

Supplementary Materials and Methods

For

Particle formation is the principal physicochemical determinant for enhancing vaccine immunogenicity by polymer-bound TLR agonists

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SUPPLEMENTARY MATERIALS AND METHODS

Chemicals

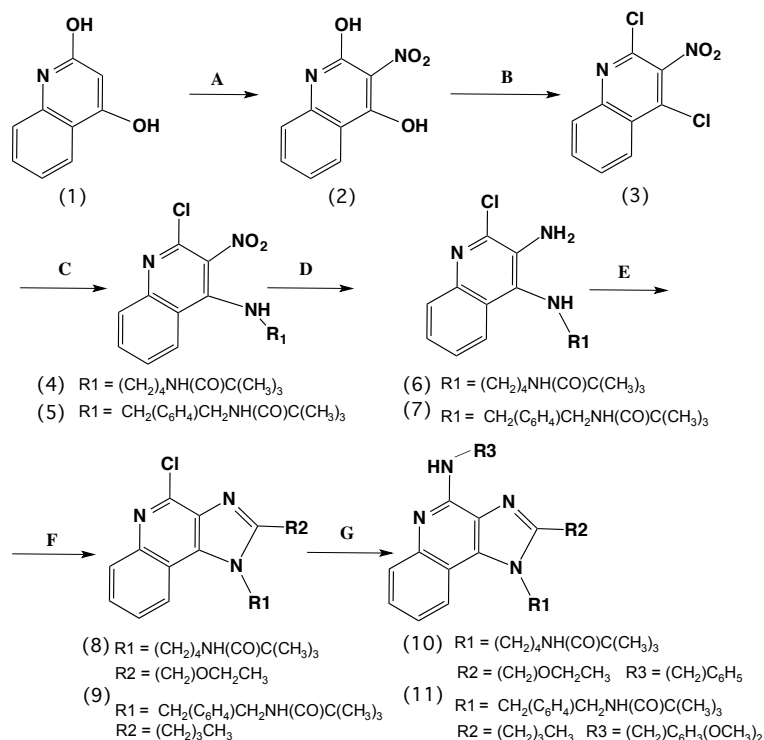
All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) as reagent grade or higher purity, unless stated otherwise. Ethoxyacetic acid was obtained from Alfa Aesar (Ward Hill, MA). Boc-15-amino-4,7,10,13-tetraoxapentadecanoic acid (PEG4) was purchased from EMD Millipore (Darmstadt, Germany). N-Boc-1,4-diaminobutane¹ and 2-Chloro-4,6-dimethoxy-1,3,5-triazine (CDMT)² were prepared as previously described. Green fluorescent reactive dyes Alexa Fluor® 488 carboxylic acid tetrafluorophenyl ester, Alexa Fluor® 488 cadaverine were purchased from Life Technologies (Carlsbad, CA) and Carboxyrhodamine 110 PEG3 azide was purchased from Alfa Aesar. Amine reactive infrared fluorescent reactive dye IRDye® 800CW NHS Ester was purchased from LI-COR (Lincoln, Nebraska). Nucleophilic Infrared fluorescent reactive dye, CruzFluor sm™ 8 amine, was purchased from Santa Cruz Biotechnology (Dallas, Texas). Dibenzocyclooctyne (DBCO) modified PEG spacer (DBCO-PEG4-Amine) was purchased from Click Chemistry Tools (Scottsdale, Arizona). Peptides were produced by solid phase peptide synthesis and were obtained from American Peptide Company (Vista, California).

Instrumentation for synthesis, purification and chemical characterization

Microwave irradiation was carried out in a CEM Discover Synthesizer with 150 watts max power. Flash column chromatography was performed on a Biotage SP4 Flash Purification system (Uppsala Sweden) using Biotage® SNAP Cartridges and SNAP Samplet Cartridges with KP-Silica 60 mm. Analytical HPLC analyses were performed on an Agilent 1200 Series instrument equipped with multi-wavelength detectors using a Zorbax Stable Bond C-18 column (4.6 x 50 mm, 3.5 mm) with a flow rate of 0.5 mL/min or 1.0 mL/min. Solvent A was 0.05% trifluoroacetic acid (TFA) in water (H₂O), solvent B was 0.05% TFA in acetonitrile (ACN), and a linear gradient of 5% B to 95% B over 10 minutes was used. ESI or APCI mass spectrometry (MS) were performed on an LC/MSD TrapXCI Agilent Technologies instrument or on a 6130 Quadrupole LC/MS Agilent Technologies instrument equipped with a diode array detector. ¹H NMR spectra were recorded on a Varian spectrometer operating at 400 MHz. Ultraviolet-Visible (UV-Vis) light spectroscopy was performed on a Lambda25 UV/Vis system from PerkinElmer (Waltham, MA) and fluorescence spectroscopy was carried out on a PerkinElmer brand Fluorescence Spectrometer, model LS 55.

Synthesis of polymer reactive small molecule TLR-7/8a

Synthesis of imidazoquinoline-based TLR-7/8a was based on previous reports³⁻⁷ and is described in more detail below.



Synthesis of imidazoquinoline-based TLR-7/8a: (A) HNO₃, heat; (B) PhPOCl₂, heat; (C) NH₂R₁, Et₃N, heat; (D) 10% Pt/c, H₂ (g) 55 PSI, Ethyl acetate; (E) R₂COOH, CDMT, NMM, EtOAc; (F) CaO, heat, MeOH; (G) NH₂R₃, Et₃N, MeOH, heat, 150 PSI; (H) H₂SO₄, heat

(1-4) The synthesis of *tert*-butyl (4-((2-chloro-3-nitroquinolin-4-yl)amino)butyl)carbamate was carried out as previously described³. ¹H NMR (400 MHz, CDCl₃) δ 8.11 (d, J = 7.6 Hz, 1H), 7.91 (dd, J = 8.4, 1 Hz, 1H), 7.74 (m, 1H) 7.52 (m, 1H), 6.40 (br s, 1H), 4.66 (br s, 1H), 3.48 (m, 2H), 3.20 (m, 2H), 1.80 (m, 2H), 1.65 (m, 2H), 1.47 (br s, 9H). MS (APCI) calculated for C₈H₂₃ClN₄O₄, *m/z*, 394.1, found 394.9 (M+H)⁺.

(5) The synthesis of *tert*-butyl (4-(((2-chloro-3-nitroquinolin-4-yl)amino)methyl)benzyl)carbamate was carried out as previously described⁶. ¹H NMR (400 MHz, DMSO-d₆) δ 8.51 (d, J = 8.5 Hz, 1H), 8.46 (t, J = 6.4 Hz, 1H), 7.88 – 7.78 (m, 2H), 7.65 (dd, J = 8.4, 5.5 Hz, 1H), 7.33 (t, J = 6.2 Hz, 1H), 7.17 (q, J = 8.2 Hz, 4H), 4.39 (d, J = 6.2 Hz, 2H), 4.07 (d, J = 6.2 Hz, 2H), 1.36 (s, 9H). MS (APCI) calculated for C₂₂H₂₃ClN₄O₄, *m/z*, 442.1, found 464.9 (M+Na)⁺.

(6) *tert*-butyl (4-(((3-amino-2-chloroquinolin-4-yl)amino)butyl)carbamate. A 23 g solution of (5) and 230 mg of Na₂SO₄ in 200 mL of ethyl acetate was bubbled with Argon for 5 minutes to remove oxygen. To this solution, 230 mg of 10% Pt/c was added and the mixture was flushed with Argon for an additional 5 minutes and then pressurized with H₂(g) 55 mm Hg. The reaction mixture was agitated with a mechanical shaker. The reaction was considered complete (~ 3 hours) once the pressure remained constant at a constant volume of H₂(g). The reaction mixture was filtered through celite and evaporated to dryness to obtain yellow oil. Trituration with 1:1 hexanes / ether yielded white crystals that were collected by filtration. Drying overnight under vacuum yielded

20.12 g (94.7 % yield) of spectroscopically pure (>95% at 254 nm) white crystals. ¹H NMR (400 MHz, DMSO-d₆) δ 8.03 – 7.95 (m, 1H), 7.70 – 7.61 (m, 1H), 7.44 – 7.34 (m, 2H), 6.73 (s, 1H), 5.14 (t, J = 6.7 Hz, 1H), 5.00 (s, 2H), 3.19 (q, J = 7.0 Hz, 2H), 2.87 (q, J = 6.5 Hz, 2H), 1.55 – 1.34 (m, 4H), 1.33 (s, 9H). MS (APCI) calculated for C₁₈H₂₅ClN₄O₂, *m/z*, 364.2, found 365.2 (M+H)⁺.

