High-throughput, aseptic production of injectable Tetra-PEG hydrogel microspheres for delivery of releasable covalently bound drugs

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SUPPLEMENTARY INFORMATION

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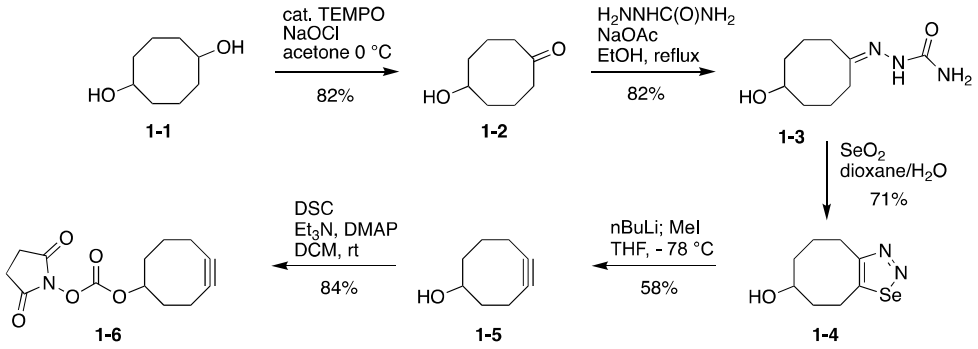
1. SYTHESIS OF STARTING MATERIALS

1.1 General Methods

HPLC analyses were performed on a Shimadzu LC-20AD HPLC system equipped with an SPD-M20A diode array detector, an Alltech 3300 ELSD, and a Phenomenex Jupiter 5 µm C18 column (300 Å, 150 x 4.6 mm). Unless otherwise noted, peaks were eluted with a 10 minute linear gradient of 20-80% acetonitrile (MeCN) in water (0.1% TFA) at 1 mL/min. LCMS analyses were obtained at the UCSF Small Molecule Discovery Center core facility using a Waters Micromass ZQTM equipped with a Waters 2795 Separation Module and a Waters 2996 Photodiode Array Detector.

1.2 5-HCO-HSC

Scheme S1. Synthesis of 5-Hydroxycyclooctyne N-hydroxysuccimidocarbonate (5-HCO-HSC, 1-6). Modifications of reported procedures were made to facilitate high-yield, large scale production.



5-Hydroxycylooctanone[1] (1-2). A 250-mL, three-necked flask equipped with a stir bar, rubber septa, addition funnel equipped with rubber septum and nitrogen inlet, and thermocouple probe was charged with 1,5-cyclooctanediol (10 g, 69 mmol, 1.0 equiv, 0.25 M final concentration), acetone (125 mL), TEMPO (217 mg, 1.40 mmol, 0.02 equiv, 5 mM final concentration) and a solution of KBr (0.5 M in water, 13.9 mL, 6.95 mmol, 0.1 equiv, 0.03 M final concentration). The reaction mixture was cooled at 0 °C (internal +2 °C) while a solution of pH adjusted bleach (commercial bleach adjusted with saturated $NaHCO_3$ to pH 8.8; ~0.7 M, 146 mL, 104 mmol, 1.5 equiv) was added dropwise via addition funnel. The reaction mixture was stirred for 20 min after addition was complete, then diluted with 5% sodium hydrosulfite (100 mL). The reaction mixture was extracted with DCM (4 x 50 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated to afford 8.80 g (92% purity; calculated yield 8.10 g, 57.0 mmol, 82%) of ketone **1-2** as a white solid.

^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm 4.34 (t, *J*=5.7 Hz, 7 H), 2.84 (br. s., 6 H), 2.02 - 2.24 (m, 2 H), 1.84 - 2.02 (m, 4 H), 1.58 - 1.80 (m, 4 H), 1.52 (dd, *J*=13.5, 5.4 Hz, 2 H)

5-Hydroxycyclooctan-1-semicarbazone (1-3). A 1-L, round-bottomed flask equipped with a stir bar and reflux condenser fitted with a rubber septum was charged with semicarbazide hydrochloride (23.6 g, 212 mmol, 1.4 equiv, 0.4 M final concentration), sodium acetate (41.9 g, 511 mol, 3.7 equiv, 0.9 M final concentration), and ethanol (600 mL). The suspension was

stirred vigorously and refluxed for 30 min. The mixture was allowed to cool to ambient temperature and was vacuum filtered through a ~4" plug of Celite over a sintered glass funnel (60 mL capacity, medium porosity) with the aid of ethanol (100 mL). A 1-L, round-bottomed flask equipped as above was charged with the resulting filtrate prepared above and 5-hydroxycyclooctanone (**1-2**) (21.6 g, 152 mmol, 1.0 equiv, 0.2 M final concentration). The reaction mixture was stirred at reflux for 2 h then allowed to cool to ambient temperature. The reaction mixture was concentrated to dryness, suspended in water (~400 mL) and the resulting solids were collected via vacuum filtration. The filtrate was concentrated to dryness and the resulting residue was suspended in water (200 mL) and filtered. This process was repeated once more (total of three cycles). The collected solids (~25 g) were recrystallized from methanol (600 mL, 2 crops) to afford 24.6 g (124 mmol, 82%) of semicarbazone **1-3** as a white solid. ^{1}H NMR spectrum was similar to previously reported data.[2]

7-Hydroxy-4,5,6,7,8,9-hexahydrocycloocta-1,2,3-selenadiazol (1-4). A 250-mL, round-bottomed flask equipped with a stir bar and rubber septum was charged with semicarbazone (**1-3**) (6.4 g, 32 mmol, 1.0 equiv, 0.4 M final concentration), dioxane (65 mL), and a solution of selenium dioxide (7.9 g, 71 mmol, 2.2 equiv, 0.8 M final concentration) in water (20 mL). The resulting mixture was stirred in the dark for 18 h. The reaction mixture was filtered through a 4" plug of Celite in a 60-mL sintered glass funnel (medium porosity) with the aid of EtOAc (100 mL). The resulting filtrate was concentrated to approximately 30 mL. The mixture was diluted with EtOAc (200 mL) and H_2O (200 mL). The aqueous phase was separated and extracted with EtOAc (5 x 100 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated to afford a red oil. Purification via silica gel chromatography (120 g SiO_2; stepwise gradient 30% (300 mL), 50% (100 mL), 60% (100 mL), 70% (100 mL), 80% (100 mL), 90% (100 mL) EtOAc/hexanes) afforded 5.00 g (21.5 mmol, 71%) of selenadiazole **1-4** as an orange oil that was stored at -80 °C.

^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm 3.65 (dddd, *J*=9.4, 7.4, 4.2, 1.8 Hz, 1 H), 3.27 - 3.46 (m, 2 H), 3.11 - 3.23 (m, 2 H), 2.12 - 2.25 (m, 1 H), 1.98 - 2.12 (m, 1 H), 1.61 - 1.95 (m, 4 H).

5-Hydroxycyclooctyne (**1-5**). A heat-gun dried, 500-mL, round-bottomed, flask equipped with a stir bar, rubber septum, nitrogen inlet needle, and thermocouple was charged with a solution of selenadiazole **1-4** (8.18 g, 35.4 mmol, 1 equiv, 0.2 M final concentration) in THF (130 mL) and cooled to – 78 °C. A solution of n-BuLi (freshly titrated: 1.4 M, 75 mL, 57.1 mmol, 2.9 equiv, 0.5 M final concentration) was added via syringe down the side of the flask at a rate such that the internal temperature did not exceed -65 °C (addition required ~25 min). The reaction mixture was stirred at -78 °C for 30 min then MeI (7.7 mL, 124 mmol, 3.5 equiv, 0.6 M final concentration) was added dropwise via syringe at a rate such that the internal temperature did not exceed -61 °C. The reaction mixture was stirred at -78 °C for 30 min and allowed to warm to ambient temperature over 30 min. The reaction mixture was cooled at 0 °C and quenched by the addition of H_2O (10 mL). The reaction mixture was partitioned between CH_2Cl_2 (200 mL) and H_2O (200 mL). The aqueous phase was separated and extracted with CH_2Cl_2 (4 x 200 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated to afford a yellow oil. Purification via column chromatography (120 g SiO_2 cartridge; stepwise gradient elution: 5%, 20% (300 mL), 30% (300 mL), 40% (500 mL), 50% (300 mL), EtOAc/hexanes) afforded 2.89 g of a pale yellow oil (88% purity by weight by ^{1}H NMR = 2.54 g, 20.4 mmol, 58%) of desired cyclooctyne **1-5**. This intermediate is used immediately in the synthesis of **1-6**. If storage is required it is stored at -80 °C in DCM.

^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm 3.87 (br. s., 1 H), 2.31 - 2.49 (m, 1 H), 2.05 - 2.31 (m, 5 H), 1.76 - 1.99 (m, 4 H)

^{13}C NMR ($CDCl_3$, 125 MHz) δ 168.8, 150.9, 94.1, 92.8, 87.8, 39.6, 37.8, 30.8, 29.8, 25.4, 19.9, 17.5

5-HCO-HSC (1-6). A 250-mL round-bottomed flask was charged with 5-hydroxycyclooctyne (**1-5**) (3.3 g, 26.6 mmol, 1.0 equiv, 0.2 M final concentration), CH_2Cl_2 (122 mL), DSC (13.6 g, 53.1mmol, 2 equiv, 0.4 M final concentration), Et_3N (8.11 mL, 58.5 mmol, 2.2 equiv, 0.4 M final concentration), and DMAP (650 mg, 5.32 mmol, 0.2 equiv, 0.04 M final concentration). The reaction mixture was stirred at ambient temperature for 24 h. The reaction mixture was diluted with CH_2Cl_2 (300 mL) and washed with saturated $NaHCO_3$, H_2O, 5% aqueous $KHSO_4$, H_2O, brine (100 mL each). The organic phases were concentrated and filtered through a cotton plug onto a silica gel column (120 g SiO_2 cartridge). Gradient elution (10%, 20%, 30%, 40%, 50% (200 mL each) acetone/hexanes) afforded 6.73 g (88% purity by weight by ^{1}H NMR = 5.92 g, 22.3 mmol, 84%) of desired HSC **1-6** as a yellow oil. Concentration from CH_2Cl_2 affords an oil which solidifies upon storage at -20 °C. The resulting white solid can be further dried under high vacuum at ambient temperature for ~ 1 h, then stored at -20 °C.

^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm 4.79 (dd, *J*=9.4, 5.3 Hz, 1 H), 2.81 (s, 4 H), 2.28 - 2.56 (m, 2 H), 2.04 - 2.28 (m, 6 H), 1.82 - 2.03 (m, 2 H)

^{13}C NMR ($CDCl_3$, 300 MHz) δ 168.8, 150.9, 94.1, 92.8, 87.8, 39.6, 37.8, 30.8, 29.8, 25.4, 19.9, 17.5

1.3 PEG_{10kDa}-$(NH_2)_4$ end group analysis

PEG_{10kDa}-$(NH_2)_4$ (5.0 ± 1.0 mg; ~ 2 μmol amine) is added to each of four 1.5 mL screw-cap HPLC vials (labeled A-D). MeCN (1 mL) and a 100 mM solution of DIPEA (40 μL, 4 μmol) in MeCN are added to each vial, and the PEGs are dissolved. A limiting amount Fmoc-OSu (10 μL of 100 mM in MeCN, 1 μmol) is added to one vial (A), and excess Fmoc-OSu (30 μL of 100 mM in MeCN, 3 μmol) is added to each of the other three vials (B-D). The reactions are kept at ambient temperature for 1 h then analyzed by C18 HPLC (266 nm) with a linear gradient of 20-80% MeCN in water (0.1% TFA) at 1 mL/min.

