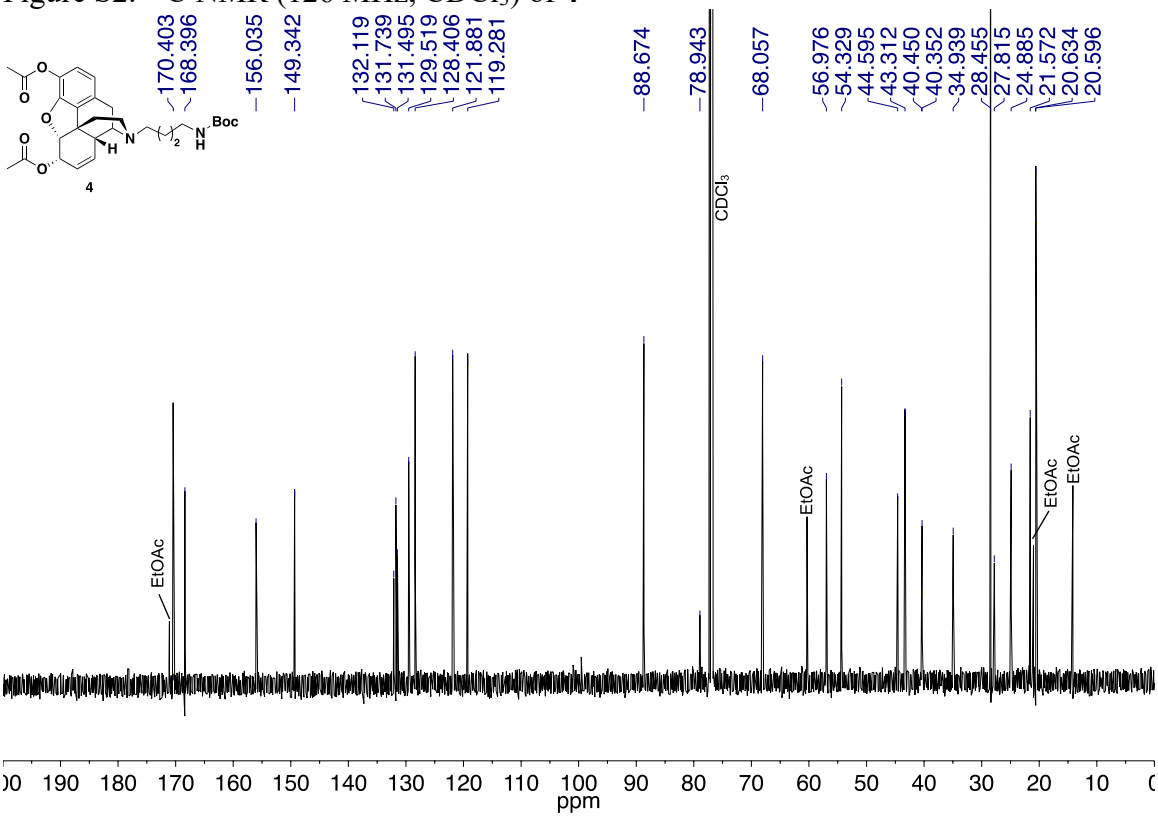
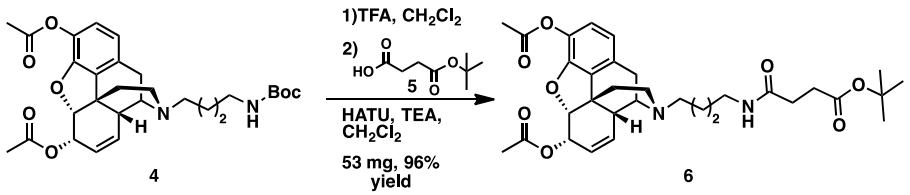


Figure S2. ^{13}C NMR (126 MHz, $CDCl_3$) of **4**

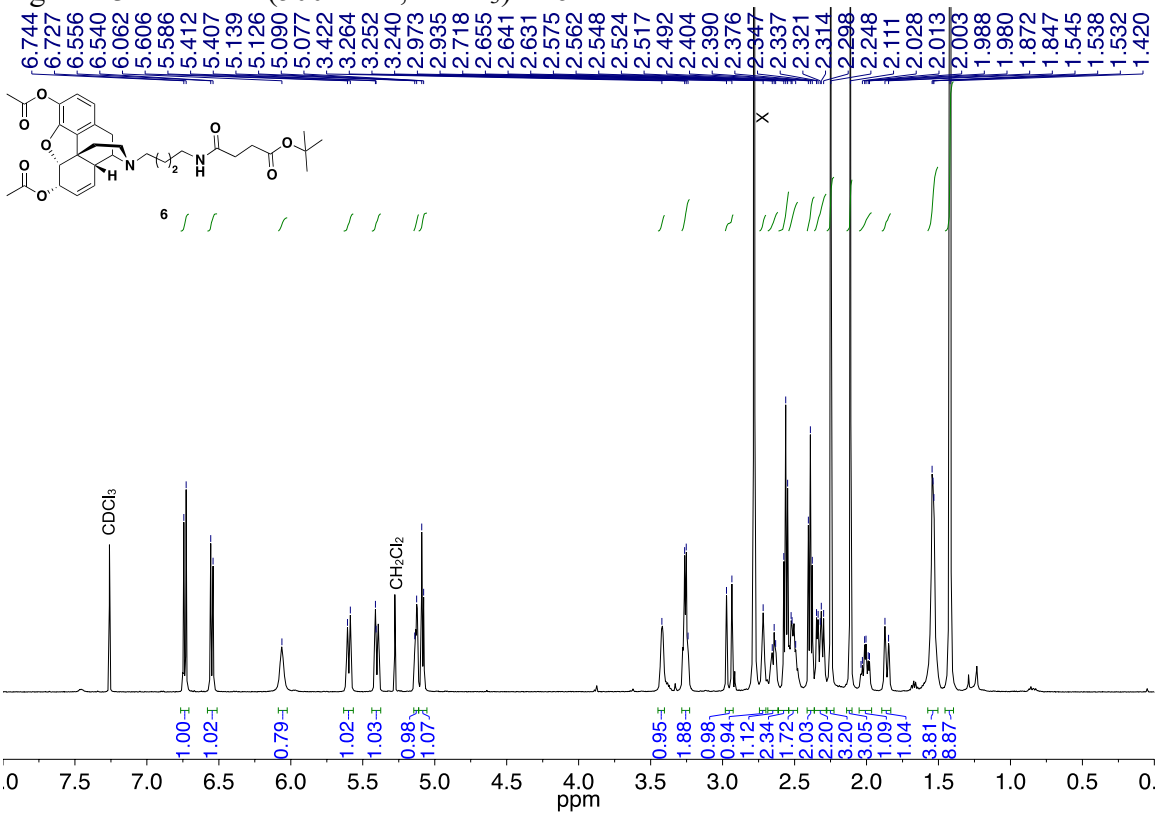


(4a*R*,7*S*,7a*R*,12b*S*)-3-(4-(4-(*tert*-butoxy)-4-oxobutanamido)butyl)-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinoline-7,9-diyl diacetate (**6**)



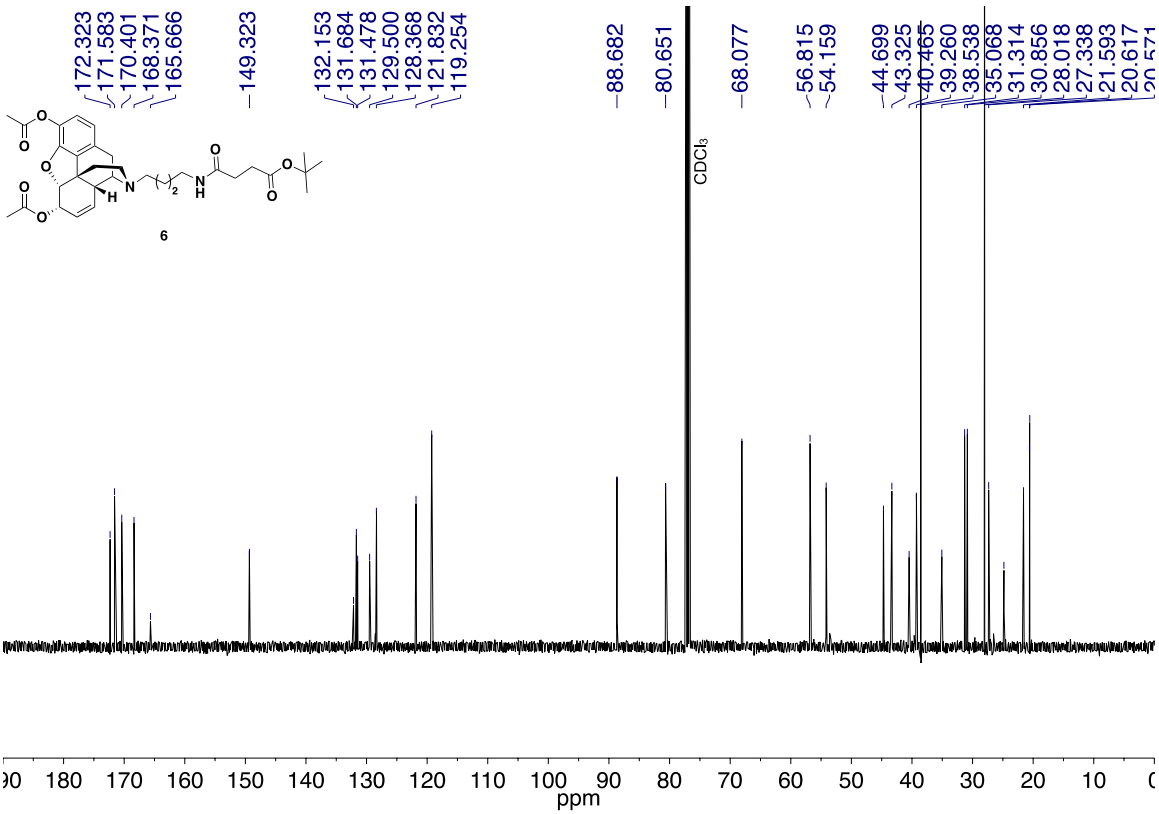
Compound **4** (50 mg, 0.09 mmol) was deprotected using 2 mL of a 1:1 solution of TFA and CH_2Cl_2. The deprotection was allowed to stir for 2 h, and was monitored by TLC (9:1 CH_2Cl_2:MeOH) and LC-MS. ESI-MS: MS (*m/z*): *calcd* for $C_{24}H_{31}N_2O_5^+$: 427.2, *found*: 427.2 $[M + H]^+$. After complete deprotection of the Boc group, the solvents were coevaporated with several portions of toluene and CH_2Cl_2. The compound was then dissolved in 1 mL of dry CH_2Cl_2 and 43 µL of TEA (0.31 mmol, 31 mg, 3.3 equiv). Mono-*t*-butyl succinate **5** (0.10 mmol, 17 mg, 1.1 equiv) and HATU (0.10 mmol, 38 mg, 1.1 equiv) were added in one portion to the solution. The reaction was allowed to stir for 3 h and monitored by TLC (9:1 CH_2Cl_2:MeOH). After complete formation of the amide (**6**), the reaction was diluted with 10 mL of CH_2Cl_2 and washed with saturated sodium bicarbonate. The organic layer was dried with Na_2SO_4 and the solvents were evaporated. The crude oil was purified by flash chromatography using 10% MeOH in CH_2Cl_2. The pure fractions were combined and solvents were evaporated to yield 53 mg of **6** (96% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.74 (d, *J* = 8.2 Hz, 1H), 6.55 (d, *J* = 8.2 Hz, 1H), 6.06 (s, 1H), 5.60 (d, *J* = 10.0 Hz, 1H), 5.41 (d, *J* = 2.3 Hz, 1H), 5.13 (d, *J* = 6.6 Hz, 1H), 5.08 (d, *J* = 6.6 Hz, 1H), 3.42 (s, 1H), 3.28 – 3.23 (m, 2H), 2.95 (d, *J* = 18.7 Hz, 1H), 2.72 (s, 1H), 2.69 – 2.62 (m, 1H), 2.56 (t, *J* = 6.8 Hz, 2H), 2.54 – 2.48 (m, 2H), 2.39 (t, *J* = 6.8 Hz, 2H), 2.36 – 2.28 (m, 2H), 2.25 (s, 3H), 2.11 (s, 3H), 2.01 (td, *J* = 12.2, 4.5 Hz, 1H), 1.86 (d, *J* = 12.6 Hz, 1H), 1.57 – 1.50 (m, 4H), 1.42 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.32, 171.58, 170.40, 168.37, 149.32, 132.15, 131.68, 131.48, 129.50, 128.37, 121.83, 119.25, 88.68, 80.65, 68.08, 56.82, 54.16, 44.70, 43.32, 40.47, 39.26, 35.07, 31.31, 30.86, 28.02, 27.34, 24.82, 21.59, 20.62, 20.57. ESI-MS: MS (*m/z*): *calcd* for $C_{32}H_{43}N_2O_8^+$: 583.3, *found*: 583.3 $[M + H]^+$.