(7) *tert*-butyl 4-(((3-amino-2-chloroquinolin-4-yl)amino)methyl)benzylcarbamate. The synthetic protocol is the same as for **(6)**, except 5 g of **(5)** was used as the starting material. Product was spectroscopically pure (>95% at 254 nm) following passage through celite. Solvent was removed under vacuum and yielded 4.57 g (93% yield) of white crystals. ¹H NMR (400 MHz, DMSO-d₆) δ 8.00 – 7.93 (m, 1H), 7.63 (dd, J = 8.0, 1.7 Hz, 1H), 7.35 (tt, J = 6.9, 5.2 Hz, 2H), 7.31 – 7.25 (m, 3H), 7.11 (d, J = 7.9 Hz, 2H), 5.79 (t, J = 7.1 Hz, 1H), 5.04 (s, 2H), 4.40 (d, J = 7.2 Hz, 2H), 4.04 (d, J = 6.2 Hz, 2H), 1.36 (s, 9H). MS (APCI) calculated for C₂₂H₂₅ClN₄O₂, *m/z*, 412.2, found 413.2 (M+H)⁺.

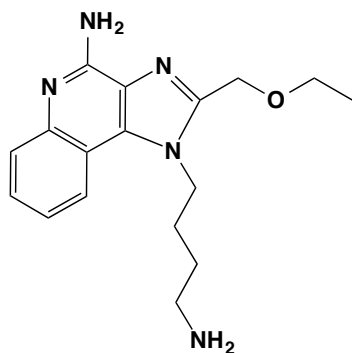
(8) *Tert*-butyl (4-(4-chloro-2-(ethoxymethyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)butyl)carbamate. To 2.5 mL of 2-ethoxyacetic acid (0.026 mol, 1.2 eq) in 150 mL of ethyl acetate were added 4.6 g (0.026 mol, 1.2 eq) CDMT, followed by dropwise addition of 6.0 mL (0.055 mol, 2.5 eq) of *N*-methylmorpholine (NMM). After 5 minutes, 8 g (0.022 mol, 1.0 eq) of **(6)** was added and the reaction was refluxed using an oil bath. A white precipitate was formed after several minutes corresponding to the NMM.Cl salt. After 16 hours, the reaction mixture was filtered and washed 3x150 mL with 1M HCl. The organic phase was dried with Na₂SO₄, filtered and evaporated to dryness. The resulting crude product was added to 20 mL of methanol with 800 mg (10 % wt/wt) CaO and then microwaved at 100°C for 3 hours. The CaO was removed by filtration and the resulting solution was evaporated to dryness to obtain an oily product that was purified by flash chromatography using a 0-6% methanol in DCM gradient, yielding 9.44 g of clear oil. Recrystallization from 5:1 Hexane / Ethyl acetate yielded 5.59 g (58.9 % yield) of spectroscopically pure (>95% at 254 nm) white crystals. ¹H NMR (400 MHz, DMSO-d₆) δ 8.37 – 8.28 (m, 1H), 8.11 – 8.04 (m, 1H), 7.81 – 7.70 (m, 2H), 6.83 – 6.75 (m, 1H), 4.84 (s, 2H), 4.65 (t, J = 7.9 Hz, 2H), 3.62 – 3.52 (m, 2H), 2.96 (q, J = 6.4 Hz, 2H), 1.85 (t, J = 7.9 Hz, 2H), 1.56 (t, J = 7.7 Hz, 2H), 1.30 (s, 9H), 1.20 – 1.12 (m, 3H). MS (APCI) calculated for C₂₂H₂₉ClN₄O₃, *m/z* 432.2, found 433.2 (M+H)⁺.

(9) *Tert*-butyl 4-((2-butyl-4-chloro-1*H*-imidazo[4,5-*c*]quinolin-1-yl)methyl)benzylcarbamate. The synthetic protocol is the same as for **(8)**, except 2 g of **(7)** was used as the starting material and pentanoic acid was used in place of 2-ethoxyacetic acid. Flash purification was not required, but the product was recrystallized from methanol to obtain 1.4 g (58% yield) of spectroscopically pure (>95% at 254 nm) yellow crystals. NMR (400 MHz, DMSO-d₆) δ 8.08 (d, J = 8.3 Hz, 1H), 8.02 (d, J = 8.4 Hz, 1H), 7.63 (dd, J = 8.2, 6.8 Hz, 1H), 7.50 (t, J = 7.7 Hz, 1H), 7.30 (t, J = 8 Hz, 1H), 7.15 (d, J = 7.9 Hz, 2H), 7.01 – 6.94 (m, 2H), 5.94 (s, 2H), 4.04 (d, J = 6.2 Hz, 2H), 2.96 (t, J = 7.7 Hz, 2H), 1.73 (q, J = 7.6 Hz, 2H), 1.38 (q, J = 7.4 Hz, 2H), 1.33 (s, 9H), 0.86 (t, J = 7.3 Hz, 3H). MS (APCI) calculated for C₂₇H₃₁ClN₄O₂, *m/z* 478.2, found 479.2 (M+H)⁺.

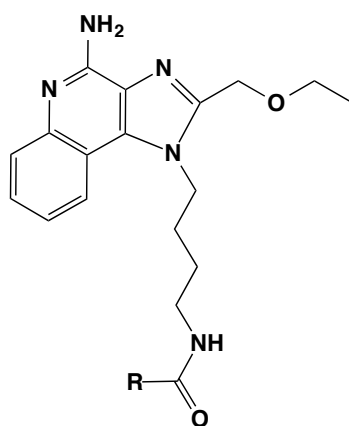
(10) *Tert*-butyl (4-(4-(benzylamino)-2-(ethoxymethyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)butyl)carbamate. 6.5 g of **(8)** (0.015 mol, 1 eq) was added to 16 mL of benzylamine (0.15 mol, 10 eq) and reacted for 6 hours at 110°C in a microwave apparatus (CEM Discover Synthesizer). After completion, the reaction mixture was cooled to room temperature and then added to 100 mL of DCM and washed 4x100 mL with 1 M HCl. The resulting yellow oil was recrystallized from 4:1 hexane / ethyl acetate to obtain 7.3g

(97.1%) of spectroscopically pure (>95% at 254 nm) white crystals. ^1H NMR (400 MHz, DMSO- d_6) δ 7.99 (d, J = 8.0 Hz, 1H), 7.66 – 7.55 (m, 2H), 7.41 (d, J = 7.3 Hz, 3H), 7.25 (td, J = 7.5, 5.6 Hz, 3H), 7.20 – 7.12 (m, 1H), 6.80 (t, J = 5.7 Hz, 1H), 4.79 – 4.72 (m, 4H), 4.53 (t, J = 7.8 Hz, 2H), 3.54 (q, J = 7.0 Hz, 2H), 2.95 (q, J = 6.5 Hz, 2H), 1.85 (m, 2H), 1.54 (t, J = 7.7 Hz, 2H), 1.31 (s, 9H), 1.15 (t, J = 7.0 Hz, 3H). MS (APCI) calculated for $\text{C}_{29}\text{H}_{37}\text{N}_5\text{O}_3$ m/z 503.3, found 504.3 (M+H) $^+$.