In the vial with limiting Fmoc-OSu (Vial A), four peaks having λ_{max} = 266 nm corresponding to PEG-Fmoc species were observed (**Figure S1 A**), and reagent-derived peaks (Fmoc-OSu and/or dibenzofulvene) eluted after the PEG-Fmoc peaks. Each PEG-Fmoc peak is assigned to PEG-$(Fmoc)_n$, where n = 1, 2, 3, or 4 based on increasing retention time. In the other three vials (Vials B, C, and D) with excess Fmoc-OSu, one major peak corresponding to PEG-$(Fmoc)_4$, and minor peaks of PEG-$(Fmoc)_3$ and PEG-$(Fmoc)_2$ were observed (**Figure S1 B-C**).

The fraction of reactive end groups was calculated as:

$$EG = x_4 + 0.75*x_3 + 0.5*x_2 + 0.25*x_1,$$

Where, EG = fractional reactive end groups, x_n = [HPLC peak area of PEG-$(Fmoc)_n$]/[total HPLC peak area of PEG-(Fmoc)]. The final EG is determined from the average values from the three vials with excess Fmoc-OSu. Using data from Vial B, commercially available PEG_{10kDa}-$(NH_2)_4$ has EG = 0.922 + 0.75*0.073 = 0.977, or 97.7%.

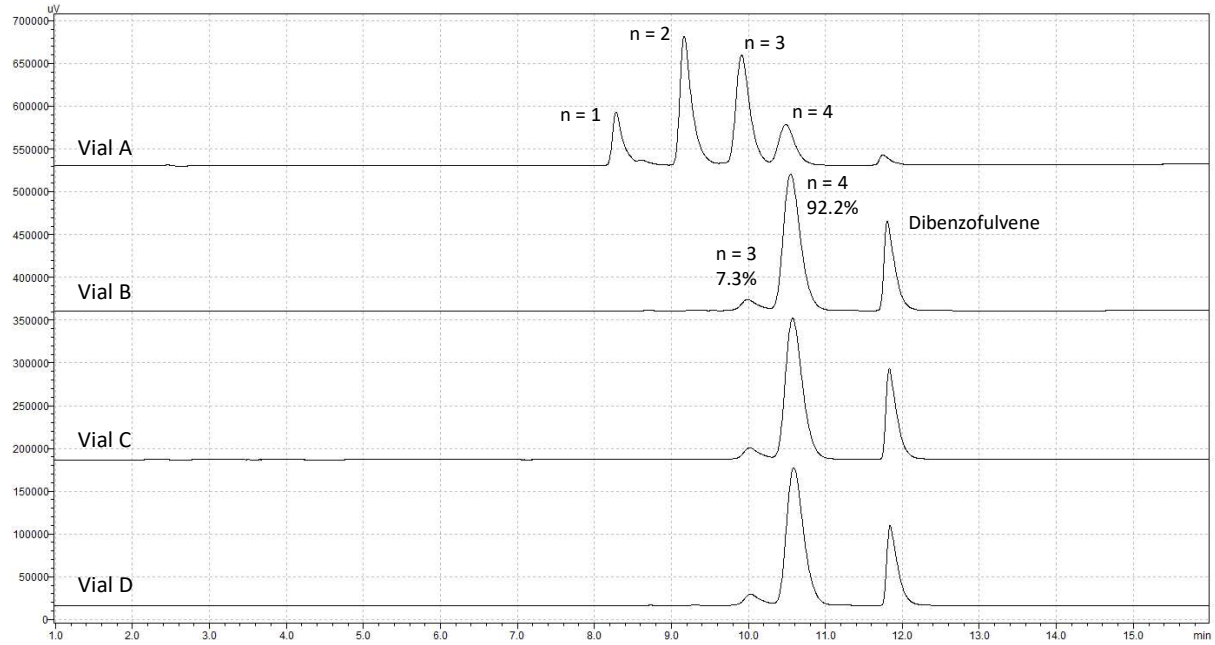
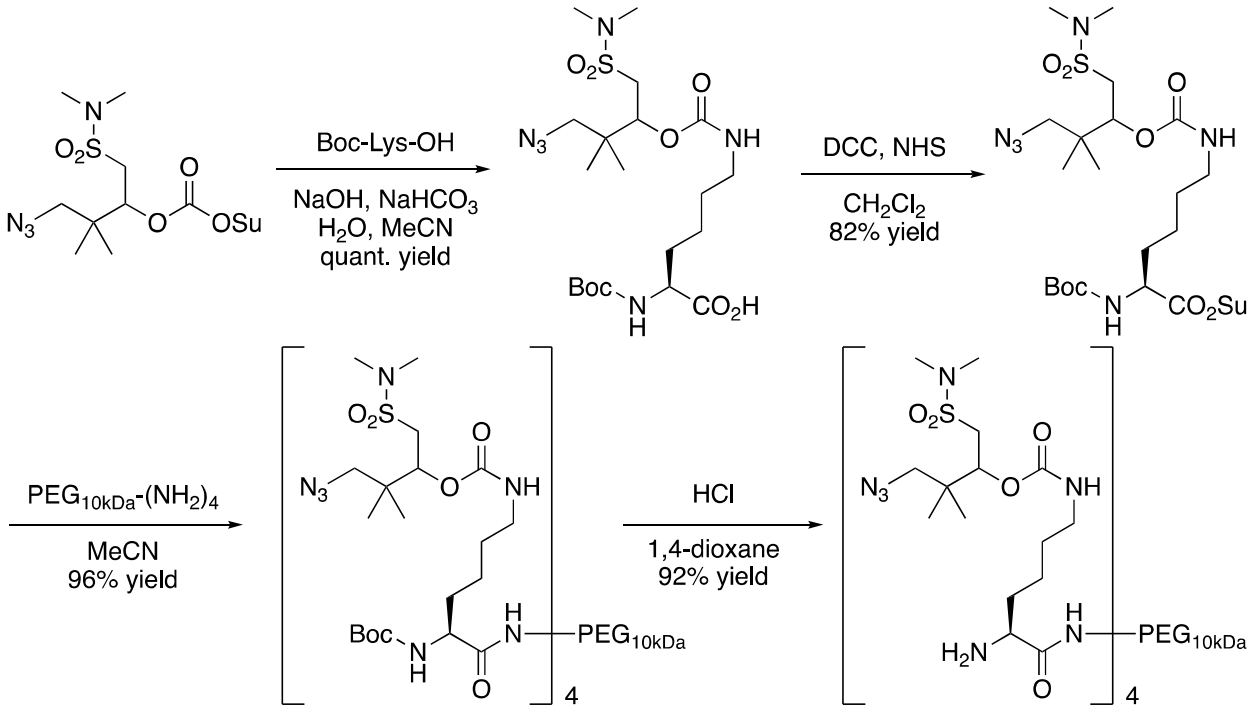


Figure S1. HPLC analysis of Fmoc derivatized PEG-$(NH_2)_4$ for determination of the number of reactive end groups. Peaks were detected at 266 nm.

1.4 Prepolymer A

Scheme S2. Synthesis of Prepolymer A



N^{α}-Boc-N^{ε}-{4-Azido-3,3-dimethyl-1-[(N,N-dimethyl)aminosulfonyl]-2-butyloxycarbonyl}-Lys-OH, Boc-Lys(L-N_3)-OH. A solution of Boc-Lys-OH (2.96 g, 12.0 mmol) in 28 mL of H_2O was successively treated with 1 M aq NaOH (12.0 mL, 12.0 mmol), 1 M aq $NaHCO_3$ (10.0 mL, 10.0 mmol), and a solution of *O*-{4-azido-3,3-dimethyl-1-[(*N,N*-dimethyl)aminosulfonyl]-2-butyl}-*O'*-succinimidyl carbonate (3.91 g, 10.0 mmol, 0.1 M final concentration) in 50 mL of MeCN. After stirring for 2 h at ambient temperature, the reaction was judged to be complete by C18 HPLC (ELSD). The reaction was quenched with 30 mL of 1 M $KHSO_4$ (aq). The mixture was partitioned between 500 mL of 1:1 EtOAc:H_2O. The layers were separated, and the aqueous phase was extracted with 100 mL of EtOAc. The combined organic phase was washed with H_2O and brine (100 mL each) then dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to provide the crude title compound (5.22 g, 9.99 mmol, 99.9% crude yield) as a white foam.
C18 HPLC, purity was determined by ELSD: 99.1% (RV = 9.29 mL).
LC-MS (*m/z*): calc, 521.2; obsd, 521.3 $[M-H]^-$.

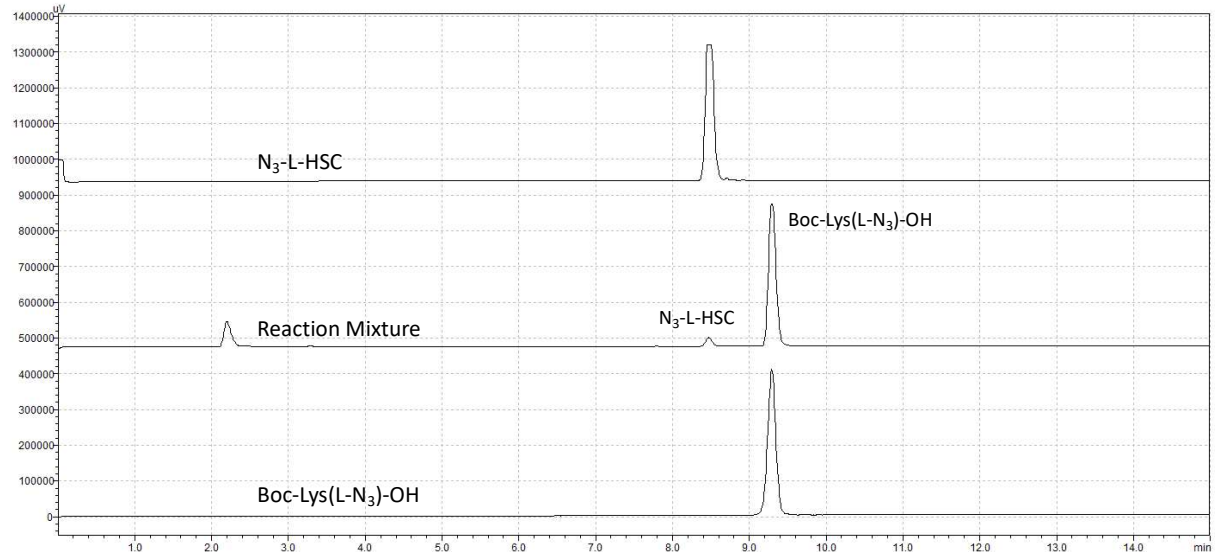


Figure S2. Boc-Lys(L-N_3)-OH: HPLC analysis of the starting N_3-L-HSC, reaction mixture, and final product. Peaks were detected by ELSD.

N^{α}-Boc-N^{ε}-{4-Azido-3,3-dimethyl-1-[(N,N-dimethyl)aminosulfonyl]-2-butyloxycarbonyl}-Lys-OSu, Boc-Lys(L-N_3)-OSu. DCC (60% in xylenes, 2.6 M, 4.90 mL, 12.7 mmol) was added to a solution of N^{α}-Boc-N^{ε}-{4-azido-3,3-dimethyl-1-[(*N,N*-dimethyl)aminosulfonyl]-2-butyloxycarbonyl}-Lys -OH (5.11 g, 9.79 mmol, 0.1 M final concentration) and *N*-hydroxysuccinimide (1.46 g, 12.7 mmol) in 98 mL of CH_2Cl_2. The reaction suspension was stirred at ambient temperature and monitored by C18 HPLC (ELSD). After 2.5 h, the reaction mixture was filtered, and the filtrate was loaded onto a SiliaSep 120 g column. Product was eluted with a step-wise gradient of acetone in hexane (0%, 20%, 30%, 40%, 50%, 60%, 240 mL each). Clean product-containing fractions were combined and concentrated to provide the title compound (4.95 g, 7.99 mmol, 81.6% yield) as a white foam.
C18 HPLC, purity was determined by ELSD: 99.7% (RV = 10.23 mL).
LC-MS (*m/z*): calc, 520.2; obsd, 520.2 $[M+H-Boc]^+$.