Figure S3. ^{1}H NMR (500 MHz, $CDCl_3$) of **6**

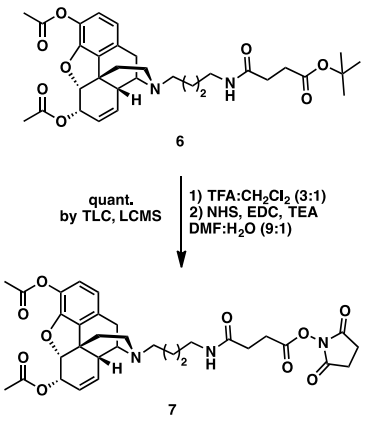


X = unidentified impurity

Figure S4. ^{13}C NMR (126 MHz, $CDCl_3$) of **6**

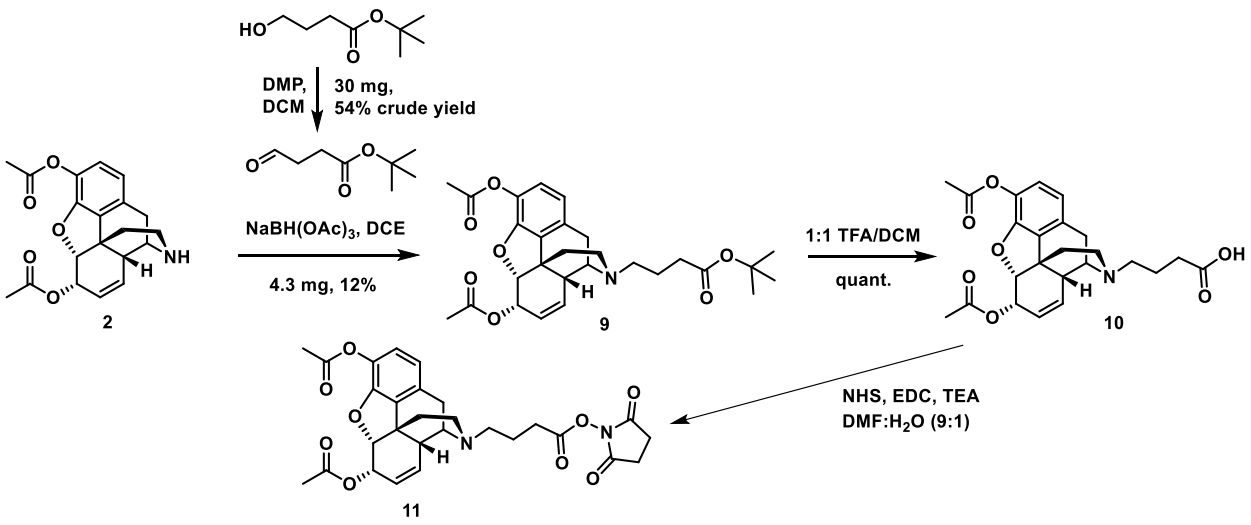


(4a*R*,7*S*,7a*R*,12b*S*)-3-(4-(4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutanamido)butyl)-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinoline-7,9-diyl diacetate (**7**)



The *t*-butyl protected heroin hapten **6** was equally divided into 5 mg aliquots and was stored in the -20 ˚C as a solid until needed for conjugation with carrier protein. To a 5 mg aliquot of **6** (0.009 mmol) was added 1 mL of a solution of TFA and CH_2Cl_2 (3:1). The deprotection was allowed to proceed overnight at rt and was monitored by TLC (9:1 CH_2Cl_2:MeOH). The deprotected hapten was then coevaporated with several portions of toluene and CH_2Cl_2. The deprotected acid was dissolved in 500 uL of a 9:1 DMF:H_2O solution, followed by addition of TEA (0.03 mmol, 3.8 µL, 3 equiv). NHS (0.09 mmol, 10.4 mg, 10 equiv) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride **(**EDC, 0.09 mmol, 17.3 mg, 10 equiv) were added in one portion. The solution was allowed to stir for an hour and was monitored by LCMS. Another 5 equiv of NHS and EDC were added in one portion to the reaction. After one hour, LCMS indicated completion of the reaction. ESI-MS: MS (*m/z*): *calcd* for $C_{32}H_{38}N_3O_{10}^+$: 624.3, *found*: 624.2 $[M + H]^+$.

(4a*R*,7*S*,7a*R*,12b*S*)-3-(4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutyl)-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinoline-7,9-diyl diacetate (**11**)



To a solution of *tert*-butyl 4-hydroxybutanoate (56 mg, 0.35 mmol) in 1 mL DCM was added DMP (273 mg, 0.64 mmol, 1.8 eq) and the solution was stirred for 2 h at rt. The reaction mixture was diluted with DCM and washed 3X with 10% sodium thiosulfate solution and once with saturated sodium bicarbonate solution to yield 30 mg crude *tert*-butyl 4-oxobutanoate (54% crude yield). Crude free-base norheroin **2** (25 mg, 0.07 mmol) was dissolved in 4 mL of dry 1,2-dichloroethane, followed by the addition of crude *tert*-butyl 4-oxobutanoate (30 mg, 0.19 mmol, 2.7 eq) and $NaBH(OAc)_3$ (22 mg, 0.11 mmol, 1.5 equiv). The reaction solution was allowed to stir for 2 h. The reaction mixture was diluted with DCM and washed twice with saturated sodium bicarbonate solution. Purification proceeded via preparative TLC using 5% MeOH in EtOAc as an eluent to yield 4.3 mg of **9** (12% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.78 (d, *J* = 8.2 Hz, 1H), 6.59 (d, *J* = 8.2 Hz, 1H), 5.66 – 5.61 (m, 1H), 5.44 (dt, *J* = 10.0, 2.7 Hz, 1H), 5.17 (dd, *J* = 5.9, 2.8 Hz, 1H), 5.12 (dd, *J* = 6.6, 1.0 Hz, 1H), 3.44 (s, 1H), 2.99 (d, *J* = 18.7 Hz, 1H), 2.78 – 2.73 (m, 1H), 2.68 (dd, *J* = 12.4, 4.7 Hz, 1H), 2.55 (d, *J* = 26.4 Hz, 1H), 2.40 – 2.35 (m, 2H), 2.35 (s, 1H), 2.32 (t, *J* = 7.2 Hz, 2H), 2.29 (s, 3H), 2.16 (s, 3H), 2.08 – 2.00 (m, 1H), 1.89 (d, *J* = 10.8 Hz, 1H), 1.84 – 1.76 (m, 2H), 1.48 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.18, 170.66, 168.62, 149.54, 131.85, 131.78, 129.86, 128.48, 121.99, 119.46, 88.96, 80.32, 68.34, 57.20, 54.19, 44.83, 43.56, 40.70, 35.33, 33.43, 28.29, 23.18, 22.03, 20.84, 20.79. HRMS (ESI-TOF): MS (*m/z*): *calcd* for $C_{28}H_{36}NO_7$ 498.2486, *found:* 498.2477.

Compound **9** was deprotected using 1 mL 1:1 TFA/DCM over 18 h to quantitatively afford **10** as the TFA salt. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.87 (d, *J* = 8.2 Hz, 1H), 6.68 (d, *J* = 8.4 Hz, 1H), 5.75 (d, *J* = 10.2 Hz, 1H), 5.42 (s, 1H), 5.22 (d, *J* = 6.8 Hz, 1H), 5.17 (s, 1H), 4.31 (s, 1H), 3.53 (s, 1H), 3.29 (s, 2H), 3.24 – 3.08 (m, 2H), 2.89 (d, *J* = 21.7 Hz, 2H), 2.52 (s, 2H), 2.28 (s, 3H), 2.12 (s, 3H), 1.25 (s, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.77, 168.65, 149.63, 133.01, 130.89, 129.28, 127.62, 125.30, 123.84, 120.09, 87.27, 66.98, 58.79, 53.96, 46.85, 41.39, 37.83, 32.46, 30.49, 29.84, 21.73, 20.65, 20.54, 19.25. HRMS (ESI-TOF): MS (*m/z*): *calcd* for $C_{24}H_{28}NO_7$ 442.1860, *found:* 442.1857 $[M + H]^+$. The procedure used for the preparation of NHS ester of **7** was also used to synthesize **11**.