(11) *Tert*-butyl 4-((2-butyl-4-((2,4-dimethoxybenzyl)amino)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)methyl)benzylcarbamate. The synthetic protocol was the same as for **(10)**, except 300 mg of **(9)** was used as the starting material and 2,4-dimethoxy benzylamine was used in place of benzylamine. Product was recrystallized from 3:1 Hexane / Ethyl acetate to obtain 272 mg (78% yield) of a spectroscopically pure product (>95% at 254 nm). ^1H NMR (400 MHz, DMSO- d_6) δ 9.64 (s, 1H), 8.16 (s, 1H), 7.91 (s, 1H), 7.60 (t, J = 7.8 Hz, 1H), 7.34 (q, J = 7.1, 6.1 Hz, 2H), 7.18 (d, J = 8.0 Hz, 3H), 7.02 (d, J = 8.0 Hz, 2H), 6.60 (d, J = 2.3 Hz, 1H), 6.49 (dd, J = 8.3, 2.4 Hz, 1H), 5.91 (s, 2H), 4.89 (s, 2H), 4.05 (d, J = 6.2 Hz, 2H), 3.77 (s, 3H), 3.74 (s, 3H), 2.92 (t, J = 7.7 Hz, 2H), 1.75 – 1.66 (m, 2H), 1.37-1.19 (m, 11H), 0.84 (t, J = 7.3 Hz, 3H). MS (APCI) calculated for $\text{C}_{36}\text{H}_{43}\text{N}_5\text{O}_4$ m/z 609.3, found 610.3 (M+H) $^+$.

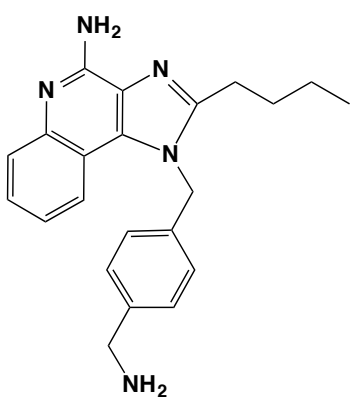


(12)
SM 7/8a

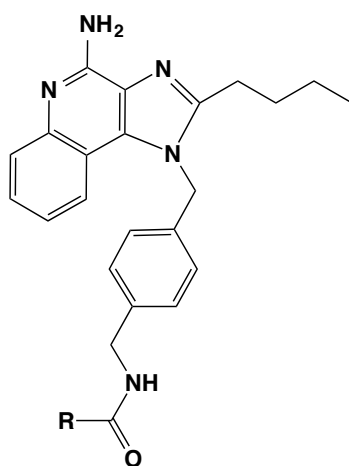


(13) R = $\text{CH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_4\text{NH}_2$ SM 7/8a-PEG

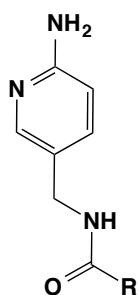
(14) R = $(\text{CH}_2)_{12}\text{NH}_2$ SM 7/8a-Alkane



(15)
SM 20x7/8a



(16) R = $\text{CH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_4\text{NH}_2$ SM 20x7/8a-PEG



(17) R4 = $\text{CH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_4\text{NH}_2$ AP-PEG

(18) R4 = $(\text{CH}_2)_5\text{N}_3$ AP-azide

Methods Figure 1.2: Nucleophilic small molecule Toll-like receptor-7/8 agonists (TLR-7/8a) and nucleophilic aromatic heterocyclic base control ligands based on aminopyridine (AP).

(12) SM 7/8a, 1-(4-aminobutyl)-2-(ethoxymethyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine. Simultaneous debenzoylation and Boc removal was achieved by adding 36 mL of 98% H₂SO₄ (36.8 N) to 7.2 g (0.014 mol) of **(10)**. The solution turned from faint yellow to cloudy orange over several minutes. Reaction progress was monitored by HPLC. After 3 hours, the reaction mixture was slowly added to 200 mL of DI H₂O and stirred at room temperature for 30 minutes. This mixture was filtered through celite and the resulting clear aqueous solution was adjusted to pH 10 using 10 M NaOH. The aqueous layer was extracted with 6x100 mL DCM. The organic layer was dried with Na₂SO₄ and then evaporated to dryness, yielding 4.03 g (89.6% yield) of a spectroscopically pure (>95% at 254 nm) white powder. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.02 (dd, *J* = 16.6, 8.2 Hz, 1H), 7.63 – 7.56 (m, 1H), 7.47 – 7.38 (m, 1H), 7.30 – 7.21 (m, 1H), 6.55 (s, 2H), 4.76 (s, 2H), 4.54 (q, *J* = 6.3, 4.4 Hz, 2H), 3.54 (q, *J* = 7.0 Hz, 2H), 2.58 (t, *J* = 6.9 Hz, 2H), 1.93-1.81 (m, 2H), 1.52 (m, 2H), 1.15 (t, *J* = 7.0 Hz, 3H). MS (APCI) calculated for C₁₇H₂₃N₅O *m/z* 313.2, found 314.2 (M+H)⁺.

(13) SM 7/8a-PEG, 1-amino-*N*-(4-(4-amino-2-(ethoxymethyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)butyl)-3,6,9,12-tetraoxapentadecan-15-amide. To 20 mL of ethyl acetate was added 500 mg (1.6 mmol, 1 eq) of **(12)**, 281 mg (1.6 mmol, 1 eq) of CDMT and 643 mg (1.8 mmol, 1.1 eq) of Boc-15-amino-4,7,10,13-tetraoxapentadecanoic acid (PEG4), followed by the dropwise addition of 441 μL (4.0 mmol, 2.5 eq) of NMM, while stirring vigorously. After 16 hours at room temperature, the reaction mixture was filtered and then washed 3x50 mL with 1 M HCl. The organic phase was dried with Na₂SO₄ and then evaporated to dryness. The resulting solid purified by flash chromatography using a 2-15% methanol / dichloromethane gradient. The resulting clear oil was added to 5 mL of 30% TFA/DCM and reacted for 1 hour at room temperature. The TFA/DCM was removed by evaporation and the resulting residue was dissolved in 1M HCl and filtered. The filtrate was made alkaline by the addition of 10 M NaOH, followed by extraction with 3x50 mL of DCM. The organic phase was dried with Na₂SO₄ and evaporated to dryness to obtain 455 mg (51% yield) of spectroscopically pure (>95% at 254 nm) clear oil. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.98 (d, *J* = 8 Hz 1H), (7.83 (t, *J* = 5.7 Hz, 1H), 7.60 (dd, *J* = 8.4, 1.3 Hz, 1H), 7.43 (dd, *J* = 8.4, 6.9 Hz, 1H), 7.25 (t, *J* = 7.7 Hz, 1H), 6.56 (s, 2H), 4.75 (s, 2H), 4.59 – 4.50 (m, 2H), 4.07 (d, *J* = 5.8 Hz, 4H), 3.59–3.39 (m, 16 H) 3.09 (q, *J* = 6.5 Hz, 2H), 2.63 (t, *J* = 5.9 Hz, 2H), 2.24 (t, *J* = 6.5 Hz, 2H), 1.83 (m, 2H), 1.56 (t, *J* = 7.5 Hz, 2H), 1.15 (t, *J* = 7.0 Hz, 3H). MS (APCI) calculated for C₂₈H₄₄N₆O₆ *m/z* 560.3, found 561.3 (M+H)⁺.

(14) SM 7/8a-Alkane, 12-amino-*N*-(4-(4-amino-2-(ethoxymethyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)butyl)dodecanamide. To 20 mL of ethyl acetate was added 200 mg (0.64 mmol, 1 eq) of **(12)**, 112 mg (0.64 mmol, 1 eq) of CDMT and 222 mg (0.70 mmol, 1.1 eq) of *N*-boc-aminododecanoic acid followed by the dropwise addition of 176 μL (1.6 mmol, 2.5 eq) of NMM while stirring vigorously. After 16 hours at room temperature, the reaction mixture was filtered and washed 3x50 mL with 1 M HCl. The organic phase was dried with Na₂SO₄ and then evaporated to dryness. The resulting solid was suspended in 5 mL of 30% TFA/DCM and reacted for 1 hour at room temperature. The TFA/DCM was removed by evaporation and the resulting residue was dissolved in 1M HCl and filtered. The filtrate was made alkaline by the addition of 10 M NaOH, followed by extraction with 3x50 mL of DCM. The organic phase was then dried with Na₂SO₄ and evaporated to dryness to obtain 279 mg (85.4% yield) of spectroscopically pure (>95% at 254 nm) white solid. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.98 (d, *J* = 8.1 Hz, 1H), 7.74 (t, *J* = 5.7 Hz, 1H), 7.60 (d, 8 Hz, 1H), 7.42 (t, *J* = 7.6 Hz, 1H), 7.24 (t, *J* = 7.5 Hz, 1H), 6.56 (s, 2H), 4.75 (s, 2H), 4.53 (t, *J* = 7.9 Hz, 2H), 3.54 (q, *J* = 7.0 Hz, 2H), 3.07 (q, *J* = 6.4 Hz, 2H),

2.60 (t, J = 7.1 Hz, 2H), 1.97 (t, J = 7.4 Hz, 2H), 1.87–1.78 (m, 2H), 1.55 (t, J = 7.6 Hz, 2H), 1.43–1.34 (m, 5H), 1.24–1.10 (m, 18H). MS (APCI) calculated for C₂₉H₄₆N₆O₂ *m/z* 510.4, found 511.4 (M+H)⁺.