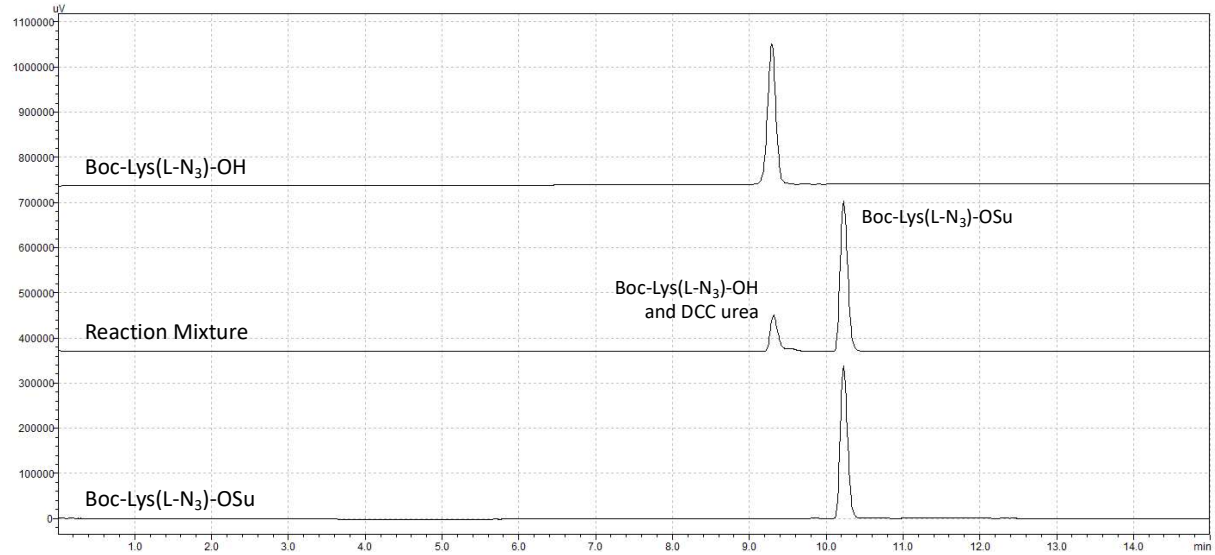


Figure S3. Boc-Lys(L-N_3)-OSu: HPLC analysis of the starting Boc-Lys(L-N_3)-OH, reaction mixture, and final product. Peaks were detected by ELSD.

(N^{α}-Boc-N^{ε}-{4-Azido-3,3-dimethyl-1-[(N,N-dimethyl)aminosulfonyl]-2-butyloxycarbonyl}-Lys)$_4$-PEG$_{10kDa}$. In a 1-L round bottom flask equipped with a Teflon-coated magnetic stir bar, PEG_{10kDa}-$(NH_2)_4$ (22.00 g, 2.209 mmol, 8.835 mmol NH_2, 20 mM NH_2 final concentration) was dissolved in 200 mL of MeCN. DIPEA (1.84 mL, 10.6 mmol) was added. Next a 48 mM solution of (N^{α}-Boc-N^{ε}-{4-azido-3,3-dimethyl-1-[(*N,N*-dimethyl)aminosulfonyl]-2-butyloxycarbonyl}-Lys)-OSu (220 mL, 10.6 mmol) in MeCN was added in two portions: 100 mL (HPLC taken to observe intermediate series) and 120 mL. The reaction solution was stirred at ambient temperature for 1 h after full addition of activated lysine. Acetic anhydride (851 µL, 8.84 mmol) was added, and the solution was stirred for 30 min. The reaction was concentrated by rotary evaporation to ~80 g then added to 600 mL of stirred MTBE. The resulting biphasic mixture was cooled in a water-ice bath and stirred for 1 h. The solid was triturated, vacuum filtered, and washed with MTBE (5x 50 mL). After air-drying for 15 min, the solid was transferred to a 250 mL HDPE packaging bottle. Residual volatiles were removed under high vacuum overnight to provide the title compound (25.416 g, 2.122 mmol, 96.06% yield) as an off-white solid.
C18 HPLC, purity was determined by ELSD: 93.3% (RV = 10.88 mL) with a 6.2% impurity (RV = 9.78) attributed to PEG-$(NH_2)_3$ contaminant in the starting PEG.

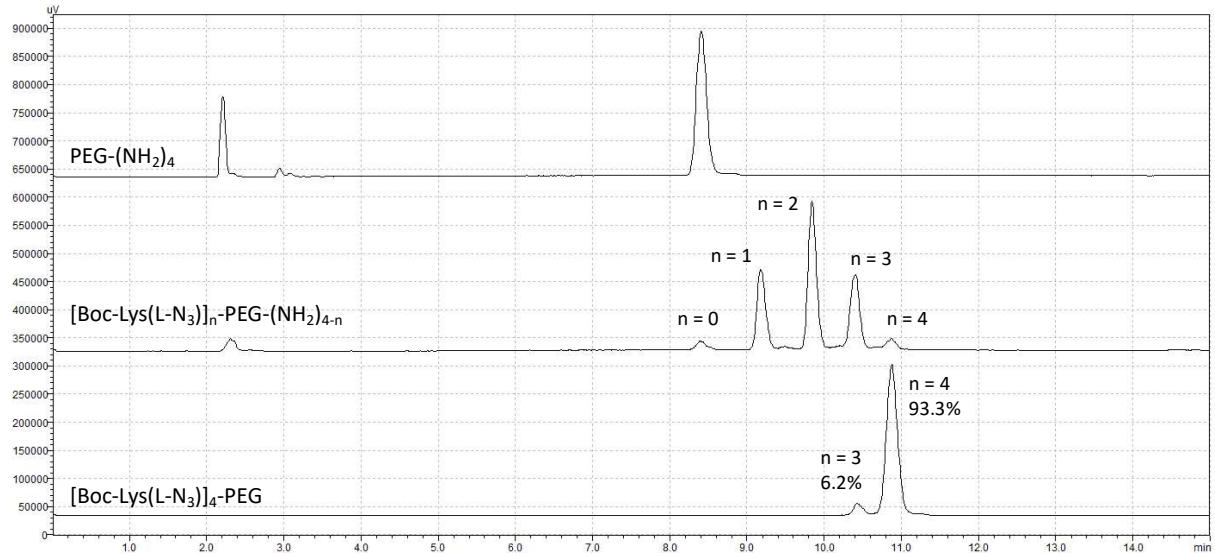


Figure S4. [Boc-Lys(L-N_3)]$_4$-PEG_{10kDa}: HPLC analysis of the starting PEG_{10kDa}-$(NH_2)_4$, reaction mixture, and final product. Peaks were detected by ELSD.

(N^{ε}-{4-Azido-3,3-dimethyl-1-[(N,N-dimethyl)aminosulfonyl]-2-butyloxycarbonyl}-Lys)$_4$-PEG_{10kDa}, Prepolymer A. In a 1-L round bottom flask equipped with a Teflon-coated magnetic stir bar, *(N^{α}*-Boc-*N^{ε}*-{4-azido-3,3-dimethyl-1-[(N,N-dimethyl)aminosulfonyl]-2-butyloxycarbonyl}-Lys)$_4$-PEG_{10kDa} (25.23 g, 2.106 mmol, 5 mM final concentration) was dissolved in 200 mL of 1,4-dioxane. A 4 M solution of HCl in dioxane (200 mL, 2 M final concentration) was added. The reaction solution was stirred at ambient temperature and periodically monitored by C18 HPLC. The starting material was converted to a single product peak via three faster eluting peaks (full series observed after 10 min). After 105 min, the reaction was complete. At t=2h, the reaction mixture was added via cannula, over 40 min, to 0.8 L of ice-cold Et_2O under N_2. The suspension was filtered, and the resulting gummy solids were washed with Et_2O (3x 100 mL) then dissolved in ~0.2 L of CH_2Cl_2. After concentrated to dryness via rotary evaporation, the product oil (~40 g) was triturated with 250 mL of Et_2O until a solid material formed throughout the sample. The suspension was kept at -20 °C overnight then decanted, and the wet solids were transferred to a 250 mL HDPE packaging bottle. Residual volatiles were removed under high vacuum (20 h) to provide the title compound (22.652 g, 1.932 mmol, 91.74% yield) as an off-white solid.
C18 HPLC, purity was determined by ELSD: 95.8% (RV = 9.36 mL) with a 4.2% impurity (RV = 9.22 mL) attributed to PEG-$(NH_2)_3$ contaminant in the starting PEG.

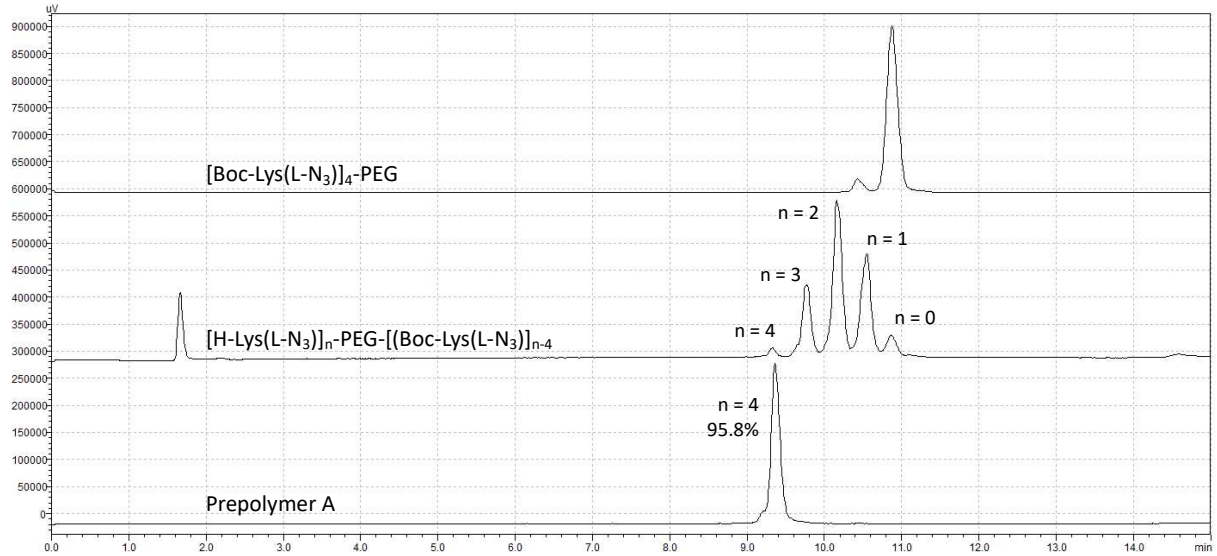
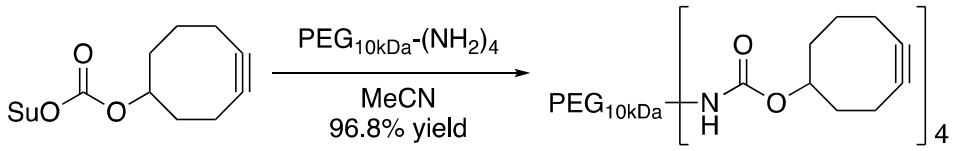


Figure S5. Prepolymer A: HPLC analysis of the starting [Boc-Lys(L-N_3)]$_4$-PEG_{10kDa}, reaction mixture, and final product. Peaks were detected by ELSD.