Figure S5. ^{1}H NMR (600 MHz, $CDCl_3$) of **9**

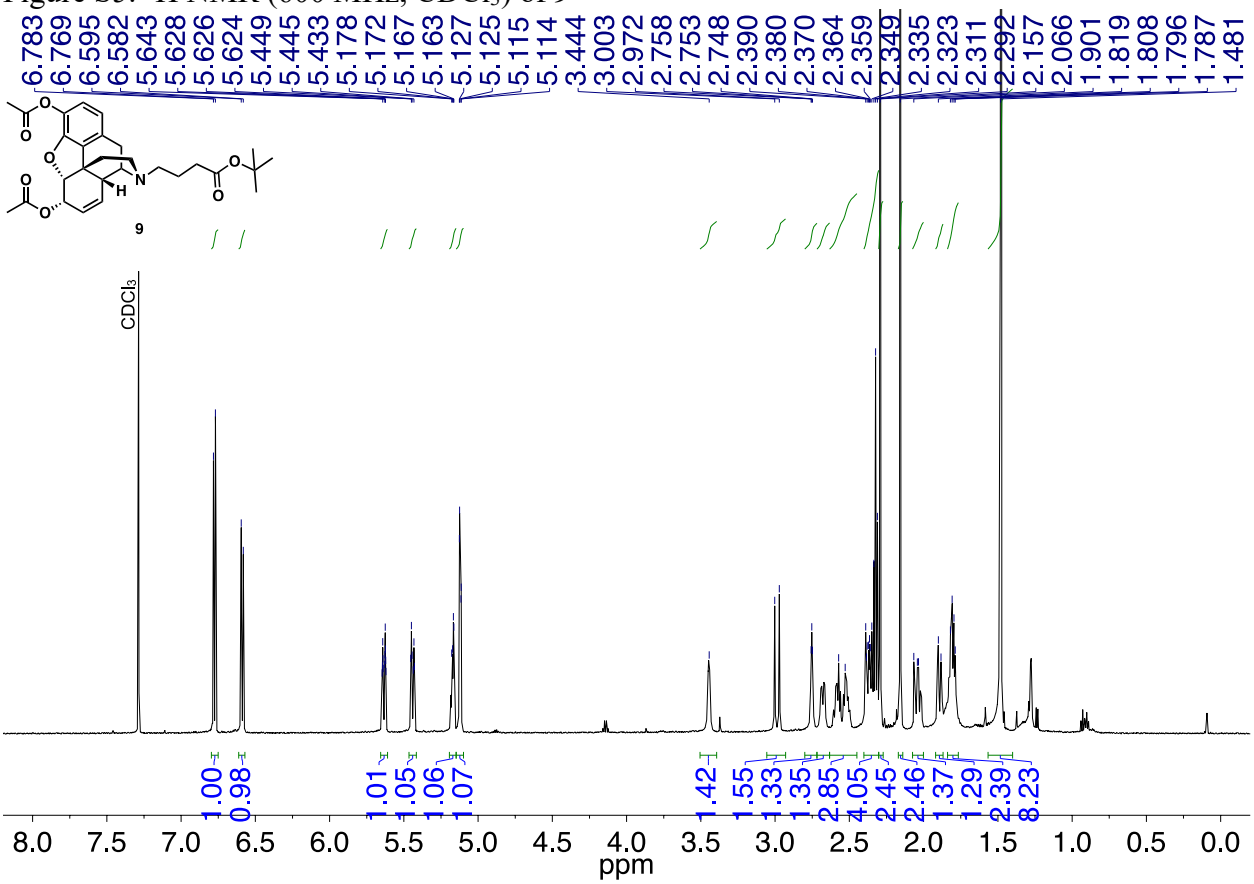


Figure S6. ^{13}C NMR (151 MHz, $CDCl_3$) of **9**

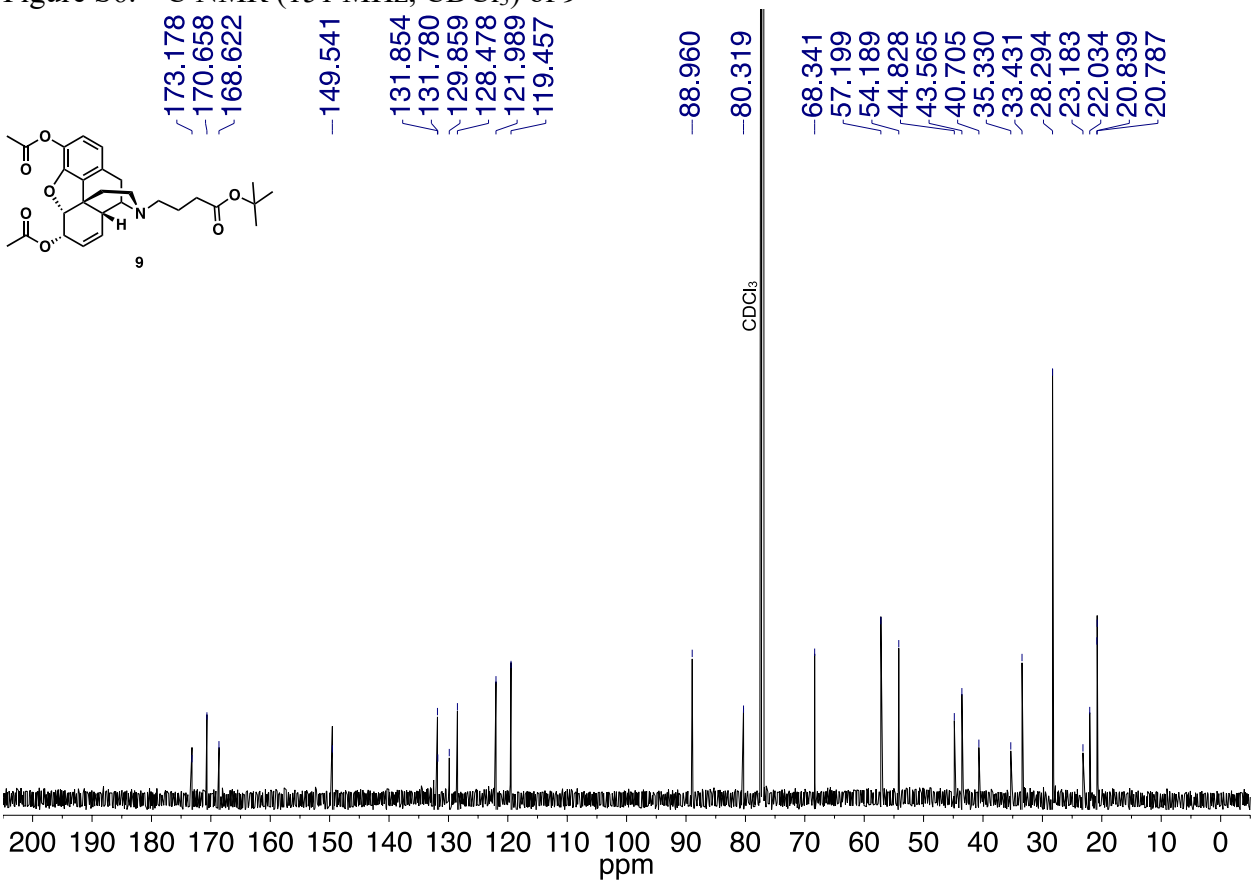


Figure S7. ^{1}H NMR (600 MHz, $CDCl_3$) of **10**

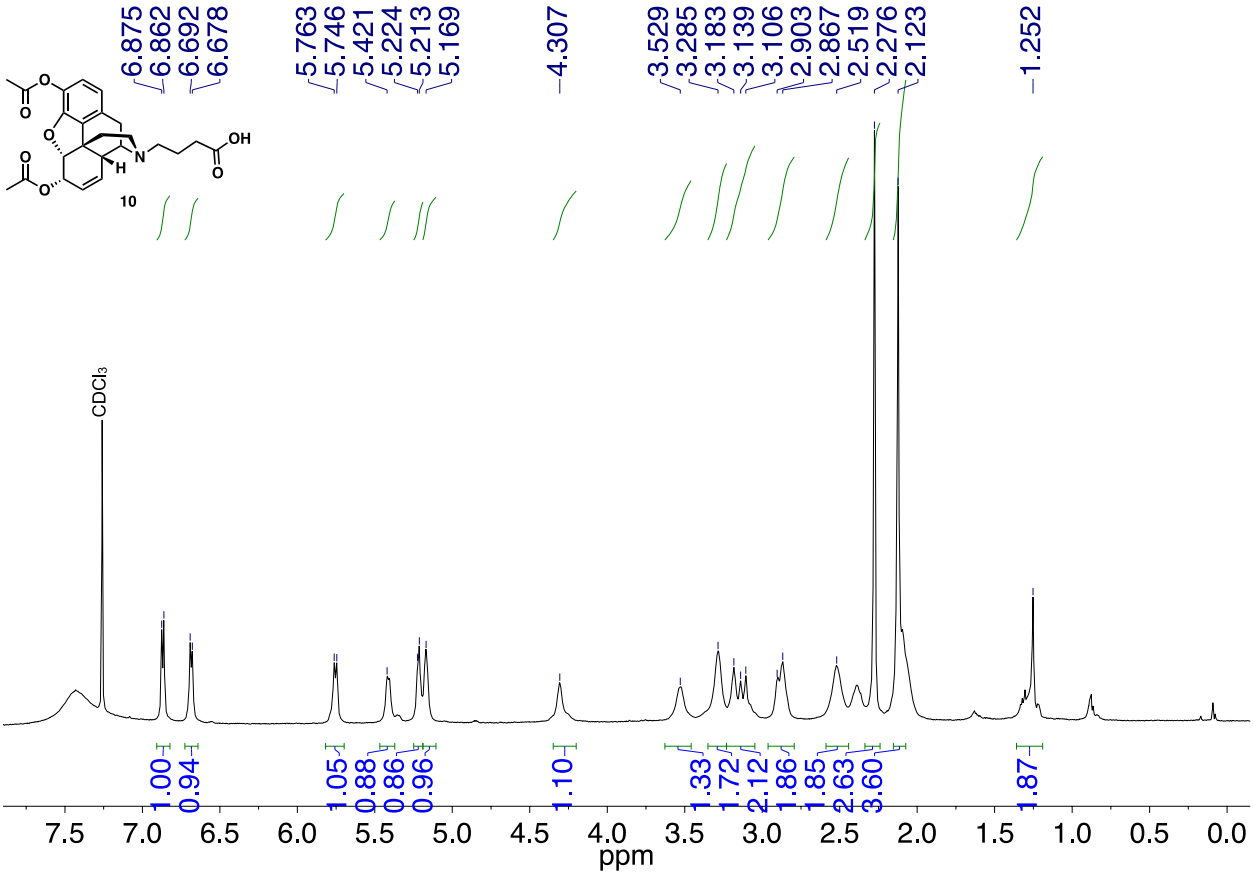
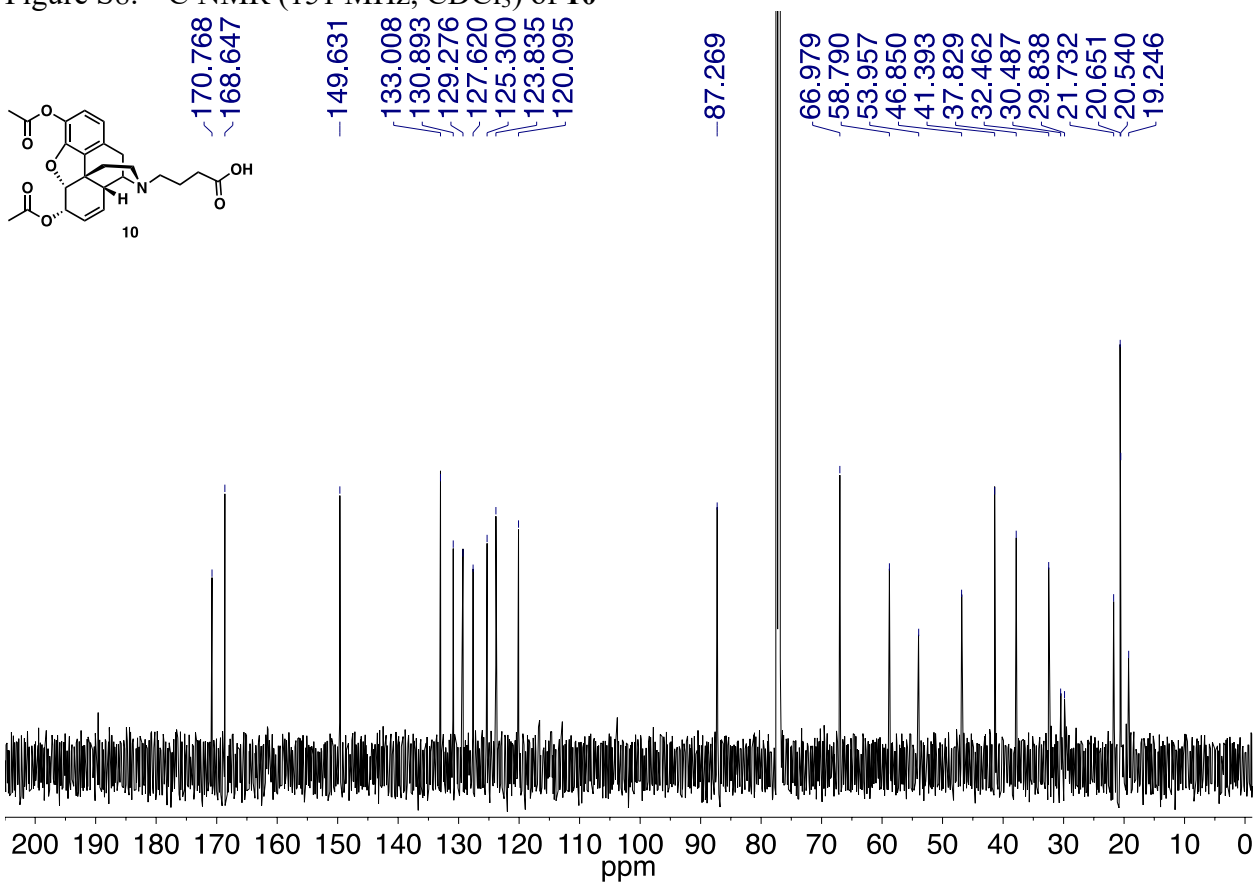
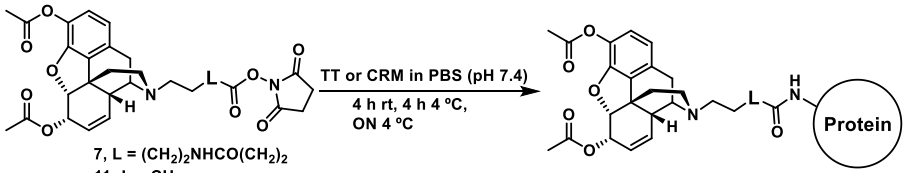


Figure S8. ^{13}C NMR (151 MHz, $CDCl_3$) of **10**



Conjugation of activated heroin haptens **7** and **11** to carrier protein tetanus toxoid (TT), diphtheria toxin mutant (CRM) or bovine serum albumin (BSA)



7, L = $(CH_2)_2NHCO(CH_2)_2$
11, L = CH_2

8a, protein = TT, hapten = 7
8b, protein = CRM, hapten = 7
8c, protein = BSA, hapten = 7
12a, protein = TT, hapten = 11
12b, protein = BSA, hapten = 11

Prior to conjugation, tetanus toxoid (TT) and mutant nontoxic form of diphtheria toxin (CRM) were dialyzed against phosphate buffered solution, pH 7.4 (PBS) using Slide-A-Lyzer™dialysis cassettes (ThermoFisher) with a 10K molecular weight cutoff. The buffer was exchanged after 2 h at rt, 4 h at 4 ˚C and overnight at 4 ˚C. A portion of the reaction solution (450 µL) of **7** or **11** was added to 4.5 mg of TT or CRM (1 mg/mL in PBS buffer, pH 7.4). Another portion (50 µL) was added to 0.5 mg of bovine serum albumin (BSA, 1 mg/mL in PBS buffer pH 7.4). The activated heroin hapten was allowed to react with the carrier proteins at rt for 4 h, followed by 16 h overnight at 4 ˚C using gentle end-over-end mixing. The reaction solutions were then dialyzed as described above. Heroin immunoconjugates were either mixed with 50% (v/v) glycerol (total immunoconjugate volume) or trehalose (total vaccine volume, % w/v) and stored in the -80 ˚C.