(15) SM 20x7/8a, 1-(4-(aminomethyl)benzyl)-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine. Deprotection of **(11)** required milder conditions as compared with **(12)** so as to avoid removal of the xylene diamine linker. Simultaneous removal of the 2,4-dimethoxybenzyl and Boc groups was achieved by adding 300 mg of **(11)** to a 30 mL solution of 40% TFA/DCM that was stirred at room temperature for 30 hours. Reaction progress was monitored by HPLC. After completion, the reaction mixture was evaporated to dryness and the resulting yellow solid was suspended in 200 mL of 1 M HCl. Insoluble material was removed by filtration and the resulting clear aqueous solution was adjusted to pH 10 using 10 M NaOH. The aqueous layer was extracted 6x100 mL using DCM as the organic phase. The organic layer was dried with Na₂SO₄ and evaporated to dryness, yielding 172 mg (89.6% yield) of a spectroscopically pure (>95% at 254 nm) white powder. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.77 (dd, J = 8.4, 1.4 Hz, 1H), 7.55 (dd, J = 8.4, 1.2 Hz, 1H), 7.35 – 7.28 (m, 1H), 7.25 (d, J = 7.9 Hz, 2H), 7.06 – 6.98 (m, 1H), 6.94 (d, J = 7.9 Hz, 2H), 6.50 (s, 2H), 5.81 (s, 2H), 3.64 (s, 2H), 2.92–2.84 (m, 2H), 2.15 (s, 2H), 1.71 (q, J = 7.5 Hz, 2H), 1.36 (q, J = 7.4 Hz, 2H), 0.85 (t, J = 7.4 Hz, 3H). MS (APCI) calculated for C₂₂H₂₅N₅ *m/z* 359.2, found 360.3 (M+H)⁺.

(16) SM 20x7/8a-PEG, 1-(4-(aminomethyl)benzyl)-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine. The same reaction conditions and purification scheme were used as for the preparation of **(13)**, except 100 mg of **(15)** was used in place of **(12)**. 126.2 mg (96% yield) of spectroscopically pure (>95% at 254 nm) clear oil was obtained. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.31 (t, J = 6.0 Hz, 1H), 7.93 (d, J = 8.4 Hz, 1H), 7.78 (d, J = 8.3 Hz, 1H), 7.71 (s, 4H), 7.61 (t, J = 7.8 Hz, 1H), 7.35 (t, J = 7.8 Hz, 1H), 7.18 (d, J = 8.0 Hz, 2H), 7.00 (d, J = 8.0 Hz, 2H), 5.92 (s, 2H), 4.20 (d, J = 5.9 Hz, 2H), 3.62–3.44 (m, 16H), 3.00–2.90 (m, 4H), 2.33 (t, J = 6.4 Hz, 2H), 1.75–1.67 (m, 2H), 1.37 (q, J = 7.4 Hz, 2H), 0.85 (t, J = 7.3 Hz, 3H). MS (APCI) calculated for C₃₃H₄₆N₆O₅ *m/z* 606.4, found 607.3 (M+H)⁺.

(17) AP-PEG, 1-amino-*N*-((6-aminopyridin-3-yl)methyl)-3,6,9,12-tetraoxapentadecan-15-amide. The same reaction conditions and purification scheme were used as for the preparation of **(13)**, except 50 mg of *tert*-Butyl 5-(aminomethyl)-2-pyridinylcarbamate was used in place of **(12)**. 73 mg (88% yield) of spectroscopically pure (>95% at 254 nm) clear oil was obtained. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.32 (t, J = 5.9 Hz, 1H), 7.76 (d, J = 2.1 Hz, 1H), 7.66 (dd, J = 9.0, 2.2 Hz, 1H), 7.51 (s, 2H), 6.81 (d, J = 9.0 Hz, 1H), 4.10 (d, J = 5.8 Hz, 2H), 3.67 – 3.37 (m, 16H), 2.96 (s, 2H), 2.53 (p, J = 1.9 Hz, 1H), 2.43 (p, J = 1.9 Hz, 1H), 2.33 (t, J = 6.4 Hz, 2H). MS (APCI) calculated for C₁₇H₃₀N₄O₅ *m/z* 370.2, found 371.2 (M+H)⁺.

(18) AP-azide, *N*-((6-aminopyridin-3-yl)methyl)-5-azidopentanamide. The same reaction conditions and purification scheme were used as for the preparation of **(13)**, except 50 mg of *tert*-Butyl 5-(aminomethyl)-2-pyridinylcarbamate was used in place of **(12)**. 21.4 mg (39% yield) of spectroscopically pure (>95% at 254 nm) clear oil was obtained. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.25 (t, J = 5.7 Hz, 1H), 7.75 (d, J = 2.2 Hz, 1H), 7.65 – 7.57 (m, 1H), 7.22 (s, 2H), 6.75 (d, J = 8.9 Hz, 1H), 4.07 (d, J = 5.8 Hz, 2H), 3.34 (s, 2H), 3.24 (s, 2H), 2.53 (p, J = 2.0 Hz, 1H), 2.43 (p, J = 1.9 Hz, 1H), 2.11 (t, J = 7.0 Hz, 2H), 1.50 (m, 4H). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.25 (t, J = 5.7 Hz, 1H), 7.75 (d, J = 2.2

Hz, 1H), 7.65 – 7.57 (m, 1H), 7.22 (s, 2H), 6.75 (d, J = 8.9 Hz, 1H), 4.07 (d, J = 5.8 Hz, 2H), 2.43 (m, 2H), 2.11 (t, J = 7.0 Hz, 2H), 1.50 (m, 4H). MS (APCI) calculated for $C_{11}H_{16}N_6O$ m/z 248.1, found 249.1 (M+H)⁺.

Synthesis of SM TLR-7/8a dye conjugates

(19) SM 7/8a-AF488.

The AF488 dye conjugate of the small molecule TLR-7/8a was synthesized by reacting 2 mg (2.3 μ moles, 1 eq) of Alexa Fluor® 488 carboxylic acid tetrafluorophenyl ester with 0.85 mg (2.7 μ moles, 1.2 eq) of (**12**) in 300 μ L of anhydrous DMSO. The reaction was monitored by HPLC and the product, (**19**), was purified by semi-prep HPLC using a 25% to 35% ACN/H₂O gradient over 16 minutes. The reaction mixture was injected over 3 runs. Fractions containing (**19**) were consolidated, frozen and lyophilized to yield 1.6 mg (85.5% yield) of spectroscopically pure (>95% at 254 nm) product. MS (ESI) calculated for $C_{38}H_{33}N_7O_{11}S_2$ m/z 827.2, found 827.7 (M+H)⁺.

(20) SM 7/8a-IRDye800

For the IR Dye conjugate of the SM 7/8a, a PEG spacer was required to increase solubility. The same reaction conditions and purification scheme were used as for the preparation of (**19**), except 4 mg (3.4 μ moles, 1 eq) of IR Dye 800cw NHS ester was used as the dye and reacted with 2.3 mg (4.1 μ moles, 1 eq) of (**13**). 3.8 mg (71% yield) of spectroscopically pure (>95% at 254 nm) product was obtained. MS (ESI) calculated for $C_{74}H_{96}N_8O_{20}S_4$ m/z 1546, found 1547 (M+H)⁺.