1.5 Prepolymer B

Scheme S3. Synthesis of Prepolymer B



Prepolymer B

(Cycloocy-4-yn-1-yloxycarbonyl)$_4$-PEG_{10kDa}, Prepolymer B. In a 1-L round bottom flask equipped with a Teflon-coated magnetic stir bar, PEG_{10kDa}-$(NH_2)_4$ (23.00 g, 2.309 mmol, 9.236 mmol NH_2, 20 mM NH_2 final concentration) was dissolved in 388 mL of MeCN. A 150 mM solution of *O*-(cyclooct-4-yn-1-yl)-*O*'-succinimidyl carbonate (73.9 mL, 11.1 mmol) in MeCN was added in two portions: 33.9 mL (HPLC taken to observe intermediate series) and, 15 min later, 40 mL. DIPEA (1.93 mL, 11.1 mmol) was added, and the reaction was stirred at ambient temperature and monitored by C18 HPLC. After 2 h, the reaction was judged to be complete and was quenched with acetic anhydride (897 µL, 9.24 mmol). The reaction solution was concentrated by rotary evaporation to ~70 g then added to 600 mL of stirred MTBE. The resulting mixture was cooled in a water-ice bath and stirred for 15 min. The solid was triturated, vacuum filtered, and washed with MTBE (3x 100 mL). After air-drying for 5 min, the solid was transferred to a 250 mL HDPE packaging bottle. Residual volatiles were removed under high vacuum until the weight stabilized (30 min) to provide the title compound (23.597 g, 2.235 mmol, 96.80% yield) as an off-white solid.
C18 HPLC, purity was determined by ELSD: 95.8% (RV = 10.49 mL) with a 3.1% impurity (RV = 10.09 mL) attributed to PEG-$(NH_2)_3$ contaminant in the starting PEG.

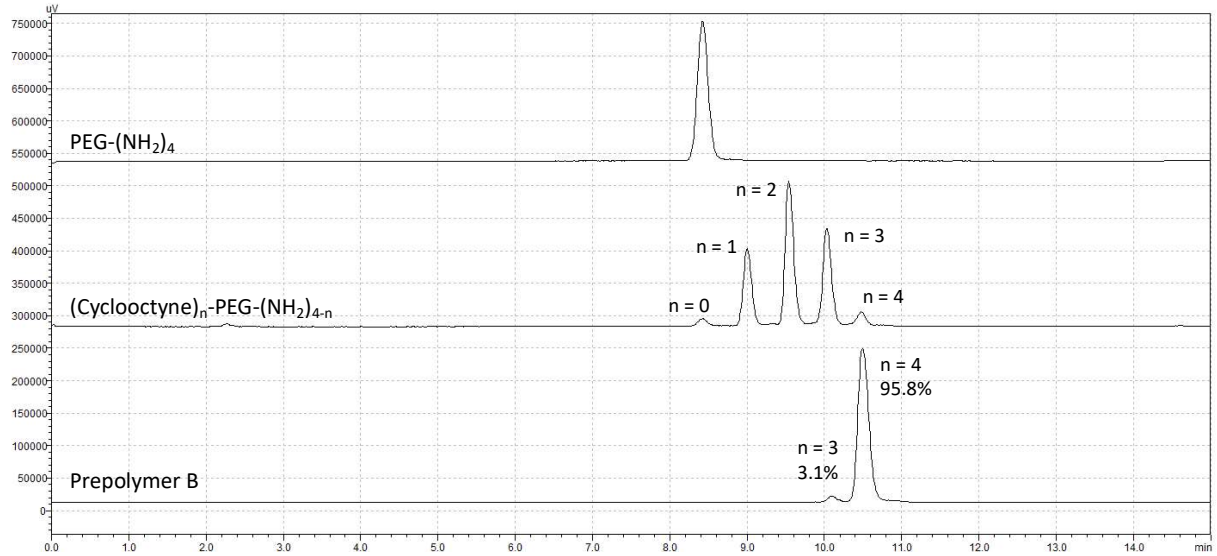


Figure S6. Prepolymer B: HPLC analysis of the starting PEG_{10kDa}-$(NH_2)_4$, reaction mixture, and final product. Peaks were detected by ELSD.

2. PREPARATION OF AMINO-MS (STAGE I)

2.1 Preparation and analysis of prepolymer feedstocks

The following procedures detail the preparation and quantitative analyses of Prepolymer A and Prepolymer B feedstock solutions used to prepare amino-MS.

2.1.1 Prepolymer A feed stock

Solid Prepolymer A (22.5 g) was dissolved in 75 mM pH 5.0 sodium acetate buffer (131 g) to give a solution of approximately 50 mM azide end group. This solution was titrated for specific azide concentration using the procedure described below, and found to be 60 ± 5 mM end group. Dilution to 25 mM end group with 75 mM pH 5.0 acetate buffer for use as a feed stock in microfluidic amino-MS synthesis was performed by weight assuming a density of 1 g/mL for the prepolymer solution and the acetate buffer according to (**Equation S1**).

Equation S1 $W_{acetate} = [(C_1 * W_1)/C_2] - W_1$

Where:

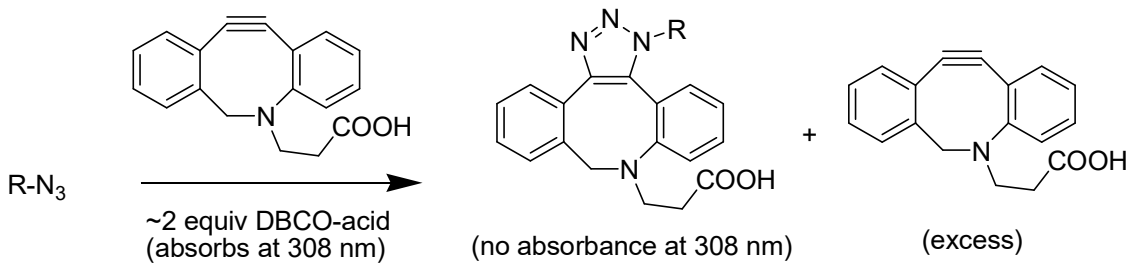
C_1 = the titrated concentration of prepolymer solution
W_1 = the weight of the prepolymer solution in grams
C_2 = 25 mM (the target feed stock concentration)
$W_{acetate}$ = the weight of acetate buffer to add for dilution

2.1.2 Prepolymer B feed stock

Solid Prepolymer B (23.5 g) was dissolved in 75 mM pH 5.0 sodium acetate buffer (154 g) to give a solution of approximately 50 mM cyclooctyne end group. This solution was titrated for specific cyclooctyne concentration using the procedure described below, and found to be 60 ± 5 mM end group. Dilution to 25 mM end group with 75 mM pH 5.0 acetate buffer for use as a feed stock in microfluidic amino-MS synthesis was performed by weight assuming a density of 1 g/mL for the prepolymer solution and the acetate buffer according to (**Equation S1**).

2.1.3 Prepolymer A end group titration

Scheme S4. Titration reaction for spectrophotometric determination of Prepolymer A azide end groups.



Azide end groups on Prepolymer A were measured by the loss of A_{308} that occurs when the azide reacts with the cyclooctyne DBCO to form a triazole (**Scheme S4).**

Reactions B: In triplicate, a sample of Prepolymer A solution (0.200 mL) was diluted with water (0.300 mL) then a sample of the resulting solution was treated with a solution of DBCO-acid (0.5 mM, 0.988 mL) in 0.1 M pH 7.6 HEPES buffer for 4-5 hours at 18-25 ºC. For analysis, a sample of the DBCO-acid reaction solution (0.125 mL) was diluted with 0.1 M pH 7.6 HEPES buffer (0.125 mL) on a 96 well microtiter plate then the A_{308} was determined using a plate reader (Molecular Devices, Spectramax i3 plate reader) configured to report absorbance values equivalent to a 1 cm path length. HEPES buffer (0.250 mL) was used for background subtraction.

Reactions A: A control reaction lacking Prepolymer A was prepared and analyzed as above using 0.075 M pH 5.0 acetate buffer (0.200 mL) in place of the Prepolymer A solution. The raw data from an example analysis and the equations used for calculation of the result (0.060 ± 0.005 M) are presented in **Figure S7**.

A

	A_{308_RA}	A_{308_RB}
Sample 1	3.817	1.809
Sample 2	3.867	1.639
Sample 3	3.847	1.952
Average	**3.844**	**1.800**
StDev	**0.025**	**0.157**

B

$$C_{\text{end group}}[\text{M}] = \frac{A_{308\_RA} - A_{308\_RB}}{\varepsilon_{\text{DBCO,308nm}}[\text{M}^{-1}\text{cm}^{-1}] * PL[\text{cm}]} * DF$$

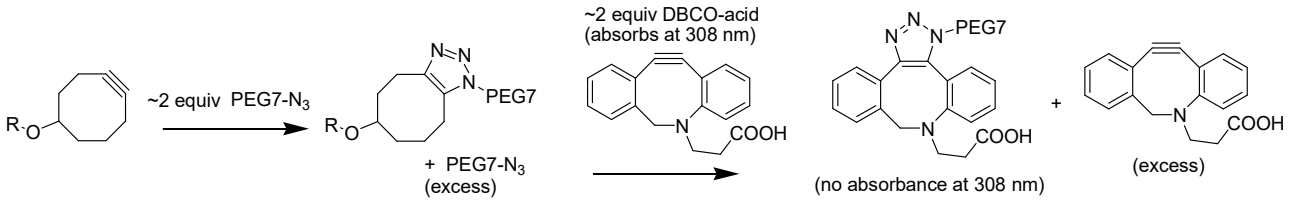
C

$$StDev[\text{M}] = \frac{\sqrt{(StDev_{\text{A308,RA}})^2 + (StDev_{\text{A308,RB}})^2}}{\varepsilon_{\text{DBCO,308nm}}[\text{M}^{-1}\text{cm}^{-1}] * PL[\text{cm}]} * DF$$

Figure S7. Determination of azide end group concentration for Prepolymer A. (**A**) Table of raw data from analytical titration reactions. (**B**) The equation used for calculation of end group concentration where: A_{308_RA} is the average absorbance value from *reactions A*, A_{308_RB} is the average absorbance value for *reactions B*, DF is the overall dilution factor for dilution of Prepolymer B into the microtiter plate (for the procedure above *DF* = 400), $\varepsilon_{308\ nm}$ = 13590 $M^{-1}cm^{-1}$ is the extinction coefficient of DBCO-acid, and *PL* = 1 cm is the path length. (**C**) The equation used for calculation of standard deviation (StDev) in end group concentration where: $StDev_{A380,RA}$ is the *StDev* in absorbance values for *reactions A*, and $StDev_{A380,RB}$ is the *StDev* in absorbance values for *reactions B.*

2.1.4 Prepolymer B end group titration

Scheme S5. Titration reactions for spectrophotometric determination of cyclooctynes.



Cyclooctyne end groups on Prepolymer B were measured spectrophotometrically by first titrating with an excess of a PEG azide, then back titrating the residual azide using the loss of

absorbance at 308 nm that occurs when the azide reacts with the cyclooctyne DBCO. The chemistry used for this spectrophotometric titration is shown above in **Scheme S5**.

Reaction A: In triplicate, a sample of Prepolymer B solution (0.200 mL) was diluted with water (0.300 mL) then a sample of the resulting solution (0.050 mL; ~20 mM) was treated with a solution of O-(2-azidoethyl)heptaethylene glycol (PEG7-N_3) in water (40 mM, 0.050 mL). The resulting *PEG7-N_3* reaction mixture was allowed to proceed for 18-24 hours at 18-25 °C, then a sample (0.0125 mL) was treated with a solution of DBCO-acid (0.5 mM, 0.988 mL) in 0.1 M pH 7.6 HEPES buffer for 4-5 hours at 18-25 °C. For analysis, a sample of the *DBCO* reaction solution (0.125 mL) was diluted with 0.1 M pH 7.6 HEPES buffer (0.125 mL) on a 96 well microtiter plate then the absorbance was measured at 308 nm using a plate reader (Molecular Devices, Spectramax i3) configured to report absorbance values equivalent of a 1 cm path length. HEPES buffer (0.250 mL) was used on the plate for background subtraction.

Reaction B: A control reaction lacking Prepolymer B was prepared and analyzed exactly as above using 0.075 M pH 5.0 acetate buffer (0.200 mL) in place of the Prepolymer B solution. The raw data from an example analysis and the equations used for calculation of the result (0.058 ± 0.001 M) are presented in **Figure S8**.