MALDI-ToF MS analysis

Heroin conjugated to BSA or CRM was run on a desalting column and then analyzed by MALDI-ToF for the hapten:carrier protein conjugation number as a surrogate for TT or CRM and for ELISAs. In order to quantify the copy number or the number of heroin haptens (Her) on BSA and CRM, the molecular weight (MW) of conjugated BSA (Her-BSA, Figures S12-13) was compared to the MW of unconjugated BSA (BSA, Figure S11) using the following formula:

$$\text{Hapten number} = \frac{(MW_{\text{Her-Protein}} - MW_{\text{Protein}})}{(MW_{\text{Her}} - MW_{H_2O})}$$

$MW_{\text{Her-BSA}}$ = 75,361 or 79,667 Da (8c); 77,827 (12b)

MW_{BSA} = 66,472 Da

MW_{CRM} = 58,417 Da

$MW_{\text{Her-CRM}}$ = 64,604 Da

MW_{Her} = 526.5 Da or 441.5 Da

MW_{H_2O} = 18 Da

Hapten: protein molar ratios =

17.5 and 25.9 for 8c

12.2 for 8b

26.8 for 12b

Figure S9. MALDI-ToF MS spectrum of unconjugated CRM

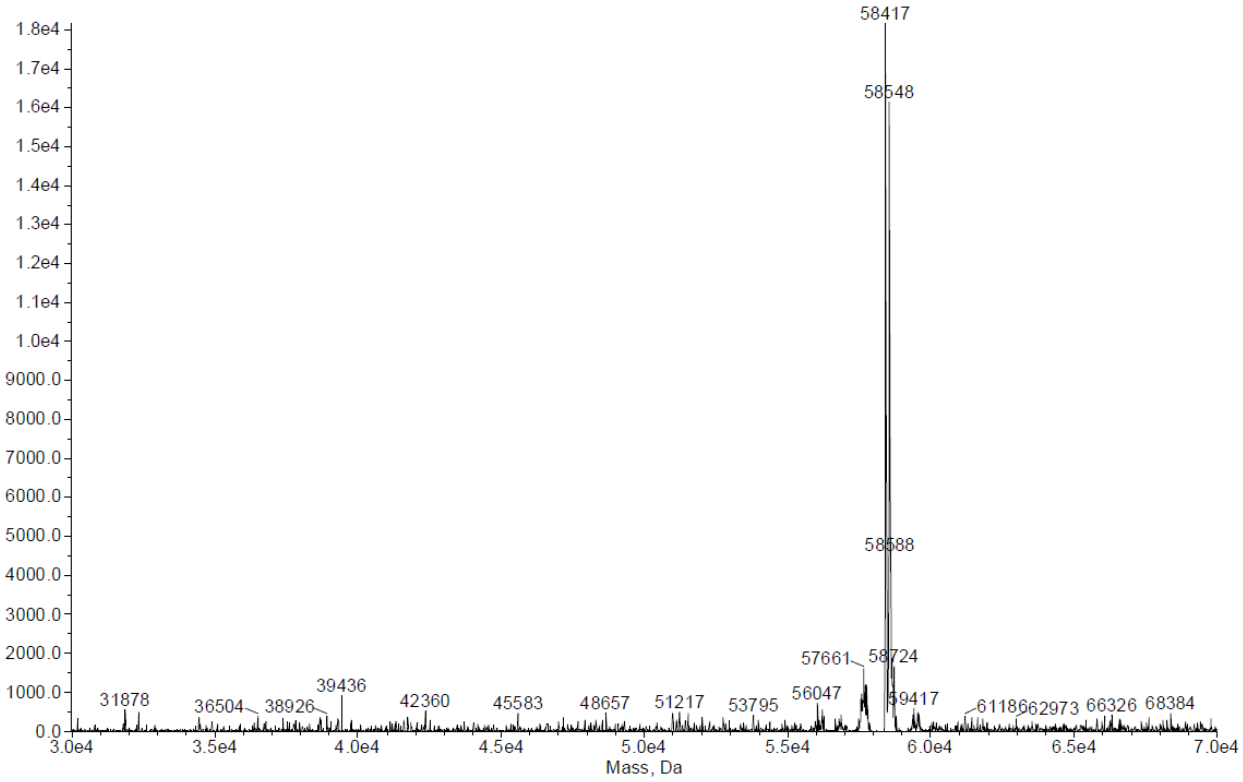


Figure S10. MALDI-ToF MS spectrum of **8b**

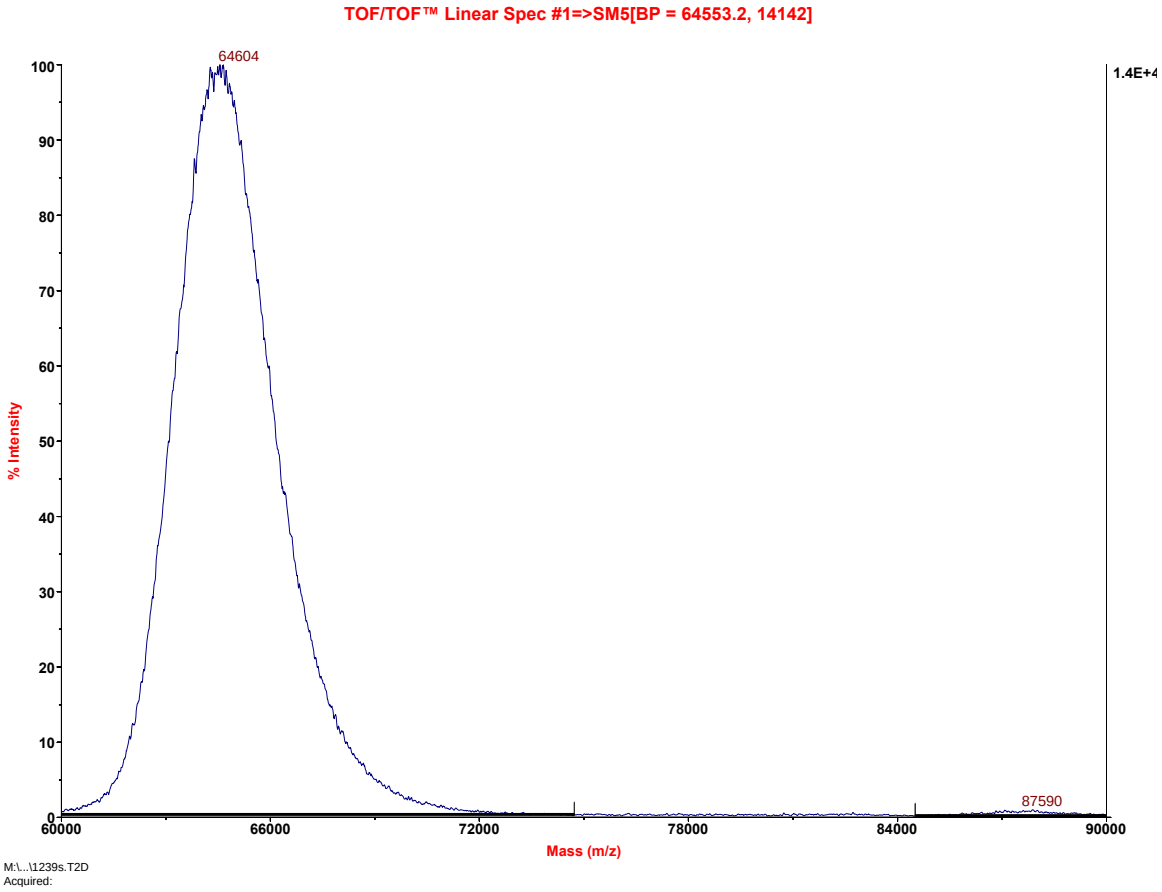


Figure S11. MALDI-ToF MS spectrum of unconjugated BSA

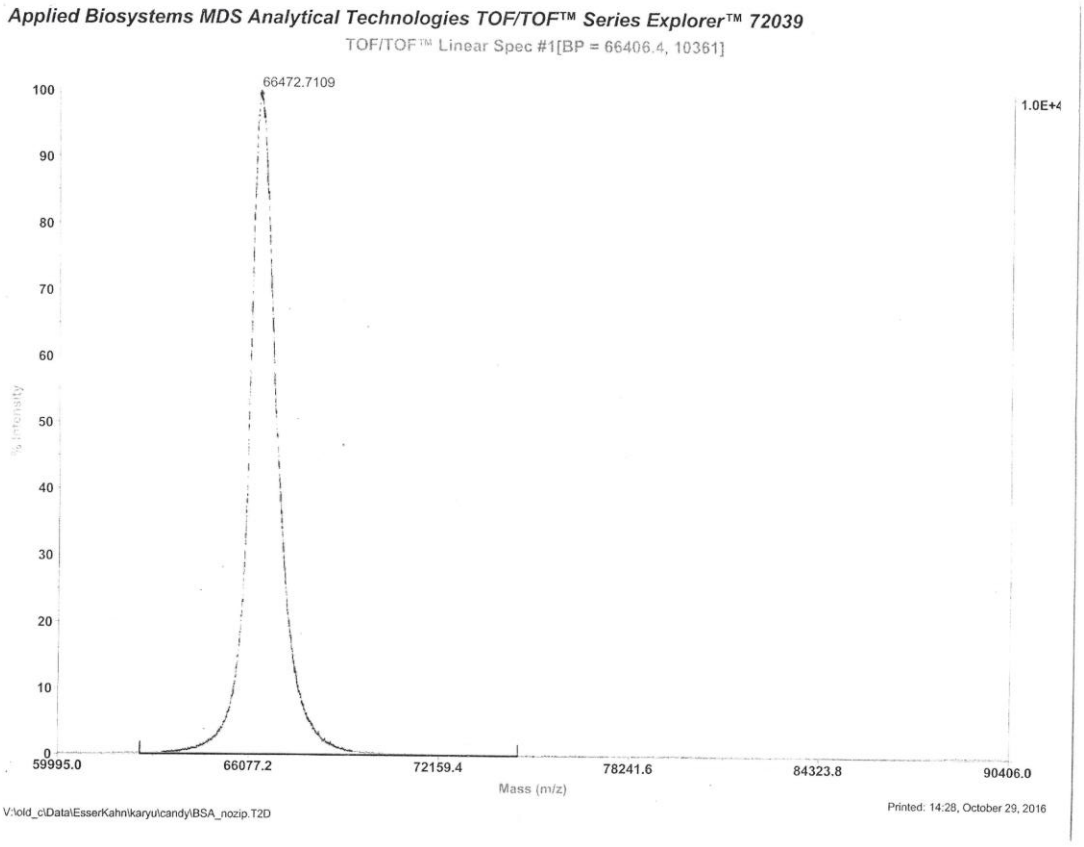


Figure S12. MALDI-ToF MS spectrum of **8c**

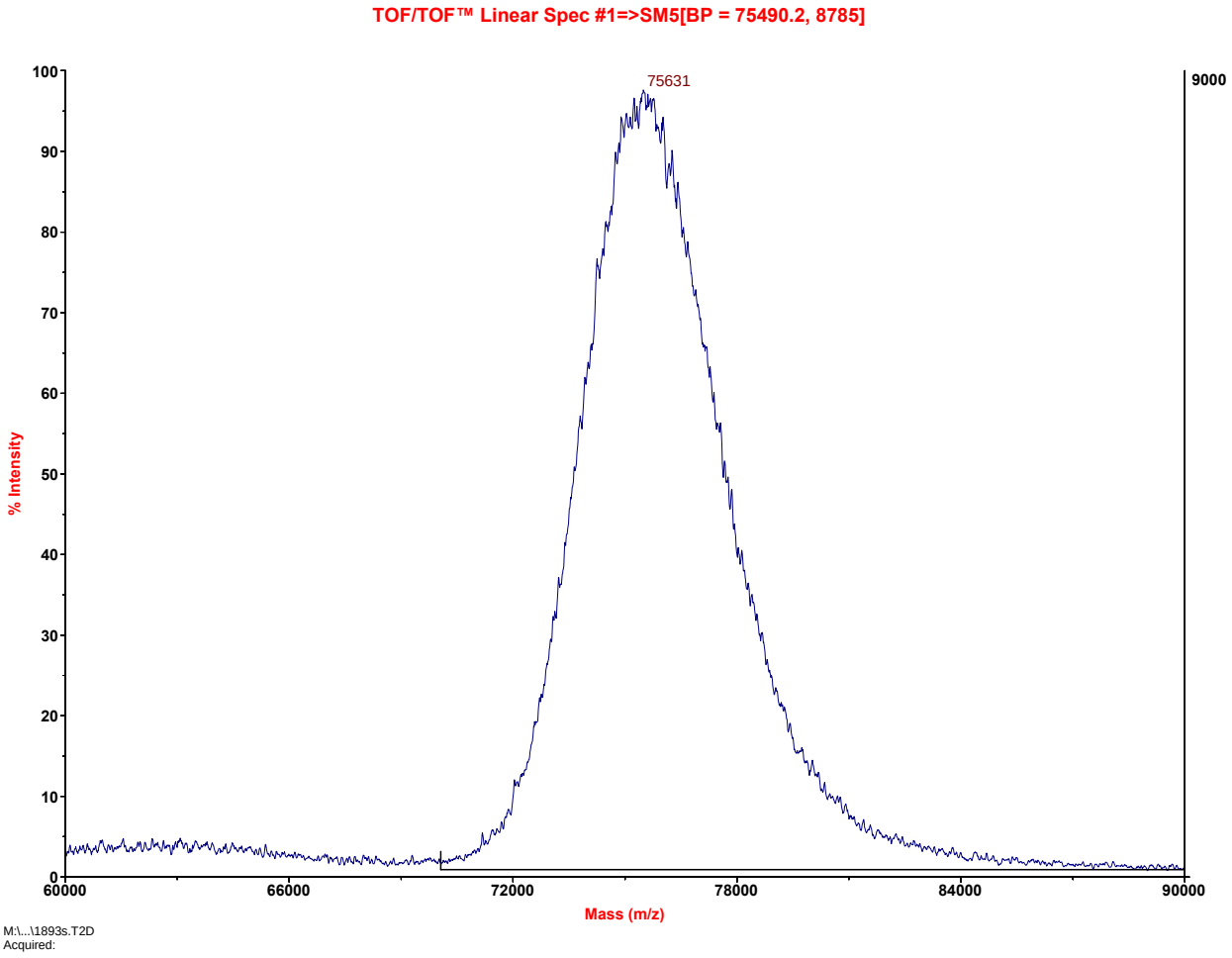


Figure S13. MALDI-ToF MS spectrum of **8c**

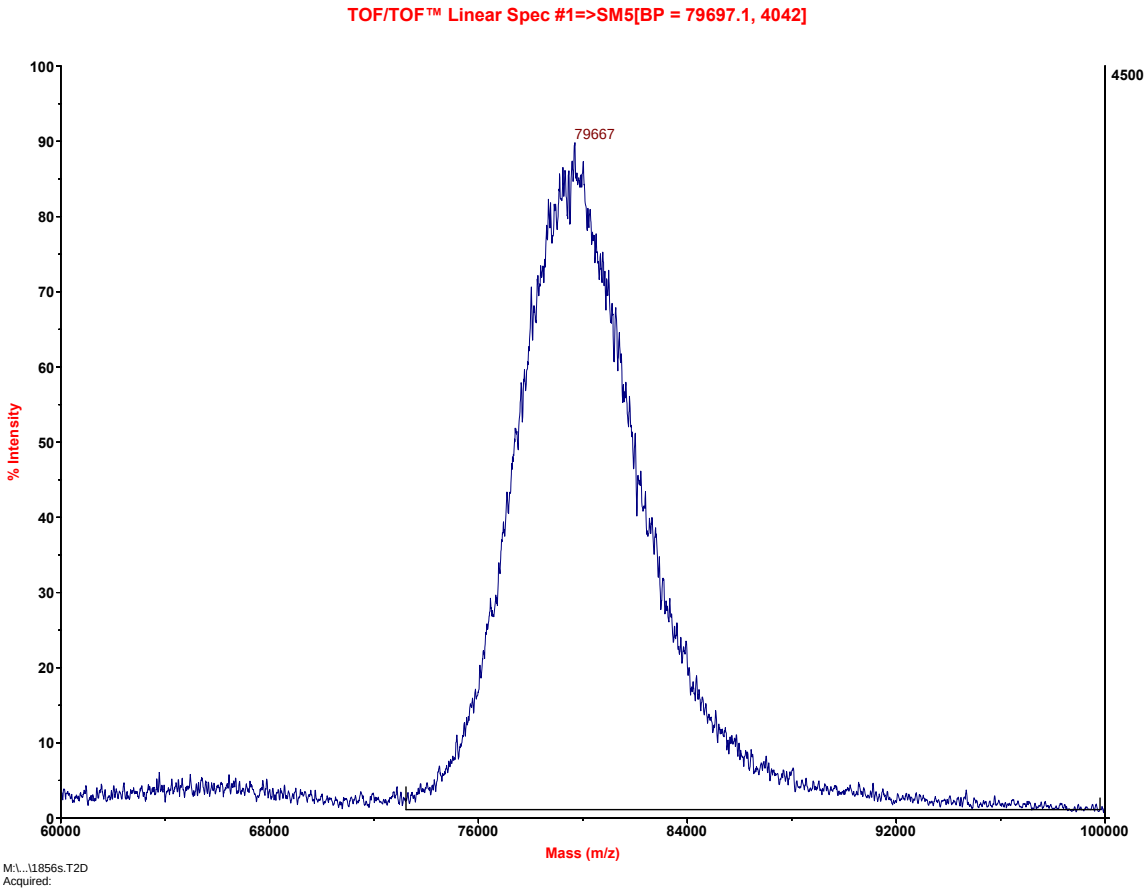


Figure S14. MALDI-ToF MS spectrum of **12b**

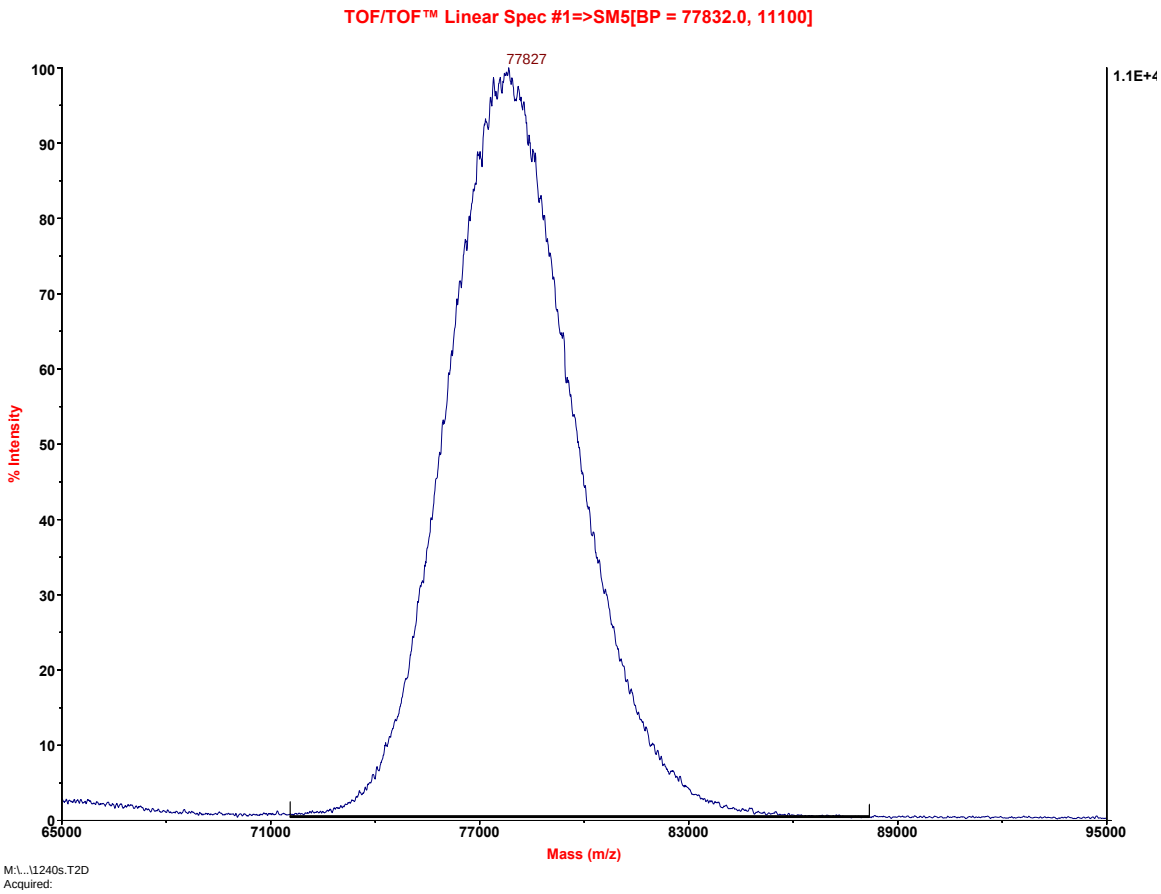


Table S1. Series A: initial screening of formulations; Series B: optimization of vaccine candidate formulations; Series C: delivery with liposome versus alum

Group	Vaccine	HerCRM/ HerTT (µg/ dose)	Alum (mg/mL)	Adjuvant	Route	Vaccination Schedule (days)	Bleeds (days)	Behavioral Assay (days)	Mice
A1	vehicle	–	1	–	s.c./s.c./s.c.	0,14,28	42	45	6
A2	H-CRM-RNA	50 µg Her-CRM	–	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	42	45	4
A3	H-CRM-Alum-RNA	50 µg Her-CRM	1	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	42	45	4
A4	H-CRM-CALV-RNA	50 µg Her-CRM	–	2.5 mg CALV + 50 µg dsRNA	s.c./s.c./s.c.	0,14,28	42	45	4
A5	H-CRM-Alum-CpG	50 µg Her-CRM	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	42	45	4
A6	H-TT-Alum-CpG	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	42	45	4
B1	H-TT-Alum-RNA	50 µg Her-TT	1	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	42,70	47	4
B2	H-TT-Alum-CpG+RNA	50 µg Her-TT	1	50 µg CpG + 50 µg dsRNA	s.c./s.c./s.c.	0,14,28	42,70	47	4
B3	H-TT-Alum-CpG+RNA-Lyo (Lyophilized and reconstituted)	50 µg Her-TT	1	50 µg CpG + 50 µg dsRNA	s.c./s.c./s.c.	0,14,28	42,70	47	6
B4	H(s)-TT-Alum-CpG (i.e., short heroin hapten)	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	42,70	47	4
B5	(IP) H-TT-Alum-CpG	50 µg Her-TT	1	50 µg CpG	i.p./i.p./i.p.	0,14,28	42,70	47	4
C1	H-TT-Alum-RNA-CALV	50 µg Her-TT	0.2	2.5 mg CALV + 50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	5
C2	H-TT-RNA-CALV	50 µg Her-TT	–	2.5 mg CALV + 50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	5
C3	H-TT-CALV	50 µg Her-TT	–	2.5 mg CALV	s.c./s.c./s.c.	0,14,28	38	42	5

s.c., subcutaneous; i.p., intraperitoneal; H(s), stands for the shorter hapten **11**

Table S2. Series D: Dose ranging dsRNA:alum ratio study design