Synthesis of amine-reactive HPMA-based copolymers

The N-(2-hydroxypropyl)methacrylamide (HPMA)-based statistical copolymer, p[(HPMA)-co-(Ma- ϵ -Ahx-TT)], was synthesized by free radical solution polymerization as previously described⁸. Briefly, a mixture of HPMA (9.8 wt%), 2-Methyl-N-[6-oxo-6-(2-thioxo-thiazolidin-3-yl)-hexyl]-acrylamide (Ma- ϵ -Ahx-TT) (5.2 wt%) and azobisisobutyronitrile (AIBN) (1.5 wt%) were dissolved in DMSO (83.5 wt%) and polymerized at 60°C for 6 hours under argon atmosphere. The polymer was precipitated from a 1:1 mixture of acetone and diethyl ether and then dissolved into methanol and precipitated from a 3:1 mixture of acetone and diethyl ether. The content of TT reactive groups determined by UV-Vis spectrophotometry was 14.8 mol% (ϵ_{305} = 10,300 L/mol); the weight- and number-average molecular weights determined by size exclusion chromatography (SEC) were M_w = 31,850 g/mol and M_n = 20,330 g/mol, respectively.

Synthesis of amine-reactive NIPAM-based (thermo-responsive) copolymers

The N-isopropylacrylamide (NIPAM)-based statistical copolymer p[(NIPAM)-co-(Ma-Ahx-TT)] was prepared by free radical solution polymerization as described elsewhere⁹. Briefly, a mixture of NIPAM (10.2 wt%), Ma- ϵ -Ahx-TT (4.8 wt%) and AIBN (1.5 wt%) was dissolved in DMSO (83.5 wt%) and polymerized at 60°C for 18 hours under argon atmosphere. The reaction mixture was diluted with an HCl aqueous solution (pH 2) and then extracted with dichloromethane (3x). The combined organic phases were dried and evaporated. The resulting residue was dissolved in methanol and precipitated into a 3:1 mixture of acetone and diethyl ether. The content of TT reactive groups determined by UV-Vis spectrophotometry was 10.2 mol% (ϵ_{305} = 10,300 L/mol); the weight- and number-average molecular weights determined by SEC were M_w = 26,830 g/mol and M_n = 17,650 g/mol, respectively.

Synthesis of polymer-TLR-7/8a (Poly-7/8a) conjugates

Example: To generate p[(HPMA)-co-(Ma- ϵ -Ahx-PEG4-7/8a)] with an agonist density of ~ 10 mol% TLR-7/8a, 10 mg (8.4 μ mol TT, 1 eq) of p[(HPMA)-co-(Ma- ϵ -Ahx-TT)] with ~ 14 mol% TT was added to 1 mL of anhydrous methanol. To this solution, 470 μ L (4.7 mg, 6.0 μ mol, 0.7 eq) of a 10 mg/ml solution of (**13**) (SM 7/8-PEG) in anhydrous DMSO was slowly added while stirring vigorously. After 16 hours, 1.25 mg (16.8 μ mol, 2 eq) of 1-amino-2-propanol was added to remove unreacted TT groups. After an additional 2 hours, the reaction mixture was dialyzed against methanol using Spectra/Por7 Standard Regenerated Cellulose dialysis tubing with a molecular weight cut-off (MWCO) of 25 kDa (Spectrum Labs, Rancho Dominguez, CA). The dialysis tube was suspended in 1000 mL of methanol and the dialysis buffer was changed twice each day for 3 days. The methanol solution containing Poly-7/8a was evaporated to dryness and yielded 11.4 mg of p[(HPMA)-co-(Ma- ϵ -Ahx-PEG4-7/8a)]. The content of 7/8a-PEG determined by UV-Vis spectrophotometry was 7.9 mol%7/8a ($\epsilon_{325} = 5,012$ L/mol); the weight- and number-average molecular weights determined by SEC were $M_w = 55,680$ g/mol and $M_n = 33,850$ g/mol, respectively.

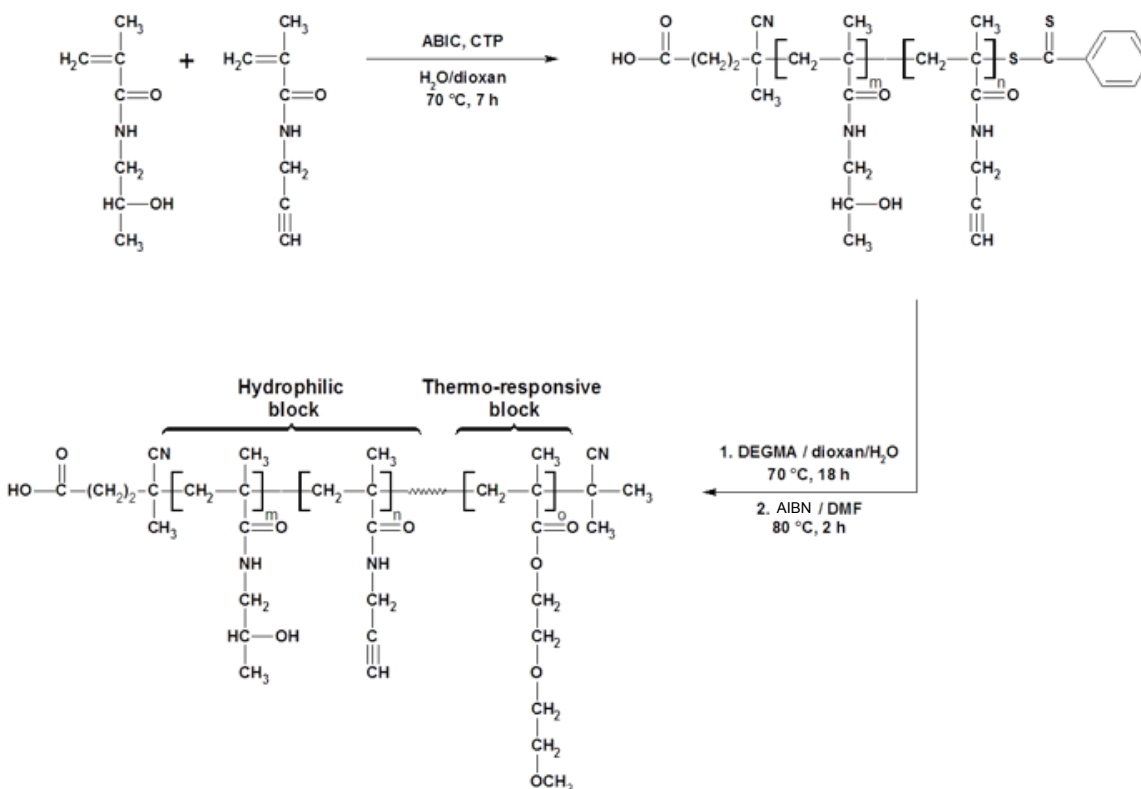
Synthesis of second-generation TRPP-7/8a with ESE coil peptide

TRPP: p[(HPMA)-co-(PgMA)]-block-p(DEGMA)

Second generation TRPP-7/8a were produced as thermo-responsive A-B type di-block copolymers by RAFT polymerization in two synthetic steps. The hydrophilic block A was prepared by copolymerizing HPMA with *N*-propargyl methacrylamide (PgMA) using 4,4'-azobis(4-cyanovaleric acid) (ACVA) as an initiator and 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTP) as a chain transfer agent in molar ratios [M]:[CTP]:[ACVA] = 142:2:1 in 1,4-dioxane / H₂O mixture. Briefly, a mixture of 7.6 mg CTP (27.3 μ mol) and 3.8 mg ACVA (13.7 μ mol) was dissolved in 647 μ L of 1,4-dioxane and added to the solution of 250.0 mg HPMA (1.75 mmol) and 23.9 mg PgMA (0.19 mmol) in 1293 μ L of DI H₂O. The reaction mixture was thoroughly bubbled with Argon and polymerized in sealed glass ampoules at 70°C for 6 h. The resulting copolymer was isolated by precipitation into a 3:1 mixture of acetone and diethyl ether and purified by gel filtration using a SephadexTM LH-20 cartridge with methanol as the eluent. The polymer solution was concentrated in vacuo and precipitated to diethyl ether yielding 131.5 mg of the p[(HPMA)-co-(PgMA)] polymer. The content of dithiobenzoate (DTB) end groups determined by UV-Vis spectrophotometry was $n_{DTB} = 0.106$ mmol/g ($\epsilon_{302} = 12,100$ L/mol) corresponding to the functionality of the polymer chain $f = 0.98$. The weight- and number-average molecular weights determined by SEC were $M_w = 9,809$ g/mol and $M_n = 9,229$ g/mol, respectively. The content of PgMA determined by ¹H NMR was 9.8 mol%.