A

	A_{308_RA}	A_{308_RB}
Sample 1	2.908	1.941
Sample 2	2.903	1.913
Sample 3	2.920	1.921
Average	**2.910**	**1.925**
StDev	**0.008**	**0.014**

B

$$C_{\text{end group}}[\text{M}] = \frac{A_{308\_RA} - A_{308\_RB}}{\varepsilon_{\text{DBCO,308nm}}[\text{M}^{-1}\text{cm}^{-1}] * PL[\text{cm}]} * DF$$

C

$$StDev[\text{M}] = \frac{\sqrt{\left(StDev_{\text{A308,RA}}\right)^2 + \left(StDev_{\text{A308,RB}}\right)^2}}{\varepsilon_{\text{DBCO,308nm}}[\text{M}^{-1}\text{cm}^{-1}] * PL[\text{cm}]} * DF$$

Figure S8. Determination of cyclooctyne end group concentration for Prepolymer B solutions. (**A**) Table of raw data from analytical titration reactions. (**B**) The equation used for calculation of end group concentration where: A_{308_RA} is the average absorbance value from reactions A, A_{308_RB} is the average absorbance value for *reactions B*, *DF* is the overall dilution factor for dilution of the Prepolymer B solution into the microtiter plate (for the procedure above *DF* = 800), $\varepsilon_{308\ nm}$ = 13590 $M^{-1}cm^{-1}$ is the extinction coefficient of DBCO-acid, and *PL* = 1cm is the path length. (**C**) The equation used for calculation of standard deviation (*StDev*) in end group concentration where: $StDev_{A380,RA}$ is the *StDev* in absorbance values for reactions A, and $StDev_{A380,RB}$ is the *StDev* in absorbance values for reactions B.

2.1.5 HPLC analysis of prepolymer feedstock solutions

A sample of Prepolymer A or B containing (0.2 mM end group) in 0.3 mM pH 5 sodium acetate buffer was analyzed by C18 reverse phase HPLC (0.010 mL injection) using a gradient of 20-80% acetonitrile in water with 0.1%TFA as the mobile phase and an ELSD detector (**Section 1.1**). From the resulting chromatogram, the % purity was determined by total integration. The peak eluting at 2.2 minutes results from buffer salts and was neglected in analysis. Prepolymer A elutes at 9.5 minutes and represents 97.3% of the total detectable peaks (**Figure S9**). Prepolymer B elutes at 10.5 minutes and represents 97.2% of the total detectable peaks (**Figure S10**).

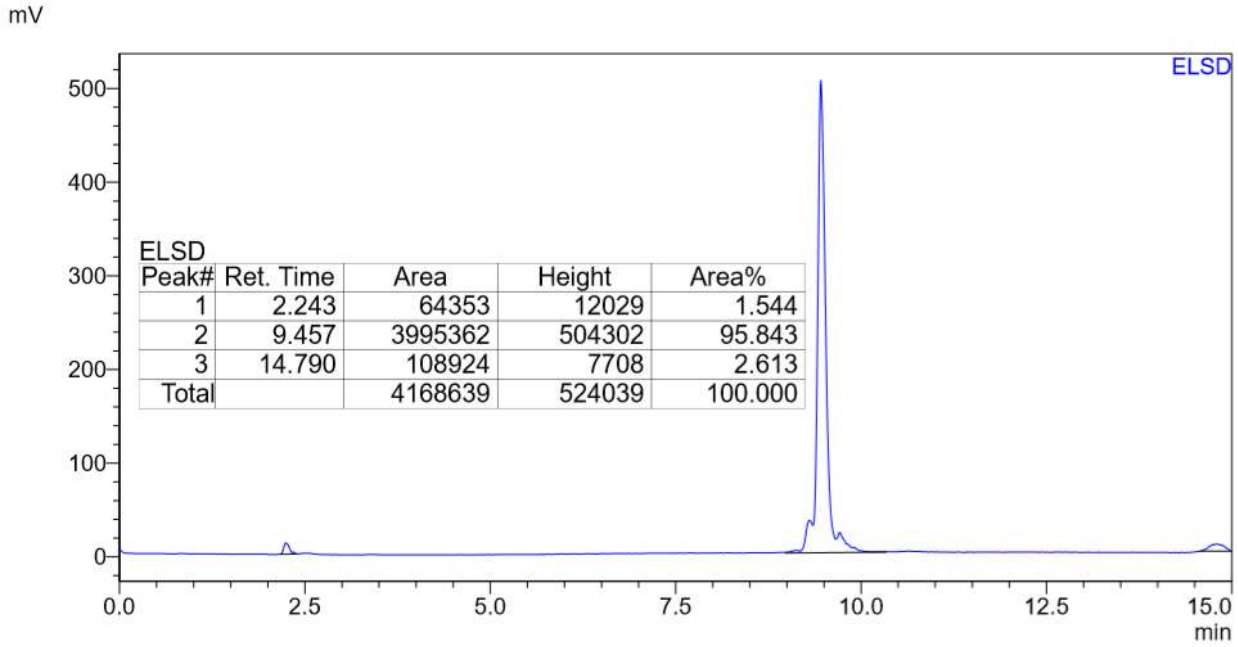


Figure S9. C18 HPLC analysis of Prepolymer A using ELSD detection
Prepolymer A elutes at 9.5 minutes and represents 97.3% of the total detectable peaks neglecting the peaks at 2.2 and 14.8 minutes that are due to buffer salts, and solvent components.

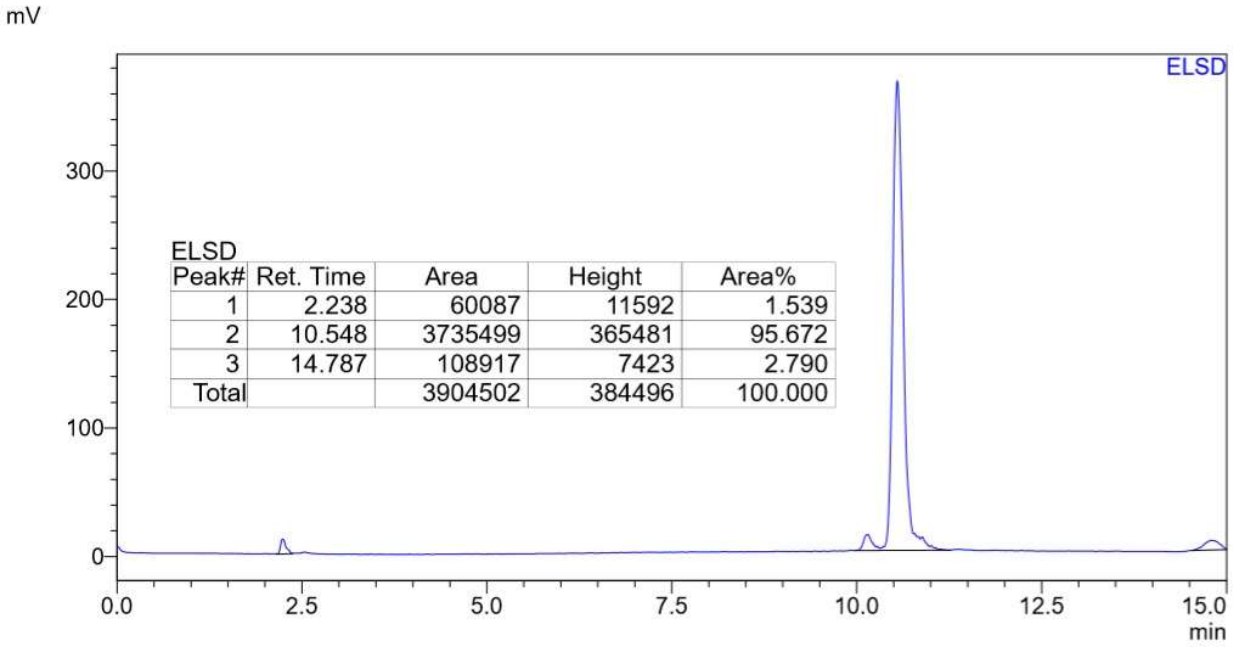


Figure S10. C18 HPLC analysis of prepolymer B using ELSD detection. Prepolymer B elutes at 10.5 minutes and represents 97.2% of the total detectable peaks neglecting the peaks at 2.2 and 14.8 minutes that are due to buffer salts, and solvent components.

2.1.5 Preparation of the continuous phase

Decane (1460 g), PGPR (20.0 g, Danisco, 451361) and Abil EM90 (20.0 g, Evonik Industries, 420095-L151) were added to a 2000 mL glass GL45 bottle. The bottle was then capped and the contents vigorously stirred until the surfactants were completely dissolved.

2.2 Microfluidic synthesis of amino-MS

The following procedures detail the microfluidic preparation and purification of amino-MS.

2.2.1 Description of equipment

The system used for production and purification of amino-MS is described in **Figure S11** and pictured in **Figure S12. Figure S11** shows all of the fluid paths, control circuits, and components of the system.

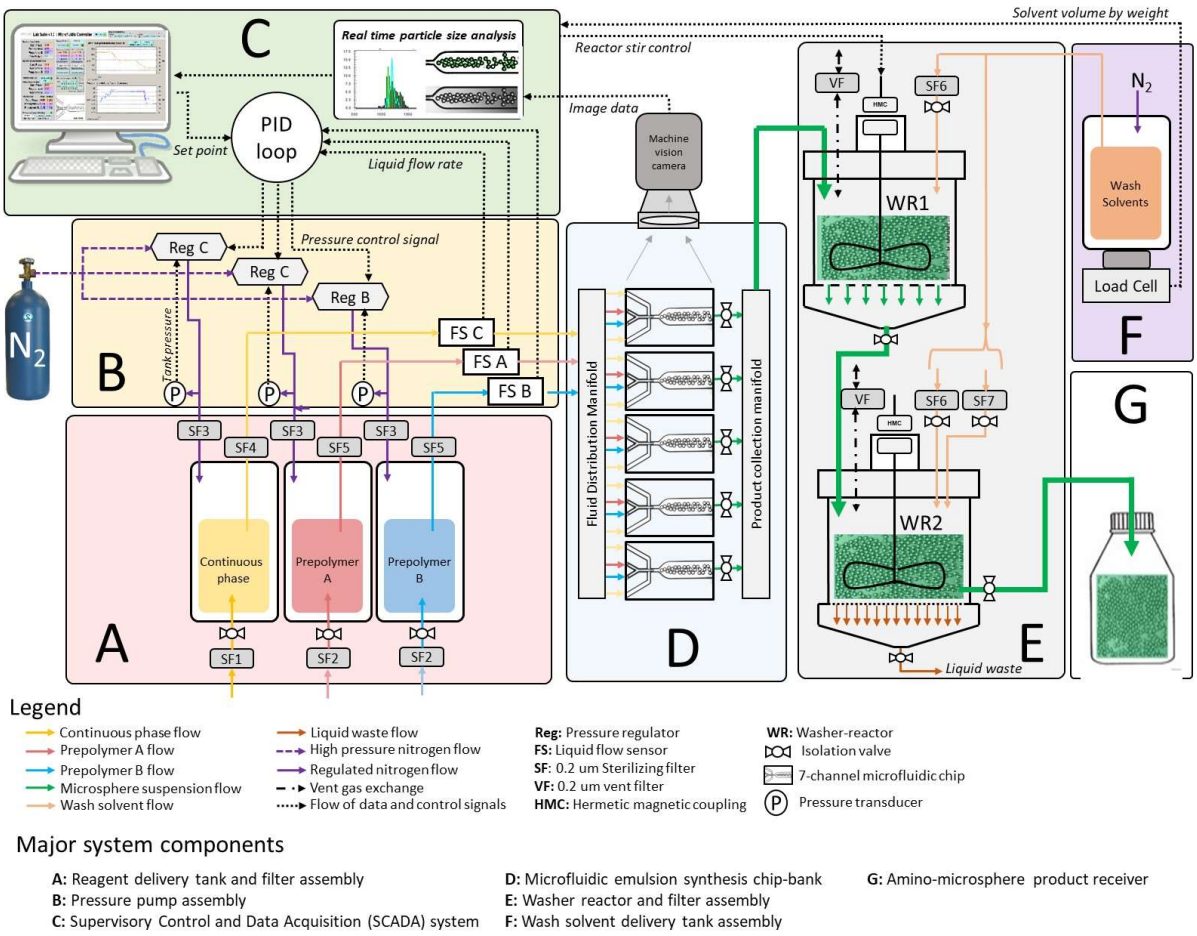


Figure S11. Schematic of the system used to produce and purify amino-MS. A photograph of this system is shown in **Figure S12**.