Group	Vaccine	HerTT (µg/ dose)	Alum (mg/mL)	Adjuvant	Route	Vaccination Schedule (days)	Bleeds (days)	Behavioral Assay (days)	Mice
D1	KLH (vehicle)	50 µg KLH	1	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	4
D2	H-TT-Alum-RNA(L)	50 µg Her-TT	1	10 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	4
D3	H-TT-Alum-RNA(M)	50 µg Her-TT	1	25 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	4
D4	H-TT-Alum-RNA(H)	50 µg Her-TT	1	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	4
D5	KLH (vehicle)	50 µg KLH	0.2	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	4
D6	H-TT-Alum(L)-RNA	50 µg Her-TT	0.2	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	4
D7	H-TT-Alum(M)-RNA	50 µg Her-TT	0.5	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	4
D8	H-TT-Alum(H)-RNA	50 µg Her-TT	1	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	4

Table S3. Series E: Dosing alum with CpG study design

Group	Vaccine	HerTT (µg/ dose)	Alum (mg/mL)	Adjuvant	Route	Vaccination Schedule (days)	Bleeds (days)	Behavioral Assay (days)	Mice
E1	KLH (vehicle)	50 µg KLH	0.5	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	4
E2	H-TT-Alum(L)-CpG	50 µg Her-TT	0.2	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	4
E3	H-TT-Alum(M)-CpG	50 µg Her-TT	0.5	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	4
E4	H-TT-Alum(H)-CpG	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	4

Table S4. Series F: dsRNA as an adjuvant for stability studies and using liposomes

Group	Vaccine	HerTT (µg/ dose)	Alum (mg/mL)	Adjuvant	Route	Vaccination Schedule (days)	Bleeds (days)	Behavioral Assay (days)	Mice
F1	H-TT-Alum-RNA (1 d) (25% trehalose, w/v)	50 µg Her-TT	0.2	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	5
F2	H-TT-Alum-RNA (30 d) (25% trehalose, w/v)	50 µg Her-TT	0.2	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	5
F3	H-TT-Alum-RNA-Lyo (25% trehalose, w/v)	50 µg Her-TT	0.2	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	5
F4	H-TT-Alum-RNA-Lyo (30 d) (25% trehalose, w/v)	50 µg Her-TT	0.2	50 µg dsRNA	s.c./s.c./s.c.	0,14,28	38	42	5

Table S5. Series G: Stability studies with CpG under lyophilized and liquid conditions

Group	Vaccine	HerTT (µg/ dose)	Alum (mg/mL)	Adjuvant	Route	Vaccination Schedule (days)	Bleeds (days)	Behavioral Assay (days)	Mice
G1	H-TT-Alum-CpG (1 d, 4˚C) (>5% trehalose, w/v)	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	5
G2	H-TT-Alum-CpG (30 d, 4 ˚C) (>5% trehalose, w/v)	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	5
G3	H-TT-Alum-CpG-Lyo (>5% trehalose, w/v)	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	5
G4	H-TT-Alum-CpG-Lyo (30 d) (>5% trehalose, w/v)	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	5
G5	H-TT-Alum-CpG (0 d) (25% trehalose, w/v)	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	5
G6	H-TT-Alum-CpG (30 d, RT) (25% trehalose, w/v)	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	5
G7	H-TT-Alum-CpG-Lyo (25% trehalose, w/v)	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	5
G8	H-TT-Alum-CpG-Lyo (30 d) (25% trehalose, w/v)	50 µg Her-TT	1	50 µg CpG	s.c./s.c./s.c.	0,14,28	38	42	5