The hydrophilic polymer block A bearing DTB terminal groups was subjected to a chain-extension polymerization through the RAFT mechanism with di(ethylene glycol) methyl ether methacrylate (DEGMA) to introduce the thermo-responsive polymer block B. Briefly, a mixture of 50.0 mg p[(HPMA)-co-(PgMA)] (5.31 μ mol ~DTB gr.), 53.0 mg DEGMA (0.28 mmol) and 0.30 mg ACVA (1.06 μ mol) was dissolved in 477 μ L of 1,4-dioxane / H₂O (2:1) solution and thoroughly bubbled with argon gas before sealing the glass ampoule reaction vessel and carrying out the reaction at 70°C for 18 h. The di-block polymer was isolated by precipitation to diethyl ether followed by re-precipitation from methanol to 3:1 mixture of acetone and diethyl ether to yield 84.4 mg of the product. The content of dithiobenzoate (DTB) end groups determined by UV-Vis spectrophotometry was $n_{DTB} = 31.1$ μ mol/g ($\epsilon_{302} = 12,100$ L/mol).

To remove the DTB end groups, the polymer and 12.9 mg of AIBN(0.79 μmol) were dissolved in 844 μL of DMF and the solution was heated to 80 $^{\circ}\text{C}$ for 2 h. The resulting polymer was isolated by precipitation in diethyl ether and purified by gel filtration using a SephadexTM LH-20 cartridge with methanol as the eluent. The polymer solution was concentrated in vacuo and precipitated in diethyl ether yielding 72.4 mg of the product. The weight- and number-average molecular weights determined by SEC were $M_w = 22,020$ g/mol and $M_n = 16,790$ g/mol, respectively. The transition temperature (TT) of the polymer, determined by DLS, was 38 $^{\circ}\text{C}$ at 1.0 mg/mL 15 M PBS (pH 7.4).

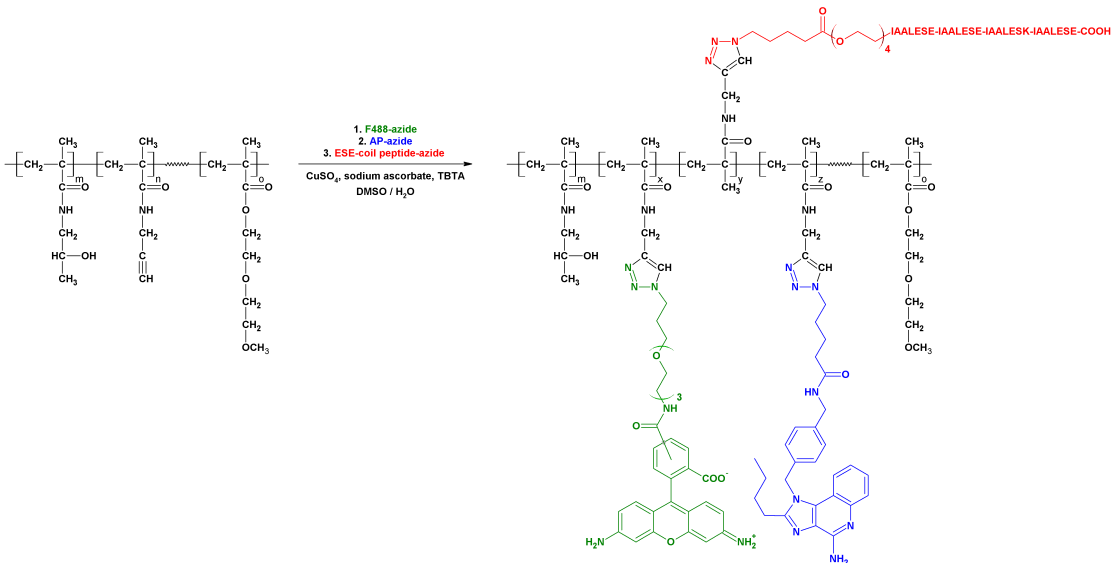


Attachment of TLR-7/8a, ESE coil peptide and fluorophore to TRPP

Different ligands (TLR-7/8a, ESE-coil peptide, scrambled peptide or fluorophore) functionalized with an azide group were attached to TRPP through the propargyl side chain moieties distributed along the hydrophilic block A of the copolymer by copper catalyzed 1,3 dipolar cycloaddition reaction. Reaction progress was monitored by HPLC.

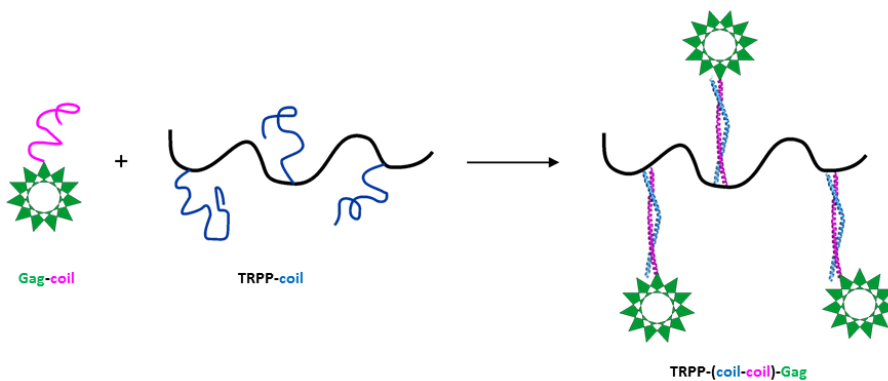
Example: A mixture of 20.0 mg TRPP (7.1 μmol propargyl group), 1.0 mg TLR-7/8a-azide (2.1 μmol), 0.4 mg Carboxyrhodamine 110-azide (0.7 μmol), 4.6 mg ESE-coil peptide-azide (1.4 μmol) and 1.1 mg TBTA (2.1 μmol) was dissolved in 460 μL of DMSO and the solution was thoroughly bubbled with Argon. Then, 0.84 mg sodium ascorbate (4.2 μmol) in 168 μL of degassed water was added. Finally, a solution of 0.54 mg CuSO₄ in 108 μL of degassed water was pipetted to the reaction mixture to initiate the “click” reaction. The reaction was performed overnight at 45 $^{\circ}\text{C}$ until no unreacted ligands were detected by HPLC. The reaction mixture was diluted (1:1) with a saturated solution of EDTA in 0.15 M PBS (pH 7.4) and the conjugate was purified by gel filtration using a

Sephadex™ PD-10 column with H₂O as the eluent. The resulting conjugate was isolated from an aqueous solution by lyophilisation yielding 18.6 mg of the product.



Attachment of HIV Gag-KSK to fluorescently labelled TRPP-ESE conjugate via the coiled coil interaction

Formation of TRPP-(coil-coil)-Gag complex was performed in PBS buffer by mixing TRPP-ESE with HIV Gag-KSK at 1.5/1.0 molar ratio (based on coil peptides). Formation of the coiled-coil complex was measured using SEC on MicroSuperose 12 column and by analytical ultracentrifugation (AUC) 1 hour after mixing.



Determination of TLR-7/8a and fluorophore content on polymers

The amount of ligand attached to the copolymers was determined by UV-Vis spectroscopy using the Beer-Lambert law relationship ($A = \epsilon \cdot c$; where A = absorption and c = mol/L). Samples were suspended in solutions of 1% triethylamine / methanol at known densities (mg/mL) and added to quartz cuvettes with a path length of 1 cm. Absorption was recorded over a spectrum from 250 – 775 nm using a Lambda25 UV-Vis spectrometer from Perkin Elmer. For example, a 0.1 mg/mL solution of Poly-7/8a in 1% triethylamine/methanol ($\lambda_{\max} = 325$ nm, $\epsilon_{325} = 5012$ L/mol) has an optical density (OD, arbitrary units) of 0.25 at 325 nm. The concentration of TLR-7/8 can be calculated by solving for c in the Beer-Lambert law relationship and is 5×10^{-5} mol/L, which can be expressed as the amount of TLR-7/8a per mass of copolymer (5×10^{-4} mmol/mg).