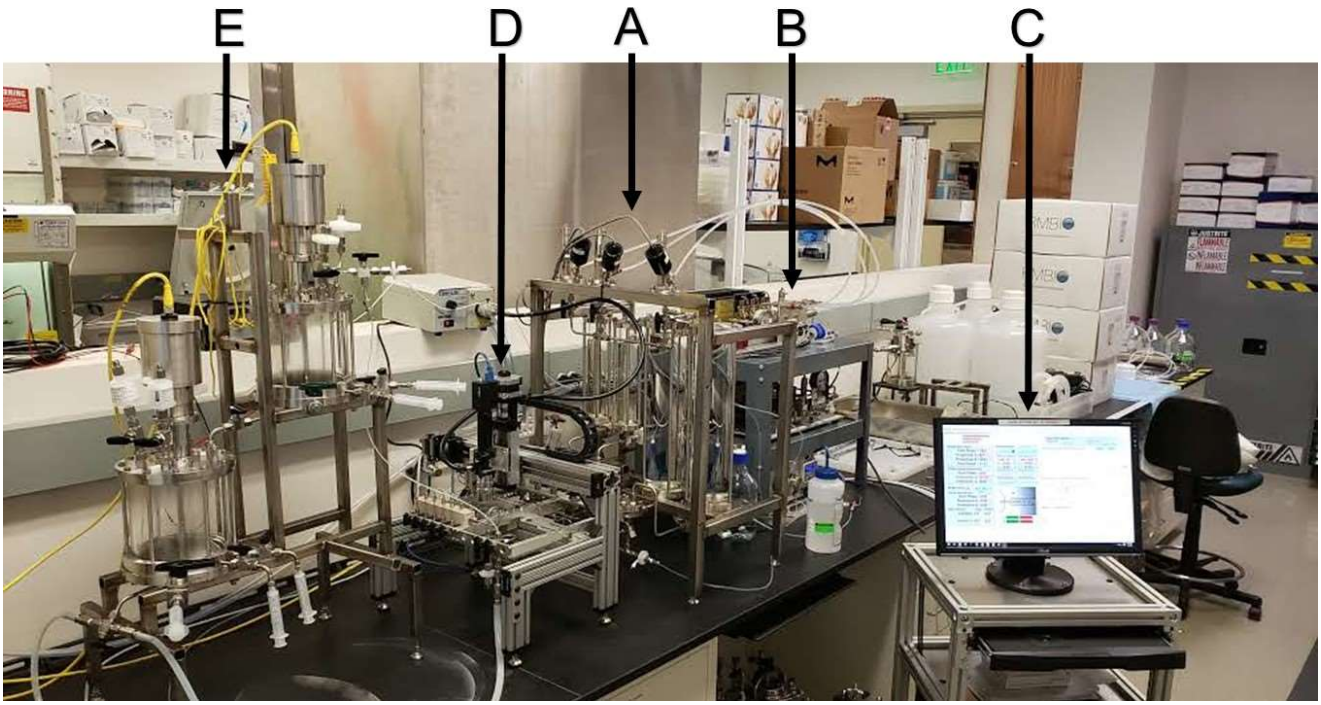


Figure S12. Photograph of the system described in **Figure S11.** The letters refer to the same sub systems as **Figure S11.** (**A**) Reagent delivery tanks and filtration assembly, (**B**) pressure pump assembly, (**C**) SCADA system, (**D**) microfluidic emulsion synthesis chip bank, (**E**) washer reactor and filter assembly.

2.2.2 Clean in place (CIP) procedure

After the system was used to produce a batch of amino-MS, a CIP procedure was used to prepare the system for production of a new batch of amino-MS. The CIP procedure consisted of removal of all filters and microfluidic chips from the system then attachment of silicone tubing to the three process contacting portions of the system: the reagent delivery tank assembly, the chip bank, and the washer reactors (**Figure S11 and S12 A, D, and E**). These three assemblies were cleaned individually. The silicone tubing was used to deliver cleaning reagents to the entire process contacting surface of an assembly in a counter-gravity fashion entering through the lowest point in the system and exiting through the upper most portion. A stainless steel centrifugal impeller pump (Chugger, TCPSSMAX-CI) was used to deliver reagents from a polyethylene carboy as shown in **Figure S13A**. A 0.2 µm PES filter (Pall, Acropak 800, 12464l) installed at the exit of the pump was used to filter cleaning fluids. After thoroughly flushing in this fashion, the equipment was drained by gravity as shown in **Figure S13B**. This process was conducted with the following cleaning solutions in this order: 99% isopropanol (USP grade), twice with CIP100 alkaline detergent (Steris) holding for 1 hours prior to draining, three times with water verifying that the effluent from the last wash was neutral to universal pH paper (Whatman, 2600-100A), and finally the equipment was dried by flushing with nitrogen gas. The washer-reactors were stirred during this process using a repeating program that would “pulse” the stirrer three times at 300 RPM for 2 seconds each pulse then stirred at 100 RPM for 1 minute.

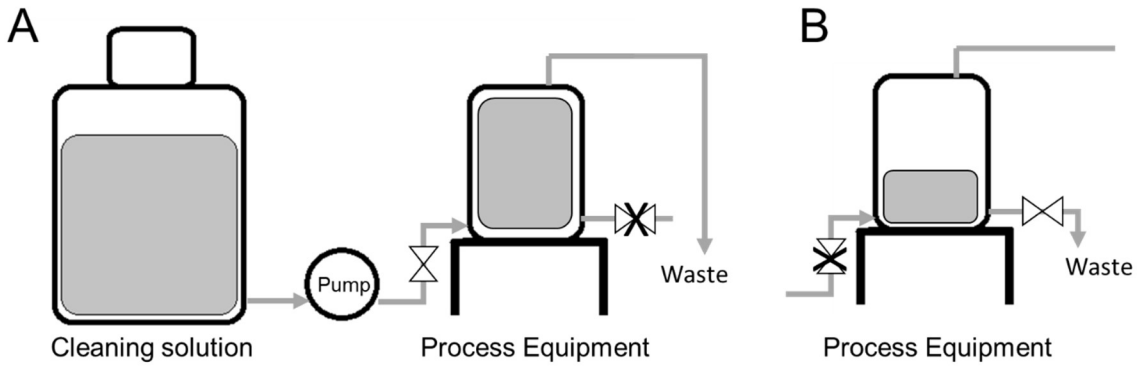


Figure S13. Flow paths for CIP of process assemblies. (**A**) Step 1. Cleaning solution is fed from a carboy and flushed through the process equipment from bottom to top. (**B**) Step 2. The cleaning solution is drained from the equipment by gravity, top to bottom. This process (Step 1 and 2) is repeated with: CIP100, water, and isopropanol.

2.2.3 Sterilizing filters

After cleaning, the system was fitted with the sterilizing filters listed in **Table S1**.

Table S1. Sterilizing filters used in the amino-MS production system.

Location [1]	Membrane	Style	Manufacturer	Part Number	Process Fluid
SF1	0.2 µm PTFE	40 mm disk membrane in molded polypropylene housing	Saint Gobain	D40CF0201N1N	Continuous phase
SF2	0.2 µm PES	40 mm disk membrane in molded polypropylene housing	Saint Gobain	D40CS0201N1N-PH	Prepolymer A and B feed stock
SF3	0.2 µm PVDF hydrophobic	47 mm disk in high pressure stainless steel housing	Millipore	GVHP04700	Nitrogen gas
SF4	0.2 µm PVDF hydrophobic	47 mm disk in high pressure stainless steel housing	Millipore	GVHP04700	Continuous phase
SF5	0.2 µm PVDF hydrophilic	47 mm disk in high pressure stainless steel housing	Millipore	GVWP04700	Prepolymer A and B feed stock
SF6	0.2 µm PTFE	Pleated membrane in polypropylene capsule	Saint Gobain	JKPF0201N1N-NO	Continuous phase and heptane
SF7	0.2 µm PES	Pleated membrane in polypropylene capsule	Saint Gobain	JKPS0201N1N-NO	Continuous phase and heptane
VF	0.2 µm PTFE	50 mm disk membrane in molded polypropylene housing	Pall	4400	Washer reactor vents / nitrogen inlets

[1] For system location references refer to **Figure S11**.

2.2.4 Calibration of flow sensors

Prior to use, the Prepolymer A and B flow sensors (**Figure S11 FSA and FSB**) were calibrated to ensure accurate mixing during amino-MS production. The sensors employed were a glass capillary type with a thermal sensing mechanism (Sensirion, SLI-1000) and contained no moving parts. The sensors were configured to produce an analog signal (5 – 10 V) proportional to flow of (0 – 1 mL/min). To calibrate the specific response of the each sensor, an HPLC style dual piston pump with a pulse damper (Cole-Parmer, LD012SFT1ACP) was used

as a standard flow source with water as the calibration fluid. The average voltage produced by the sensor over 80 seconds was recorded for flow rates of: 0.00, 0.20, 0.40, 0.60, 0.80, and 0.90 mL/min, then liner regression was used to determine the response equation: ***volts = slope(flow)+intercept*** from a plot of voltage vs. flow (**Figure S14**). To test the accuracy and precision of the calibrations, the sensors were tested at two additional flow rates near the upper and lower limits of the calibration curve (0.15 and 0.85 mL/min) that bracket the process conditions. The voltage produced at those two flow rates must match the voltage calculated for those flows from the calibration response equation within 1% for the calibrations to be accepted. The system control software employs the response equation for conversion of sensor volts to flow during the amino-MS production process.

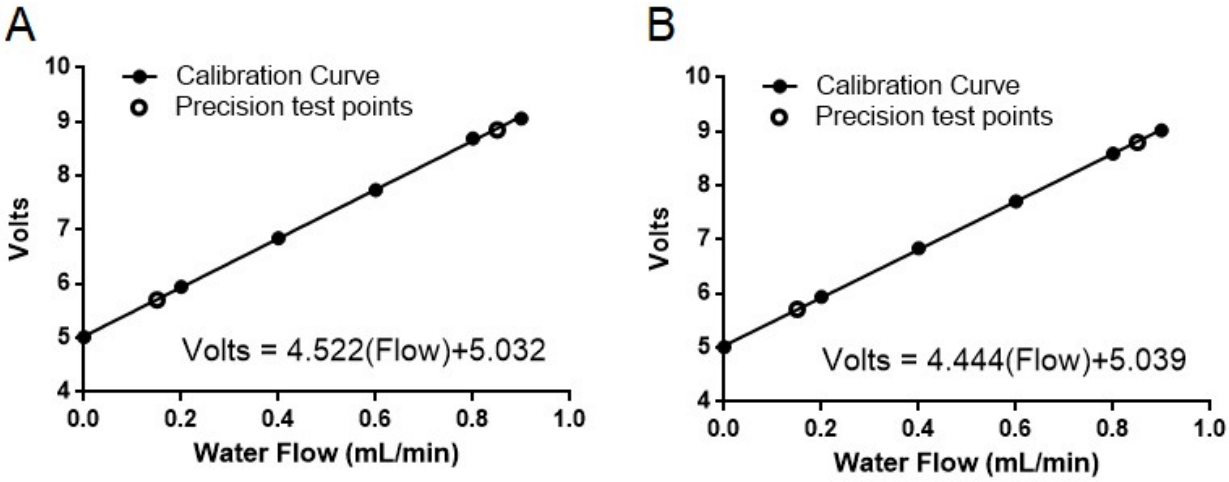


Figure S14. Calibration curves for prepolymer feed stock flow sensors including precision test points. (**A**) Calibration of the Prepolymer A feed stock sensor. (**B**) Calibration of the Prepolymer B feed stock sensor. The precision test points must agree within 1% of the expected values for the calibrations to be accepted.