Figure S15. Standard timeline of vaccination schedule and related behavioral experiments

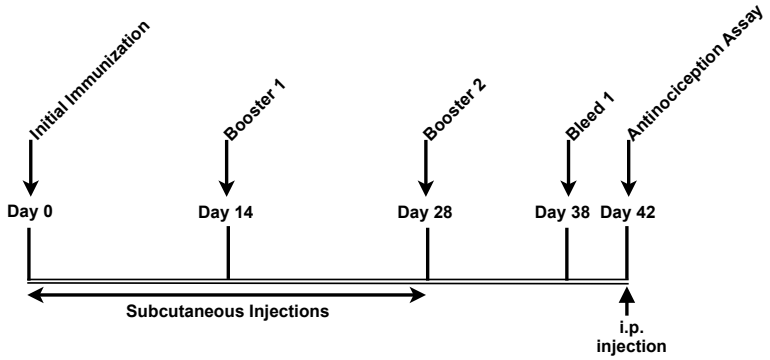


Figure S 16. Timeline of vaccination schedule for stability studies and related behavioral experiments

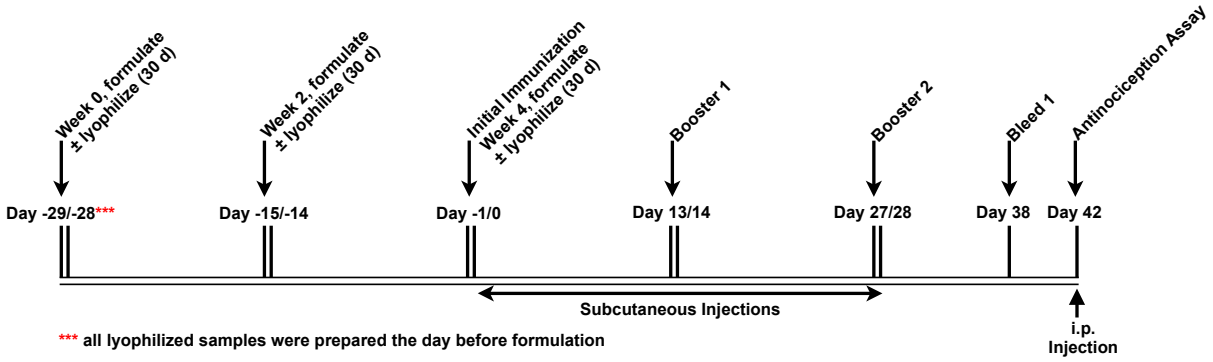
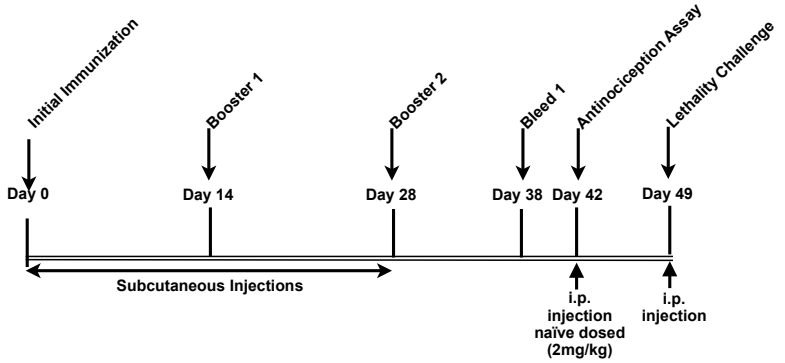


Figure S 17. Timeline of vaccination schedule for lethality study and related behavioral experiments



Hot plate and tail immersion antinociceptive testing

The day of each behavioral assay is indicated for each series in Tables S1-5. Mice were tested for cumulative heroin response in primarily suprapinal (hot plate) and spinal (tail flick) behavioral tests as previously described.[3] The hot plate test was measured by placing the mouse in an acrylic cylinder (14 cm diameter × 22 cm) on a 54 ˚C surface and timing latency to perform one of the following nociceptive responses: licking of hind paw, shaking/withdrawal of hind paw or jumping. Typical baseline latency was between 10 to 20 s with a cutoff of 35 s to prevent tissue damage. After response or reaching the cutoff time, mice were immediately removed from the hot plate. The tail immersion test was administered by lightly restraining mice in a small pouch constructed from absorbent laboratory underpads and dipping 2 cm of the tip of the tail into a heated water bath, with the time of withdrawal measured. Typical baseline response was 0.4 – 1 s and a cutoff of 10 s was used to prevent tissue damage. Since tail flick immersion is a more reflexive behavior, testing order always hot plate first followed by tail immersion. Immediately following completion of both antinociceptive assays, heroin (0.4 mg/kg in saline) was immediately injected intraperitoneally. The heroin doses tested were 2, 4, 6, 8, 10, 14 and 18 mg/kg to generate a full dose-response curve. Testing was repeated at roughly fifteen minutes intervals, following each injection and this cycle of testing and injections was repeated with increase cumulative dosing until full antinociception (cut off times surpassed) was observed in both assays. Antinociception data were transformed from time to percent maximum possible effect (%MPE), which is calculated as:

$$\%\,\mathrm{MPE} = \frac{(\text{test-baseline})}{(\text{cutoff-baseline})} \times 100$$

These data were then fit using a log(agonist) vs. normalized response non-linear regression in GraphPad PRISM 6. The ED_{50} values and 95% confidence intervals were determined for each antinociception test and individual treatment groups to determine ED_{50} values.

ELISA procedure

Costar 3690 plates with half-area, high-binding 96-well microtiter plates were coated with 25 ng of Her-BSA (25 µL of 1 µg/mL Her-BSA conjugate in PBS) per well overnight at 37 ˚C. The

plates were blocked with 5% skim milk in PBS buffer (pH 7.4) for one hour at rt. Mouse serum was serially diluted 1:1 in 2% BSA in PBS buffer (pH 7.4) across twelve columns starting at 1:200. After two hours incubation at rt, the plates were washed ten times with PBS, and donkey anti-mouse IgG horseradish peroxidase (HRP) secondary antibody (Jackson ImmunoResearch) at 1:10,000 dilution in 2% BSA in PBS buffer (pH 7.4) was added and incubated overnight at 4 ˚C. After washing ten times with PBS buffer, a 1:1 solution of 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2 substrate (Thermo Pierce) were added. Plates were incubated for 8 minutes and then quenched with 2.0 M H_2SO_4. Plates were read at 450 nm. Using GraphPad PRISM 6, absorbance values were normalized to the highest absorbance value per set, and a curve was fit using the log(inhibitor) vs. Normalized response – variable slope equation to determine midpoint titer and standard error. Serum from non-vaccinated mice did not contain any detectable anti-heroin titers. Data were tested for statistical outlier evaluation using Grubbs test and significant outliers were removed.

For the experiment using 6AM-BSA as a coating antigen, 6AM-BSA and Her-BSA were run simultaneously on separate plates and run in the same manner as described above, except for modification to the serial dilution and final TMB incubation steps. The mouse serum was serially diluted 1:3 in 2% BSA in PBS buffer (pH 7.4) across twelve columns starting at 1:400. After the final wash step, the plates were incubated with TMB and H_2O_2 for fifteen minutes each.

Figure S18. Antibody titers over time
Geometric endpoint titers against Her-BSA. ELISA assay was run using our heroin hapten conjugated to BSA. Blood was collected at week 6 and 10. Bars represent means ± SEM, where 1 represents 6 weeks; 2 represents 10 weeks

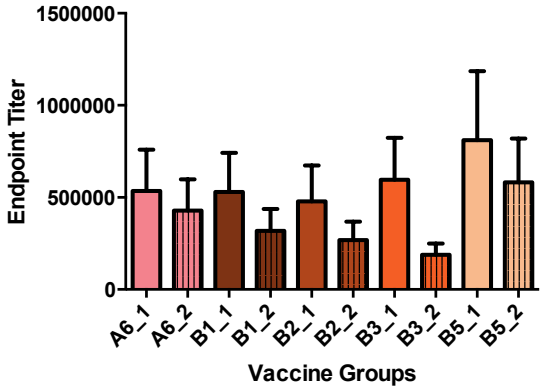


Figure S19. Midpoint titers for anti-heroin antibodies as determined by ELISA
A) Structure of the coating antigen with different hapten linker lengths. B) Midpoint titer data for A6 and B4. C) The ELISA assay was performed using either the heroin hapten (Her-BSA) or 6-monoacetyl morphine conjugated to BSA (6-AM-BSA). D) The Her-BSA midpoint titer data shown for G1-G8 differ for from previously reported midpoint titers in Table 1, due to a different 1:3 dilution set-up used for this assay. Blood was collected at week 6. Bars represent means ± SEM.