The Beer-Lambert relationship was used to determine the amount of ligand molecules and dyes attached to the polymers based on known extinction coefficients.

Ligand	Max absorption (λ_{\max} , nm)	Extinction coefficient, (L/mol) 1% triethylamine / methanol	A_{325} / A_{\max}
Aminopyridine	305	3,511	---
TLR-7/8a (SM 7/8a)	325	5,012	1.00
AF488	495	167,415	0.12
Cruz Fluor 8	775	71,493	0.09

Methods table 1: Absorption maxima and extinction coefficients were determined for different ligand and dye molecules in 1% triethylamine/methanol. Note that for copolymers with both TLR-7/8a and dye (AF488 or Cruz Fluor 8), the contribution of absorption at 325 nm by the dye was determined using the relationship described by A_{325}/A_{\max} .

Agonist density (mol% 7/8a) determination

UV-Vis can be used to estimate the agonist density (mol%) of co-monomers. Mol% of co-monomer y , for a statistical copolymer comprised of monomers x and y is estimated

using the following relationship:
$$\text{mol}\%_y = \left(\frac{1}{1 + \left(\frac{\rho \times \epsilon}{A \times Mw_x} - \frac{Mw_y}{Mw_x} \right)} \right) * 100$$

$\text{mol}\%_y$ (agonist density) = percentage of copolymer that is y (e.g., TLR-7/8a containing monomer), for copolymer comprised of x and y monomers

ρ = volumetric mass density (mg/mL) of copolymer during UV-Vis measurement

ϵ = molar extinction coefficient for monomer y (e.g. for TLR-7/8a = 5,012)

A = Absorbance

Mw_x = molecular weight (g / mol) of majority monomer

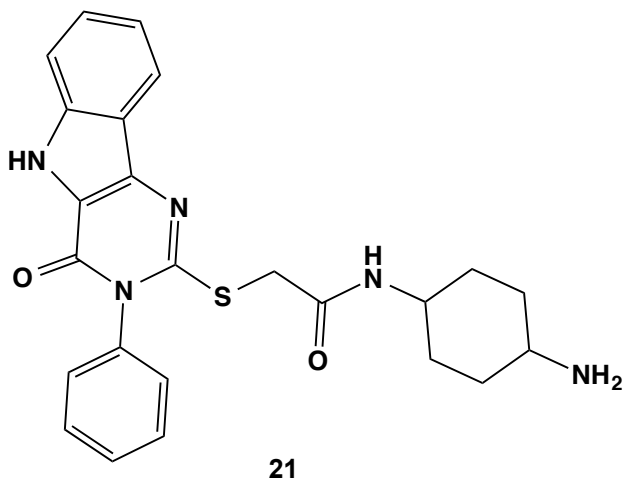
Mw_y = molecular weight (g / mol) of minority monomer

Example calculation:

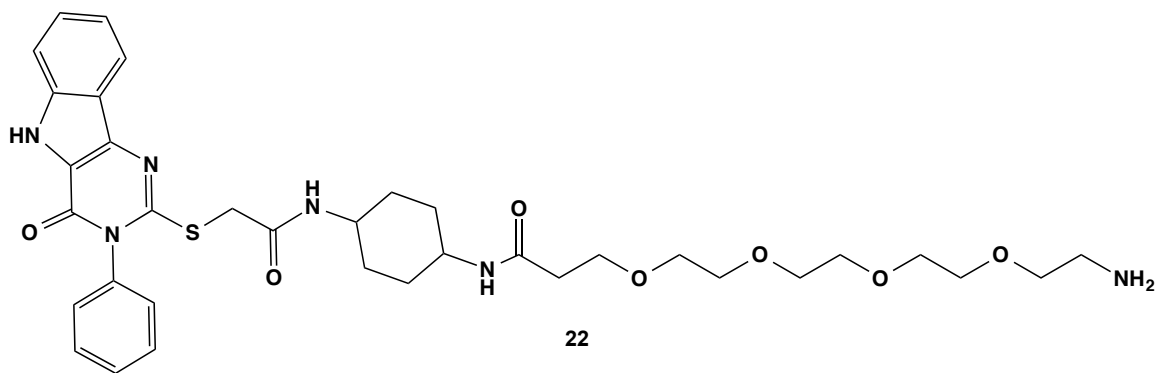
For poly-7/8a comprised of the majority monomer HPMA ($Mw_{\text{HPMA}} = 143.2$) and minority monomer containing the TLR-7/8a (MA-Ahx-PEG4-7/8a; $Mw_{\text{MA-PEG4/7/8a}} = 741.9$) that is suspended in methanol at 0.1 mg / mL and measures an average absorbance of 0.25 at 325 nm, the mol% of the minority unit, MA-PEG4-7/8a is:

$$\text{mol}\%_{\text{MA-Ahx-PEG4-7/8a}} = \left(\frac{1}{1 + \left(\frac{0.1 \times 5012}{0.25 \times 143.2} - \frac{741.9}{143.2} \right)} \right) * 100 = 10.2\%$$

Synthesis of conjugatable TLR-4 agonists



(21) PI-NH₂, *tert*-butyl (4-(2-((4-oxo-3-phenyl-4,5-dihydro-3*H*-pyrimido[5,4-*b*]indol-2-yl)thio)acetamido)cyclohexyl)carbamate. The pyrimidoindole carboxylic acid precursor (2-((4-oxo-3-phenyl-4,5-dihydro-3*H*-pyrimido[5,4-*b*]indol-2-yl)thio)acetic acid) was prepared as recently described¹⁰. 100 mg of this compound (0.28 mmol, 1 eq) and 67.1 mg (0.31 mmol, 1.1 eq) of *N*-Boc-*trans*-1,4-cyclohexanediamine were then added to 2 mL of DMF with triethylamine 80 μ L Et₃N (0.56 mmol, 2 eq). A solution of 118 mg (0.31 mmol, 1.1 eq) of HATU in 400 μ L of DMF was then added to the reaction mixture. The reaction was stirred at RT for 24 h. The solution was concentrated and recrystallized from methanol to provide the Boc-protected product as a white solid (108 mg, 70% yield). ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.1 (s, 1H), 8.08 (d, *J* = 8, 1H), 7.63-7.61 (br m, 2H), 7.53 (t, *J* = 8, 2H), 7.50-7.48 (br m, 4H), 7.30 (t, *J* = 6, 1H), 6.72 (d, *J* = 8, 1H), 3.89 (s, 2H), 3.43 (br s, 1H), 3.17 (br s, 1H), 1.76 (br t, *J* = 13, 4H), 1.38 (s, 9H), 1.30-1.14 (br m, 4H). ¹³C NMR (500 MHz, DMSO-*d*₆) δ 166.4, 155.4, 153.0, 139.4, 137.7, 136.6, 130.0, 129.7, 129.4, 128.5, 127.8, 120.8, 120.6, 119.7, 114.7, 113.3, 77.9, 48.1, 46.2, 37.2, 31.7, 31.6, 28.8. TLC: 100% Ethyl acetate, R_f 0.7. HRMS: *m/z* calcd for C₂₉H₃₃N₅O₄S [M+Na]⁺ 570.2, observed 570.2. 50 mg of the resulting Boc protected compound was then added to 5 mL of 30% TFA/DCM and reacted for 1 hour at room temperature. The TFA/DCM was removed by evaporation and the resulting residue was dissolved in 1M HCl and filtered. The filtrate was made alkaline by the addition of 10 M NaOH, followed by extraction with 3x50 mL of DCM. The organic phase was dried with Na₂SO₄ and evaporated to dryness to obtain 33 mg (80.8% yield) of a spectroscopically pure (>95% at 254 nm) white solid. MS (ESI) calculated for C₂₄H₂₅N₅O₂S *m/z* 447.17, found 448.2 (M+H)⁺.