2.2.5 Microfluidic production of emulsion

Final equipment setup: After cleaning, installation of sterilizing filters, and flow sensor calibration as described above, five microfluidic chips (Dolomite, Telos, 7 x 50 µm drop forming channels each) were installed into the chip bank (**Figure S15 A**), using oval shaped FFKM "O-rings" (Dolomite, 3200371) to seal the chips to the PEEK chip-manifold. The fluidic lines were then connected between the major assemblies to complete the flow paths shown in **Figure S11**.

Charging of reagents: The prepolymer feed stock solutions (250 mL each) and continuous phase (3 L) were filtered into the reagent delivery tanks through inlet filters (**SF1 and SF2, Figure S11**, **Table S1**). Solutions were delivered to the filters from glass GL45 laboratory bottles (Duran, pressure plus) at constant pressure (20 PSIG, nitrogen) from FEP dip tubes.

Production of emulsion: Production of emulsion was then carried out using the computer controlled pressure pump system to drive the flow of reagents through the microfluidic chips. This system (**Figure S11C**), composed of a custom application written in DAQ Factory (Azeotech), runs a software PID loop for each process fluid: continuous phase, Prepolymer A and Prepolymer B. The PID loops take the intended flow rates as the set point, the voltage from the flow sensors (mathematically converted to flow within the software) as the process

variables, and output a control voltage (0-10 V) to precision pressure regulators. The pressure regulators (Proportion Air, MPV2PBNEE2P150PSGAXL) regulate nitrogen pressure to the reagent delivery tanks (≤ 100 PSIG for continuous phase and ≤ 150 PSIG for prepolymers) to drive and maintain process fluid flow. The pressure of each tank was monitored at the outside of the nitrogen inlet filters (**Figure S11 SF3**) with an analog pressure transducer (Proportion Air, DSTEX06ZP150 PSGB, 0 – 10 V for 0 - 150 PSIG) attached to the input of pressure regulators. A USB data acquisition card (Lab Jack, U6 PRO) fitted with two analog output (two 14 Bit, 0-10 V outputs each) daughter boards (Lab Jack, LJTick-DAC) was used for analog input and output to the computer. Typical process flow rates were: 60 mL/h for the continuous phase and 10 mL/h for Prepolymers A and B. Needle valves directly before the microfluidic chip manifold were used set the pressure required to 50 PSIG for the continuous phase and 75 PSIG for the prepolymers at the beginning of the run.

Process monitoring: During production of emulsion, a custom DAQ Factory software application was used to log the operating pressures and flow rates of the three process fluids and acquire images of the microfluidic drop forming channels using a custom robotic microscope (**Figure S15 B**). The robotic microscope was constructed using a two axis linear actuator system. The X axis actuator (Thomson, MS25LF0N0425-050N001A0A08) was used for positioning the microscope over the microfluidic channels, and the Z (perpendicular) axis actuator (Thomson, MS25LB0N0146-031N001A0A08) was used for the focal plane. Both actuators were powered with stepper motors fitted with 200 pulse/rev quadrature encoders (Oriental Motor, PKP264D28A-R2E) for true closed loop operation. Motion control was achieved with motor control boards (All Motion, EZHR23ENHC) that accepted commands from our DAQ Factory application through an RS485 serial communication interface. The microscope consisted of a monochrome 1/1.8" format 3.2 megapixel CMOS USB 3.0 camera (FLIR, GS3-U3-32S4M-C), attached to an infinity corrected tube lens (Infinity, InfiniTube FM-200), a coaxial illumination adapter (Infinity, 991168), and a 5x Plan APO objective (Edmund, 59876). Illumination was provided by a fiber optic white light source (Dolan-Jenner, Fiber-Lite DC950). Images of each drop forming channel (**Figure S15 C**) were recorded every 5 minutes of the run for quality control. Periodically a channel would fail and produce large drops (~200 µm, **Manuscript Figure 3**). To restore performance the prepolymer flows were stopped, allowing continuous phase to flow for 5 minutes, then prepolymer flows were resumed. If that procedure failed to restore performance, all flows would be stopped and the failed chip was replaced.

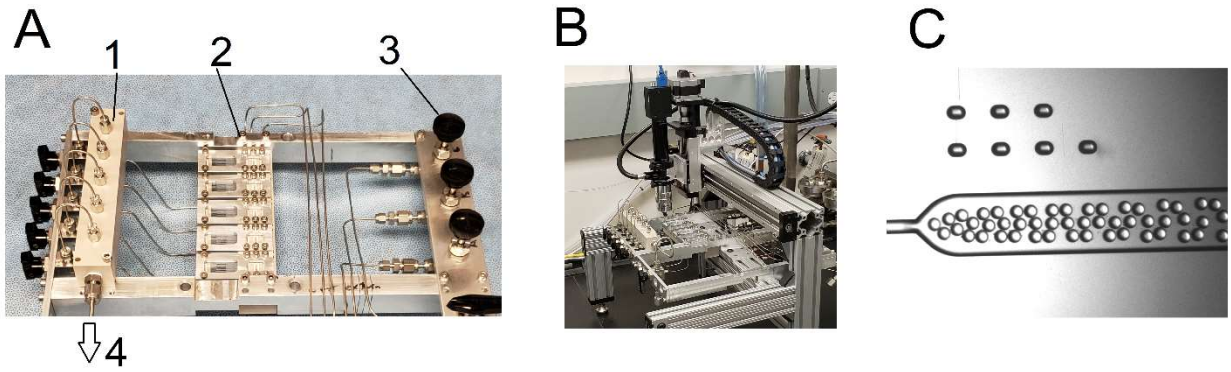


Figure S15. Microfluidic chip bank and imaging system. (**A**) Chip-bank showing: 1) emulsion collection manifold with chip isolation valves, 2) microfluidic chips in the chip-bank manifold, 3) needle valves used to control the fluidic resistance for the three feed stock solutions (Prepolymer A, Prepolymer B and the continuous phase). (**B**) The robotic microscope used for imaging the drop formers. (**C**) A micrograph of a functioning drop former.

2.2.6 Polymerization and purification of amino-MS

Polymerization: The entire batch of emulsion was collected in washer reactor 1 (**Figure S11, WR1)** without stirring as stirring prior to polymerization leads to greater polydispersity presumable due to shear and or coalescence of droplets. This consisted of 500 mL of dispersed prepolymer A/B mix, and ~1500 mL of continuous phase collected over 25 hours. To polymerize (fully crosslink) the emulsion into microsphere hydrogel particles, the emulsion was heated to 40 ^{o}C for 18 h. Heating was achieved with a 3"x18" silicone band heater (BenchMark Thermal, 5 watts/in^2, EFH SH-3X18-5-115) attached to the outside of the glass reactor body. The temperature of the reaction was controlled using a PID temperature controller (Omega, Platinum Series CS8DPT-C24-EIP-A) and a stainless steel sheathed type K thermocouple probe (McMaster Carr,3856K86) mounted in the upper bulkhead of the reactor with the sensing tip inside of the emulsion.

Purification of amino-MS: After polymerization, the amino-MS in washer reactor 1 were stirred at 100 RPM to form a homogenous suspension. While stirring, washer reactor 1 was pressurized to 5 PSIG with nitrogen and the suspension was drained through the stainless steel sieve cloth (McMaster PN 9419T13, Dutch Weave 80 um) in the base of washer reactor 1 into washer reactor 2. The excess continuous phase was drained through the sieve cloth (McMaster 9419T38, Dutch Weave 20 um) in the base of washer reactor 2 while stirring at 100 RPM and pressurizing washer reactor 2 to 10-20 PSIG with nitrogen. Washer reactor 1 was then rinsed twice with 400 mL continuous phase by filtering the solution in through SF6 (**Figure S11, WR1)**, by dip tube transfer from a 2L GL45 bottle at 20 PSIG with nitrogen, while stirring at 100 RPM, then draining through the sieve into washer reactor 2. The excess continuous phase was again drained from washer reactor 2 as described above. The thick slurry in washer reactor 2 was then washed six times with heptane (1.4 L per wash) to remove continuous phase and surfactants. Heptane was filtered into washer reactor 2 through SF6 (**Figure S11, WR2**), by dip tube transfer from a stainless steel tank (**Figure S31A)** at 20 PSIG with nitrogen, then the washer reactor was stirred at 50-100 RPM, and pressurized to 12 PSIG with nitrogen while draining the solvent through the sieve in the base. After heptane washing, the slurry was washed using the technique described for heptane using filter **SF7**, six times with ethanol (190 proof, 1 L per wash), twice with water (1 L per wash), then six times with storage buffer (100 mM, pH 4.0, sodium acetate / acetic acid, 1 L per wash). After washing with ethanol and water, the slurry swells to twice its initial volume (~1 L after swelling). After removal of excess buffer, the slurry (~1 L) was drained from washer reactor 2 under a pressure of 2 PSIG with nitrogen, through the 3/8" port above the sieve in the lower bulkhead and collected into a 2 L GL45 glass laboratory bottle. Washer reactor 2 was then rinsed twice with storage buffer (350 mL per rinse), and the rinsed combined with the slurry in the bottle to give 1400 mL of dilute amino-MS suspension. This suspension was analyzed as described below, and stored at 4 ^{o}C ready for autoclave sterilization prior to further use.

2.3 Analysis of amino-MS – Table of results

The following procedures describe the analytical methods used for characterization of amino-MS as a slurry in pH 4 acetate buffer. The results of all methods are given below in **Table S2**.

Table S2. Analytical Release of Amino-MS Slurry in pH 4.0 Acetate Buffer [1]

Assay Parameter	Analytical Method	Engineering Batch Value	Acceptance Specification
Appearance	Visual inspection	Thick clear translucent slurry [2]	No discoloration, volume change, or growth [3]
[Amine]/[PEG]	free-amine/PEG content	275 ± 11 nmol free NH_2 / mg of PEG [2]	253 – 297 nmol free NH_2/mg of PEG [3]
Dissolution time (t_{RG})	Kinetics of dissolution at pH 9.4, 37 ºC	19.1 ± 0.3 hours [2]	18.5-25.3 hours [3]
Particle size	Microscopy and image analysis	67 ± 7.5 µm mean diameter [2]	10 - 80 µm distribution [3]
pH	pH electrode	4.1 [2]	3.5 - 4.5 [3]
Residual alkyl-azide	Spectrophotometric determination	57 ± 14 µM [2]	To be reported
Residual surfactant PGPR 90	As ricineolic acid after digest, by HPLC-ELSD	< 1.6 PPM [2]	To be reported
Residual Abil EM90	As total Si by ICP MS	58.7 PPM Si [2]	To be reported
Residual solvents	USP <467>	To be determined	Decane < 5000 [4,5] Heptane < 5000 ppm [4] Ethanol < 5000 ppm [4] Isopropanol < 5000 ppm [4]
Elemental Impurities	UPS <232>	To be determined	Class I As 15 ppm [6] Cd 2 ppm [6] Hg 3 ppm [6] Pb 5 ppm [6] Class IIA Co 5 ppm [6] V 10 ppm [6] Ni 20 ppm [6] Class IIB Se 80 ppm [6,7]
Bioburden	USP <61>	Total Aerobic microbes <10 CFU/g of slurry Combine yeast/mold <10 CFU/g of slurry [2]	<10 CFU/g of slurry [3]
Endotoxin	USP <85>	0.34 ± 0.09 EU/mL [2]	<310 EU/mL [8]

1 Analytical procedures are given below.

2 From the batch of amino-MS reported here, reported errors are: SD n=4 for [Amine]/[PEG], range/2 n=2 for t_{RG}, SD n > 100 for particle size, SD n=3 for endotoxin.

3 Preliminary acceptance specifications are estimates of those anticipated.

4 Residual solvent limits are set to the Permitted Daily Exposure limits according to ICH: GUIDELINE FOR RESIDUAL SOLVENTS Q3C(R6) 2016. The listed limits are for finished drugs assuming a 1 mL daily dose.

5 Unlisted in ICH guidance, chemically analogous to Heptane, Pentane (< 5000 ppm).

6 Elemental impurities are set to the Permitted Daily Exposure limits for parenteral administration assuming a 1mL dose as in ICH: GUIDELINE FOR ELEMENTAL IMPURITIES Q3D 2014.