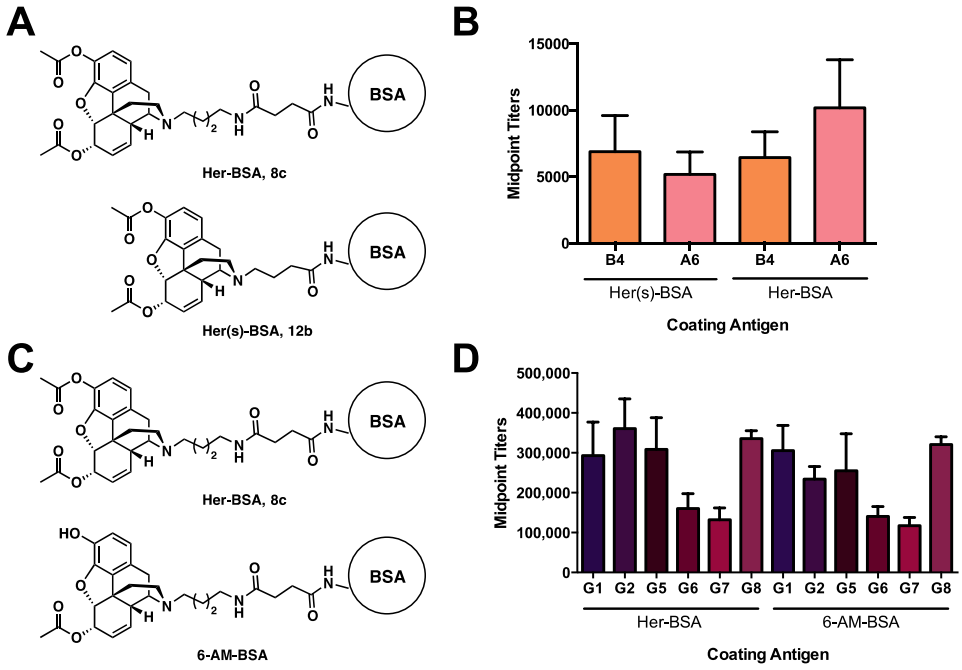


Figure S20. All behavioral and antibody data for Series A, B, and C

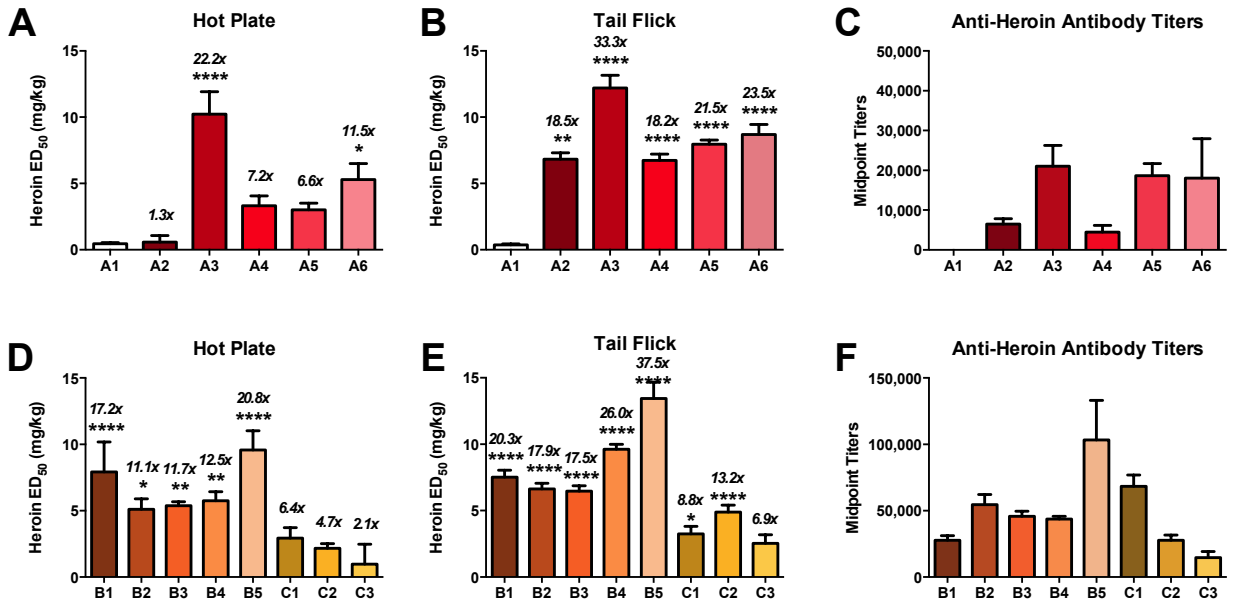
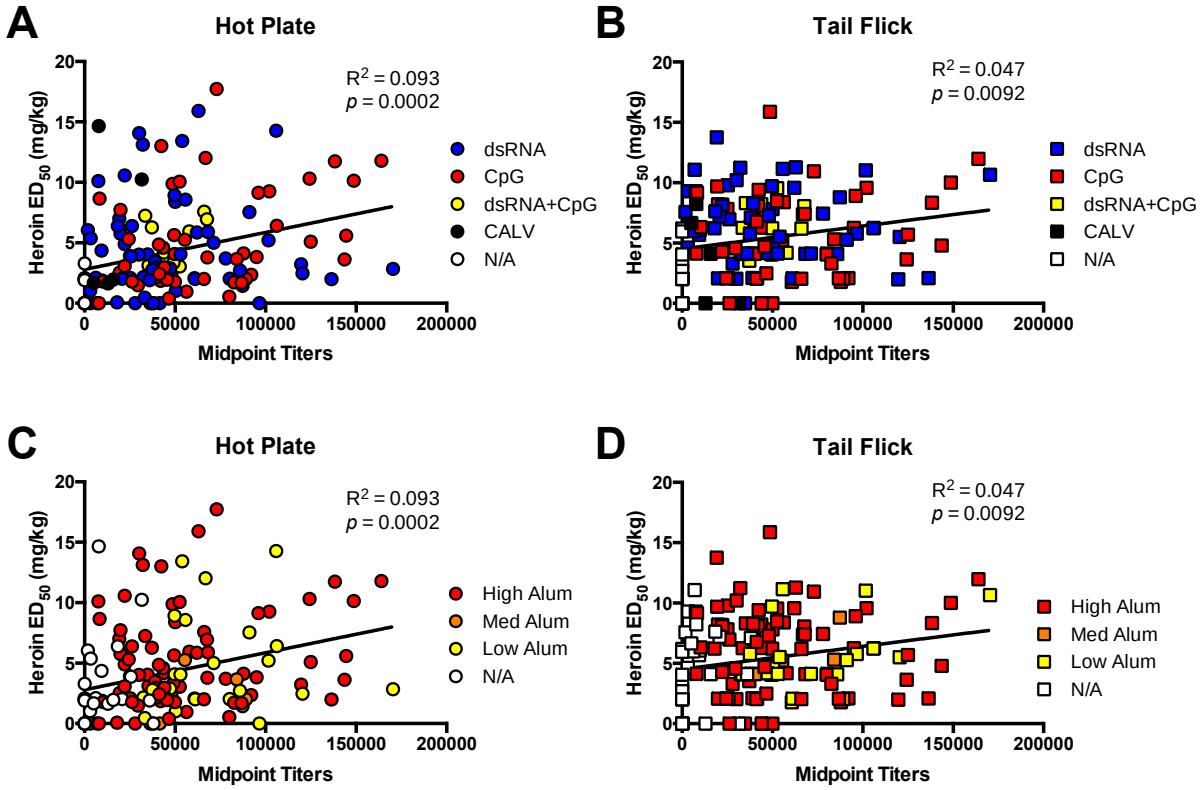


Figure S21. Pearson's Correlation coefficient for titers vs behavioral data



Although some controls contained specific amounts of adjuvants, in these plots controls were arbitrarily marked with N/A.

Results from chemical stability studies under various vaccine storage conditions

Table S6. Sample preparation and concentration of each individual component

No.	Sample Description	CpG (ug/uL)	dsRNA (ug/uL)	TT (ug/uL)	Alum (ug/uL)	Trehalose (w/v)	H_2O	PBS	Total Volume
1	*CpG in H_2O*	0.333							600 uL
2	*CpG in PBS*	0.333							600 uL
3	*CpG + TT*	0.333		0.333					600 uL
4	*CpG + TT + Alum + Trehalose*	0.333		0.333	6.667	25%			600 uL
5	*dsRNA in H_2O*		0.333						600 uL
6	*dsRNA in PBS*		0.333						600 uL
7	*dsRNA + TT*		0.333	0.333					600 uL
8	*dsRNA + TT+ Alum +Trehalose*		0.333	0.333	6.667	25%			600 uL

Greyed out boxes indicate the absence of that vaccine component. Each vaccine was prepared to a volume of 600 µL. Aliquots of 100 µL of sample were taken out for each additional room temperature and lyophilization stability experiments. The remaining liquid was stored as a liquid at 4 ˚C. Lyophilized samples were frozen in liquid nitrogen and then lyophilized overnight. Lyophilized samples were stored as a dry solid at room temperature over 7 days. The solids were resuspended in water and vortexed for twenty minutes prior to analysis. Results from the stability studies are displayed in Figures S22-28.

Figure S22. Monitoring TT stability as a liquid at 4 ˚C.

A) SDS-PAGE using NuPAGE 12% Bistris (BT) with MOPS (1x) as the running buffer with Coomassie staining. Lanes correspond to 1) SeeBlue® Plus 2 prestained standard ladder; 2) TT stock undialyzed; 3) TT dialyzed in PBS; 4) TT + CpG in PBS, t = 7d, liquid at 4 ˚C after dialysis; 5) TT + CpG + Alum + trehalose in PBS, t = 7d, liquid at 4 ˚C after dialysis; 6) TT + CpG in PBS, t = 7d, liquid at 4 ˚C; 7) TT + CpG + Alum + trehalose in PBS, t = 7d, liquid at 4 ˚C. It should be noted that samples with alum exhibited natural separation from alum over time; protein in the supernatant was collected for gel analysis.
B) Qualitative color analysis of the precipitation generated in the CpG + TT + Alum + Trehalose sample (middle) compared to Alum alone (right), may explain the decrease in protein signal seen in lanes 4 and 6 in Figure S22A. Protein may have coprecipitated out with the alum based on the brownish hue in the precipitate. Initial gel analysis in the presence of alum precipitate obscured any signal in an SDS gel indicating alum may interfere with protein migration during electrophoresis.

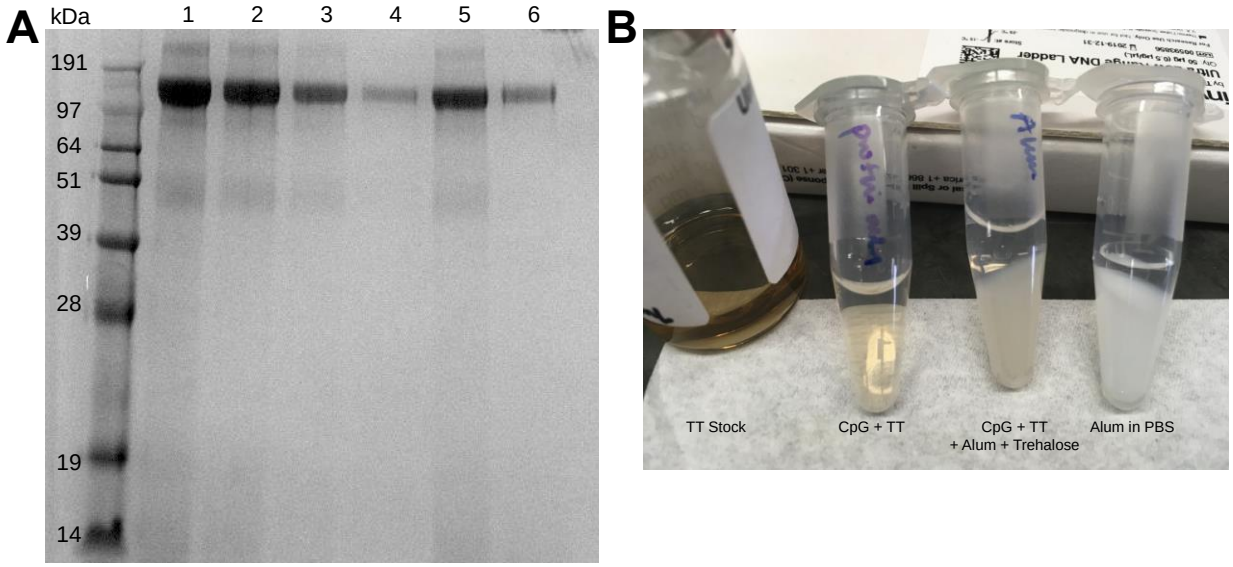


Figure S23. Monitoring TT stability as a liquid and lyophilized with dsRNA and CpG

A) SDS-PAGE using NuPAGE 12% Bistris (BT) with MOPS (1x) as the running buffer with Coomassie staining. Lanes correspond to 1) SeeBlue® Plus 2 prestained standard ladder; 2) TT + dsRNA in PBS, t = 7d, liquid at 4 ˚C; 3) TT + dsRNA + Alum + trehalose in PBS, t = 7d, liquid at 4 ˚C; 4) TT + dsRNA in PBS, t = 7d, lyophilized and resuspended in water; 5) TT + dsRNA + Alum + trehalose in PBS, t = 7d, lyophilized and resuspended in water; 6) TT + CpG in PBS t=7d, liquid at RT; 7) TT + CpG + Alum + trehalose in PBS, t= 7d, liquid at RT; 8) TT + CpG in PBS, t=7d, lyophilized and resuspended in water; 9) TT + CpG + Alum + trehalose in PBS, t= 7d, lyophilized and resuspended in water.

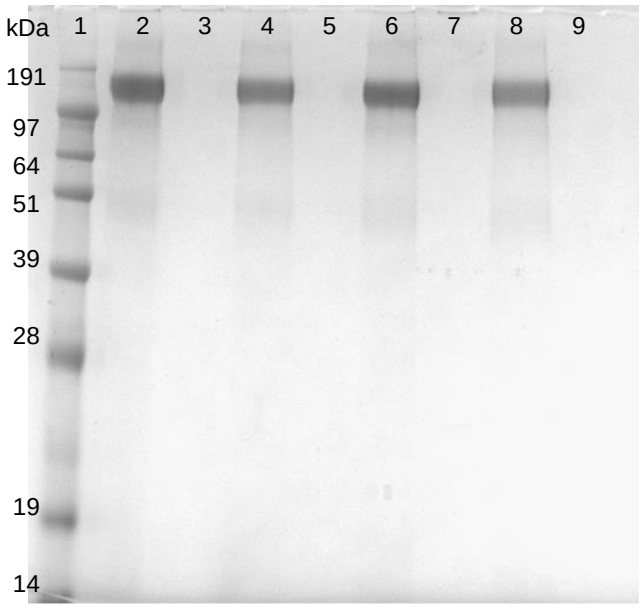


Figure S24. Monitoring stability of CpG as a liquid or lyophilized

A) Gel 1: Lanes correspond to 1) GeneRuler Ultra Low Range DNA ladder, double stranded DNA standards; 2) Single stranded MuLink VH3 22-mer DNA primer; 3) CpG in water; 4) CpG in PBS, t = 7d, liquid at 4 ˚C; 5) CpG + TT in PBS, t = 7d, liquid at 4 ˚C; 6) CpG + TT + alum + trehalose in PBS, t = 7d, liquid at 4˚C; 7) CpG in water, t = 7d, liquid at RT; 8) CpG in PBS, t = 7d, liquid at RT; 9) CpG + TT in PBS, t = 7d, liquid at RT; 10) CpG + TT + alum + trehalose in PBS, t = 7d, liquid at RT;
B) Gel 2: Lanes correspond to 1) GeneRuler Ultra Low Range DNA ladder, double-stranded DNA standards; 2) Single-stranded MuLink VH3 22-mer DNA primer; 3) Single-stranded USF1ALL 16-mer DNA primer; 4) CpG in water, t = 7d, lyophilized and resuspended in water; 5) CpG in PBS, t = 7d, lyophilized and resuspended in water; 6) CpG + TT in PBS, t = 7d, lyophilized and resuspended in water; 7) CpG + TT + alum + trehalose in PBS, t = 7d, lyophilized and resuspended in water.