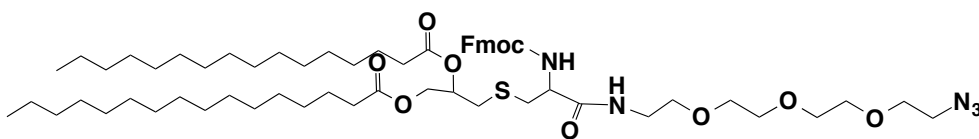


(22) PI-PEG, 1-amino-*N*-(4-(2-((4-oxo-3-phenyl-4,5-dihydro-3*H*-pyrimido[5,4-*b*]indol-2-yl)thio)acetamido)cyclohexyl)-3,6,9,12-tetraoxapentadecan-15-amide. To a 1:2 solution of 5 mL of methanol/DCM was added 15.0 mg (0.03 mmol, 1 eq) of **(21)**, 5.9 mg (0.03 mmol, 1 eq) of CDMT and 18.4 mg (0.05 mmol, 1.5 eq) of Boc-15-amino-4,7,10,13-tetraoxapentadecanoic acid (PEG4), followed by the dropwise addition of 9.25 μ L (0.08 mmol, 2.5 eq) of NMM, while stirring vigorously. After 16 hours at room temperature, the reaction mixture was filtered and then washed 3x50 mL with 1 M HCl. The organic phase was dried with Na₂SO₄ and then evaporated to dryness to yield solid that was purified by semi-prep HPLC using a 33-55% ACN/H₂O gradient over 14 minutes. 11 mg (41% yield) of white solid was obtained and then added to 1 mL of 30% TFA/DCM and reacted for 1 hour at room temperature. The TFA/DCM was removed by evaporation and the resulting residue was dissolved in 1M HCl and filtered. The filtrate was made alkaline by the addition of 10 M NaOH, followed by extraction with 3x50 mL of DCM. The organic phase was dried with Na₂SO₄ and evaporated to dryness to obtain 7 mg (73 % yield) of spectroscopically pure (>95% at 254 nm) white solid. MS (APCI) calculated for C₃₅H₄₆N₆O₇S *m/z* 694.3, found 695.3 (M+H)⁺.

Synthesis of Polymer-TLR4a conjugates (PP-PI)

Example: The polymer-particle forming TLR-4a conjugate (PP-PI) described in this study was prepared by reacting **(22)** with amine reactive p[(HPMA)-*co*-(Ma- β -Ala-TT)]. In short, 5 mg (3.7 μ mol, TT, 1 eq) of p[(HPMA)-*co*-(Ma- β -Ala-TT)] with ~ 11.7 mol% TT was added to 500 μ L of anhydrous methanol. To this solution was added 2.6 mg (3.7 μ mol, 1 eq) of a 10 mg/ml solution of **(22)** in anhydrous DMSO while stirring vigorously. After 16 hours, 2 eq of 1-amino-2-propanol was added to remove unreacted TT groups. After an additional 2 hours, the reaction mixture was dialyzed against methanol using Spectra/Por7 Standard Regenerated Cellulose dialysis tubing with a molecular weight cut-off (MWCO) of 25 kDa (Spectrum Labs, Rancho Dominguez, CA). The dialysis tube was suspended in 1000 mL of methanol and the dialysis buffer was changed twice each day for 3 days. The methanol solution containing Poly-PEG-PI was evaporated to dryness and yielded 6.7 mg of p[(HPMA)-*co*-(Ma- β -Ala-PEG-PI)]. The content of PI-PEG determined by UV-Vis spectrophotometry was 6.3 mol% (ϵ_{340} =7,272 L/mol).

Synthesis of conjugatable Pam2cys (TLR-2/6a)



23

(23) Pam2Cys-PEG-N3 14-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-1-azido-13-oxo-3,6,9-trioxa-16-thia-12-azanonadecane-18,19-diyl dipalmitate, 1-amino-*N*-(4-(2-((4-oxo-3-phenyl-4,5-dihydro-3*H*-pyrimido[5,4-*b*]indol-2-yl)thio)acetamido)cyclohexyl)-3,6,9,12-tetraoxapentadecan-15-amide. To a 20 mL solution of 1:1 DCM/Methanol, was added 100 mg (0.11 mmol, 1 eq) of Fmoc-protected Pam2Cys-COOH (Fmoc-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH) (Bachem, Bubendorf, Switzerland) 27 mg (0.12 mmol, 1.1 eq) of Amino-11-azido-3,6,9-trioxaundecane and 20 mg (0.11 mmol, 1 eq) of CDMT, followed by the dropwise addition of 25 μ L (0.22 mmol, 2.0 eq) of NMM, while stirring vigorously. After 16 hours at room temperature, the reaction mixture was filtered and then washed 3x50 mL with 1 M HCl. The organic phase was dried with Na₂SO₄ and then evaporated to dryness to yield a white solid that was further purified by flash column chromatography using 0-10% methanol / DCM gradient. Fractions were combined and evaporated to dryness to obtain 75.6 mg (62 % yield) of spectroscopically pure (>95% at 254 nm by TLC) white solid. MS (APCI) calculated for C₆₁H₉₉N₅O₁₀S *m/z* 1093.7, found 1113 (M+H₃O)⁺ and 1208 (M+TFA)⁺

Synthesis of Polymer-2/6 conjugates (PP-Pam2Cys)

Example: The polymer-particle forming TLR-2/6a conjugate described in this study was prepared by reacting (**23**) with amine reactive p[(HPMA)-*co*-(Ma- β -Ala-TT)] in a 3 step reaction. In the first step, 5 mg (3.7 μ mol TT, 1 eq) of p[(HPMA)-*co*-(Ma- β -Ala-TT)] with ~ 11.7 mol% TT was added to 500 μ L of anhydrous methanol. To this solution was added 98 μ L (1.96 mg, 3.7 μ mol, 1 eq) of a 10 mg/ml solution of the cross-linker, DBCO-PEG₄NH₂, in anhydrous DMSO while stirring vigorously. After 2 hours, 204 μ L (2.04 mg, 3.7 μ mol, 1 eq) of a 10 mg/mL solution of (**23**) was then added while stirring the reaction mixture vigorously. After 16 hours, 2 eq of 1-amino-2-propanol was added to remove unreacted TT groups. After an additional 2 hours, the reaction mixture was dialyzed against methanol using Spectra/Por7 Standard Regenerated Cellulose dialysis tubing with a molecular weight cut-off (MWCO) of 25 kDa (Spectrum Labs, Rancho Dominguez, CA). The dialysis tube was suspended in 1000 mL of a 1:1 methanol/DCM solution and the dialysis buffer was changed twice over 1 day. The methanol solution containing Poly-PEG-Pam2Cys(Fmoc) was evaporated to dryness and then suspended in a 1 mL solution of 20% Piperidine/DMF for 1 hour to remove the Fmoc group. The reaction mixture was then dialyzed again against a solution of 1:1 methanol/DCM and the dialysis buffer was changed after 15 minutes, and then twice per day for 3 days. The methanol solution containing Poly-PEG-Pam2Cys was evaporated to dryness and yielded 8.1 mg of p[(HPMA)-*co*-(Ma- β -Ala-PEG-Pam2Cys)]. The content of Pam2Cys-PEG determined by UV-Vis spectrophotometry was 4.5 mol% Pam2Cys as determined using the TNBSA assay to measure primary amine content (ϵ_{420} = 11,500 L/mol).

Formulation of MPL (TLR-4a) and CpG (TLR-9a) with particulate carriers

Both Monophosphoryl Lipid A (MPL) and CpG ODN 1826 were purchased from Invivogen as vaccine grade adjuvants. Alum/MPL for immunizations was comprised of a solution of PBS with 0.1 mg/mL MPL and 1 mg/mL Aluminum Hydroxide (Alhydrogel, Invivogen) that was allowed to incubate at room temperature for 2 hours prior to

immunization. Polymer/CpG poly(plex) particles were prepared by formulating 16 kD Poly(L-lysine hydrochloride) (Alamanda Polymers, Huntsville, Alabama, USA) linear polymers with CpG ODN 1826 at 20:1 N:P in PBS.

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