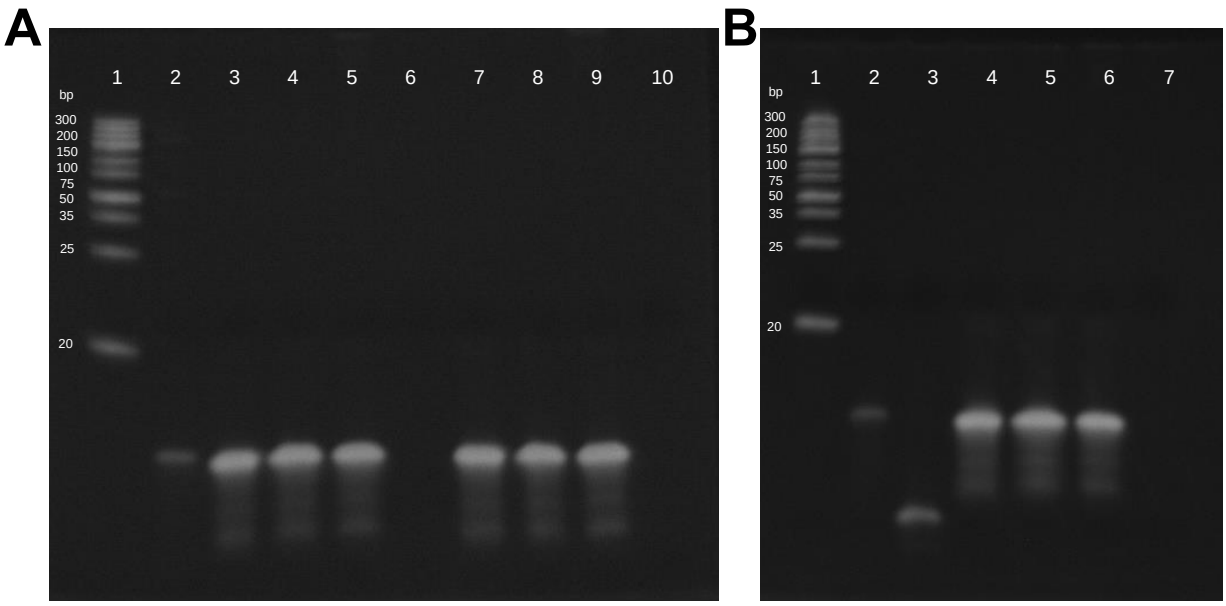
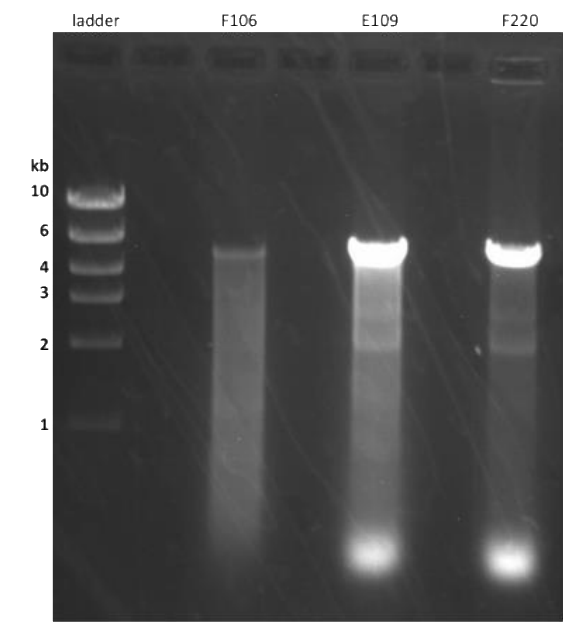


Figure S25. Long-term stability analysis of dsRNA at 4 ˚C, liquid

A) Agarose gel of dsRNA;
B) Data table with UV-Vis measurements over time. Samples were diluted 1:500 in 10 mM Tris Buffer (pH 7.5)

A



B

Date measured	Storage time	Absorbance (260nm)
6-Jul-16	0-1 days	0.6559
18-Nov-16	134 days	0.6735
5-Dec-16	152 days	0.6657

Figure S26. Monitoring dsRNA stability after lyophilization or as a liquid at 4 ˚C

Lanes correspond to M) DNA HyperLadder 1kb; 1) dsRNA in water; 2) dsRNA in PBS, t = 7d; 3) dsRNA + TT in PBS, t = 7d; 4) dsRNA + TT + alum + trehalose in PBS, t = 7d; 5) dsRNA in water, t = 7d, lyophilized and resuspended; 6) dsRNA in PBS, t = 7d, lyophilized and resuspended; 7) dsRNA + TT in PBS, t = 7d, lyophilized and resuspended; 8) dsRNA + TT + alum + trehalose in PBS, t = 7d, lyophilized and resuspended; 9) no sample run; 10) test run with CpG in water. Wells were filled with 8 µL of sample + 2 uL ethidium bromide (20 µg/mL).

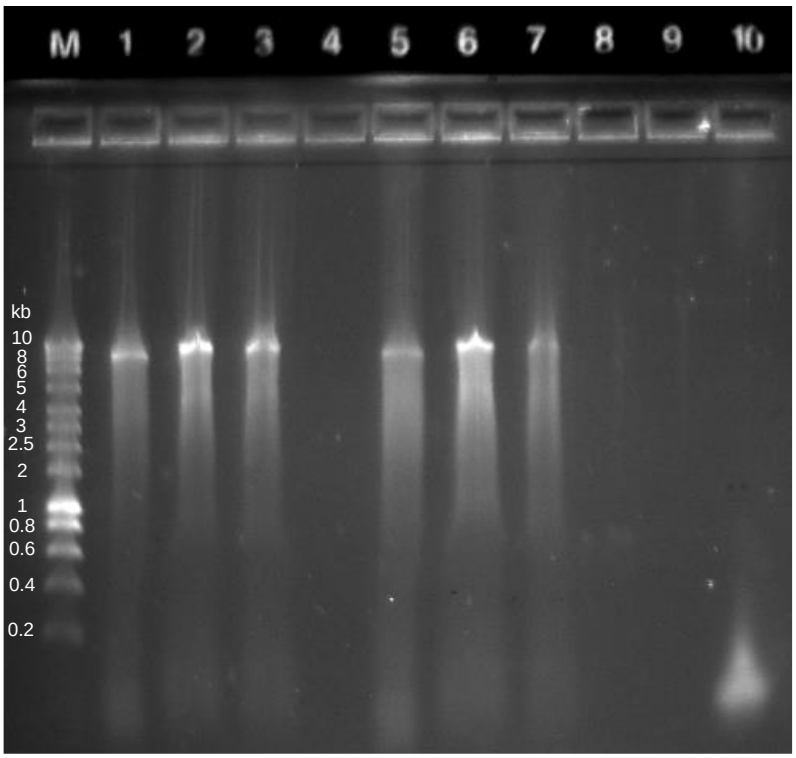
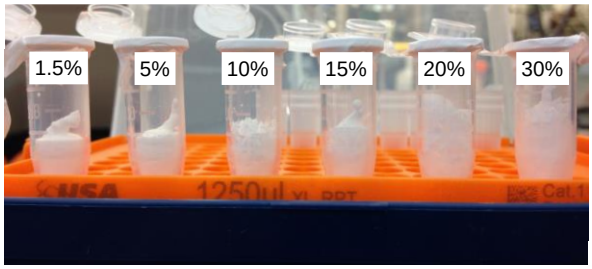


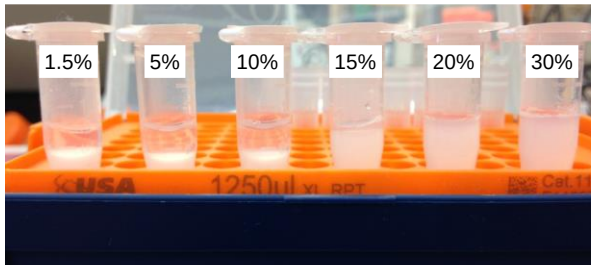
Figure S27. Qualitative analysis of alum in solution after lyophilization and resuspension at different alum and trehalose concentrations

Samples contain 250 ug of BSA, and variable amounts of alum and trehalose in PBS buffer. Samples were frozen in liquid N_2, lyophilized overnight, resuspended in H_2O and vortexed for twenty minutes.

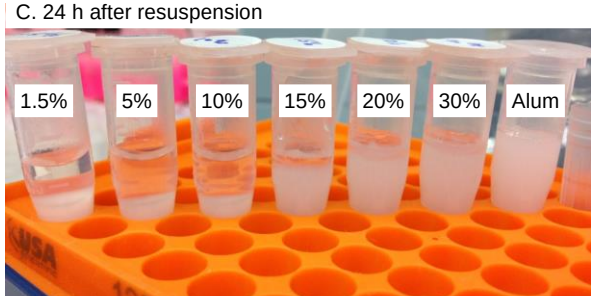
A. 1 mg Alum after lyophilization



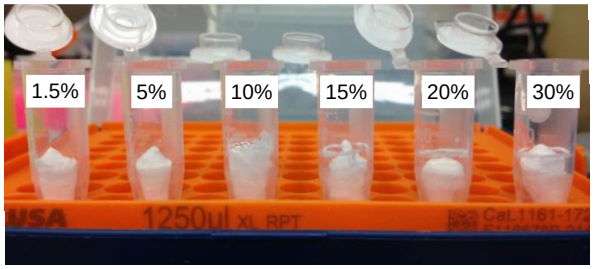
B. 2 h after resuspension



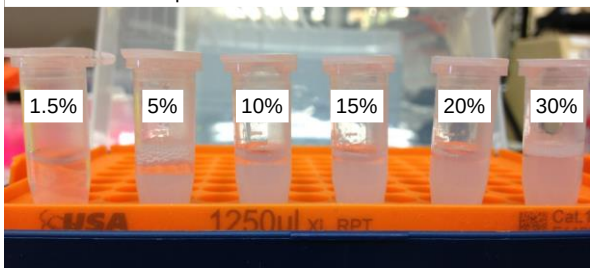
C. 24 h after resuspension



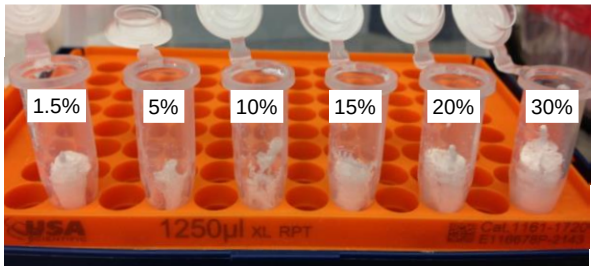
A. 0.5 mg Alum after lyophilization



B. 2 h after resuspension



A. 0.2 mg Alum after lyophilization



B. 2 h after resuspension

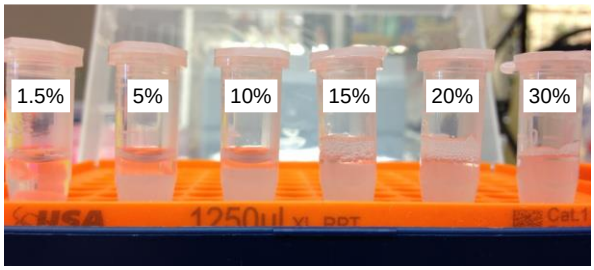
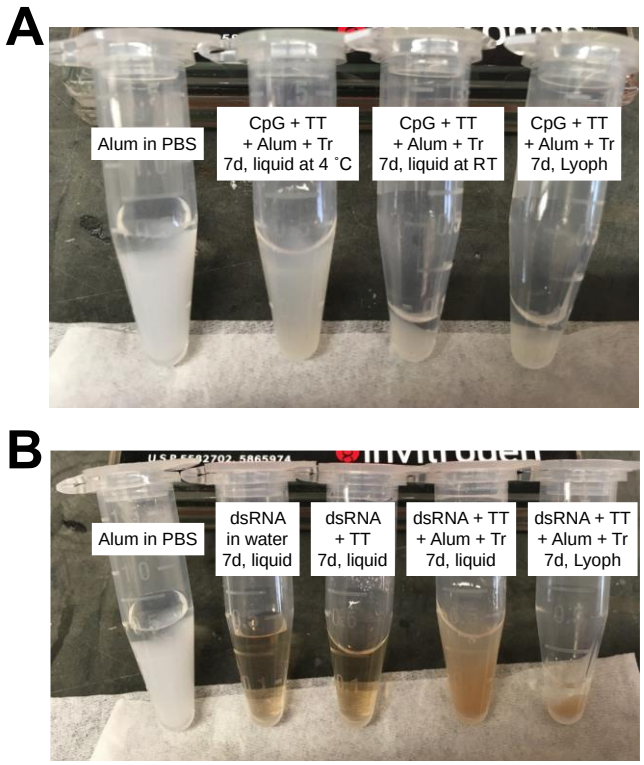


Figure S28. Qualitative analysis of alum in solution after storage as a liquid or lyophilization and resuspension with A) CpG series or B) dsRNA series

A very common phenomenon observed in gels run in the presence of alum, suggests that adjuvants and carrier protein are bound to alum and coprecipitate with it. The lack or decrease of TT, dsRNA and CpG samples containing alum indicate alum binds these components, and therefore components are not present in the supernatant. This is further corroborated with the color observation in the alum (originally white) precipitate for TT (brown) and dsRNA (reddish brown). CpG is colorless and therefore cannot be qualitatively observed. Tr in the figure stands for trehalose.



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