7 Selenium is used in the synthesis of a precursor to Prepolymer B.

8 Specification based on limit dose of 5 EU/kg (USP <85>) assuming a dose of 1 mL.

2.3.1 Appearance of amino-MS slurry

For fresh lots and stability samples: a container (GL45 glass media bottle) of amino-MS slurry was visually inspected for: color (inspector's interpretation of the slurry's color), volume within the container (mL), and growth (observable growth of bacteria, fungus, etc.). A photo of the slurry (in the container) was taken against a black background and saved for record (**Figure S16**).

Figure S16. Photo of a 60 mL stability sample of amino-MS slurry in pH 4 acetate buffer. The sample in the photo was stored at 4 ± 2 °C for 4 months. The color of this sample was recorded as "*standard – translucent white slurry,*" no observable growth was present, and the volume of the slurry was 60 mL unchanged from its original volume.

2.3.2 [Amine]/[PEG]

[amine]/[PEG]: The amine to PEG content ratio (*Ratio*$_{amine/PEG}$) of the amino-MS was calculated from individual measurements of the amine (nmol_amine/gram_slurry) and PEG (mg PEG/gram_slurry) content of amino-MS slurry using the equation shown in **Figure S17A**. The standard deviation in the *Ratio*$_{amine/PEG}$ is given by the equation shown in **Figure S17B**. The spectrophotometric methods used for measuring the amine and PEG content of the amino-MS slurry are given below. From the results of those assays given below, the results were *Ratio*$_{amine/PEG}$ = 275 ± 11 nmol amine / mg PEG.

A

$$\text{Ratio}\left[\frac{\text{nmol amine}}{\text{mg PEG}}\right] = \frac{AVG_{amine}\,[\text{nmol amine / g slurry}]}{AVG_{PEG}\,[\text{mg PEG / g slurry}]}$$

B

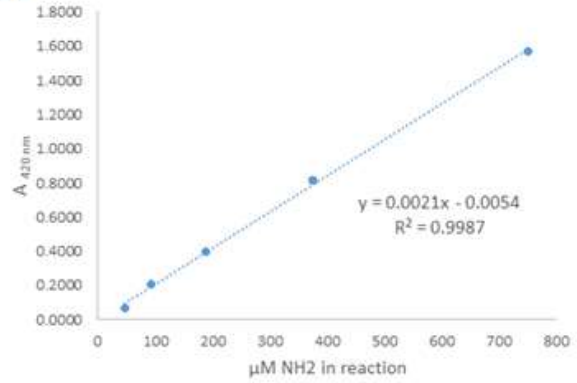
$$StDev_{\text{amine/PEG}} = Ratio_{\text{amine/PEG}} * \sqrt{\left(\frac{SD_{\text{amine}}}{Avg_{amine}}\right)^2 + \left(\frac{SD_{\text{PEG}}}{Avg_{\text{PEG}}}\right)^2}$$

Figure S17. Equations used to calculate the amine to PEG content ratio of amino-MS. (**A**) Equation for the *Ratio*$_{amine/PEG}$ where *AVG*$_{amine}$ and *AVG*$_{PEG}$ are the average values from quadruple determinations of the amine and PEG content of an amino-MS slurry. Where amine concentration in µM is equivalent to (nmol amine / g slurry). (**B**) Equation for the standard deviation (*StDev*$_{amine/PEG}$) in the *Ratio*$_{amine/PEG}$ where: *SD*$_{amine}$ and *SD*$_{PEG}$ are StDev from quadruple determinations of the amine and PEG content of an amino-MS slurry and *AVG*$_{amine}$, *AVG*$_{PEG}$ are the values described in part A.

Preparation of amino-MS digest: In quadruplicate, amino-MS slurry (0.090 - 0.110 g) was dissolved in sodium hydroxide (50 mM, NaOH, 9.000 mL per g of slurry) for 1 h at 18-25 ºC to prepare *amino-MS digest.*

Determination of amine content. Amines were measured spectrophotometrically in amino-MS digests by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) followed by quantitation of the resulting adduct at 420 nm.[3] In quadruplicate, a sample of amino-MS digest (0.060 mL) was treated with a solution of sodium borate (100 mM, pH 9.3, 0.090 mL) and a solution of TNBS (0.04%, 0.150 mL) in sodium borate (100 mM, pH 9.3). After 3.5 hours at 31ºC, the absorbance of the solution was measured at 420 nm using a plate reader (Molecular Devices, Spectramax i3 plate reader) set to report absorbance values equivalent to a 1 cm path length. The amine concentration was calculated using a linear standard curve of (A_{420} vs. [amine]) that was generated by this same procedure with a solution of lysine (0.047 - 0.75 mM) in place of the MS digest (**Figure S18**). The absorbance of an identical solution containing only TNBS (no amines) was used for background subtraction, for MS and lysine samples.

A



B

Sample	$A_{420\ nm}$	Conc.
A	1.272	3019
B	1.165	2766
C	1.271	3017
D	1.246	2958
Average	---	**2940**
StDev	---	**119**

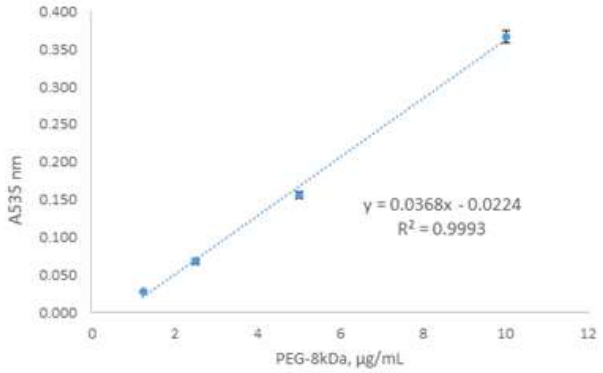
C

$$C\left[\frac{\text{nmol amine}}{\text{g slurry}}\right] = \frac{A_{420nm} - b}{m} * DF * CF$$

Figure S18. Determination of free amine content of amino-MS slurry. (**A**) Lysine standard curve. (**B**) Table of raw data values from a representative analysis showing a result of 2940 ± 119 (nmol amine/g slurry). (**C**) Equation used to calculate free amine content where: $A_{420\ nm}$ the average absorbance value from four samples, b is the y-intercept from the linear standard curve, m in the slope of the linear standard curve, *DF* = 10 is the dilution factor for dilution of amino-MS slurry into sodium hydroxide, *CF* = 0.5 is a conversion factor to convert total amine content of the digest to free amines present on the amino-MS.

Spectrophotometric determination of PEG content: PEG content was measured spectrophotometrically in amino-MS digests using a modification of a reported procedure that determines PEG as a complex with barium and iodide ions.[4] In quadruplicate, a sample of amino-MS digest (0.020 mL) was diluted with water (0.980 mL) to give *diluted MS digest*. A sample of the resulting solution (0.200 mL) was acidified with perchloric acid (0.5 M, 1.000 mL), then a sample of the acidified solution (0.200 mL) was treated with a solution (0.075 mL) containing 3.33% BaCl2, 0.03% I_2 and 0.06 % KI w/v. After 5 min, the absorbance of the solution was measured at 535 nm using a plate reader (Molecular Devices, Spectramax i3 plate reader) set to report absorbance values equivalent to a 1 cm path length. The amount of PEG (10.7 ± 0.1 mg PEG/g slurry) was calculated using a linear standard curve of (A_{535} vs. [PEG]) that was generated by this same procedure with a solution of 8000 MW linear PEG (7.5 -60.0 µg/mL) in place of the diluted MS digest (**Figure S19**). The concentration of the PEG standard was determined by quantitative ^{1}H-NMR using DMF as a standard using a previously reported method.[5]

A



B

Sample	$A_{535\ nm}$	Conc.
A	0.111	10.76
B	0.109	10.60
C	0.111	10.76
D	0.112	10.83
Average	---	**10.7**
StDev	---	**0.1**

C

$$C\left[\frac{\text{mg PEG}}{\text{g slurry}}\right] = \frac{A_{535\ nm} - b}{m} * DF * CF$$

Figure S19. Determination of the PEG content of amino-MS slurry. (**A**) PEG standard curve with error bars showing StDev of three replicates per point. (**B**) Table of raw data values from a representative analysis showing a result of 10.7 ± 0.1 (mg PEG/g slurry). (**C**) Equation used to calculate PEG content where: $A_{535\ nm}$ is the average absorbance value from four samples, *b* is the y-intercept of the linear standard curve, m is the slope of the linear standard curve, *DF* = 3000 is the dilution factor for dilution of amino-MS slurry into sodium hydroxide and water, and *CF* = $1x10^{-3}$ is a conversion factor to convert (µg PEG/mL slurry) to (mg PEG/ g slurry) assuming the density of the slurry is 1 g/mL.

2.3.3 Amino-MS Dissolution time (t_{RG})

Dissolution cells and heat block*:* Custom dissolution cells and a heat block were used for dissolution reactions. The PEEK dissolution cell was composed of a hexagonal cylinder and two flanges held together by stainless steel screws. The flanges were used to seal nylon mesh (335 x 335 mesh size, 0.0015", McMaster Carr 9318T24) to the top and bottom of the cylinder, forming a ~1 mL chamber capable of retaining MS while being freely permeable to buffer and dissolution products (**Figure S20A)**. The side of the cylinder had a threaded hole used for filling the cell with MS that was sealed with a stainless steel set screw (**Figure S20B**). The dissolution cell was placed into a 25 mL centrifuge tube containing the dissolution buffer, a magnetic stir bar (VWR, 58949-276), and a septa cap (Syringa Lab Supplies, SeptaSecure 1J920.20) to form the dissolution reaction vessel (**Figure S20C**). A custom heat block with sockets for six 25 mL reaction vessels was machined from 6061-T6 aluminum. The heat block (**Figure S20C**) allowed for complete immersion of the tubes to prevent condensation from collecting in the upper portion of the tubes, and fit on top of a six position magnetic stirrer (2mag AG, MIXdrive 6 MTP). The temperature was controlled using PID controller (Omega Platinum series CS8DPT-C24-EIP-A), connected to a 3" x 18," 2.5 watt/in^2 adhesive back silicon band heater (Benchmark Thermal) wrapped around the heat block, and a type K thermocouple with a 6"x1/8" stainless steel sheath (McMasterCarr, 3856K912) placed into one of the 25 mL tubes containing 25.0 mL of water and a stir bar.

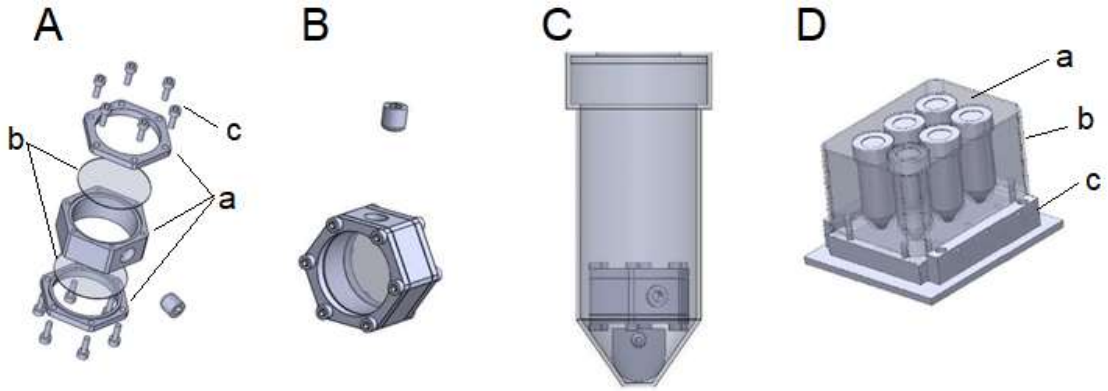


Figure S20. CAD model of the dissolution cell, dissolution reaction vessel and heat block with magnetic stirrer. (**A**) Individual parts of the cell showing: (a) PEEK body, (b) nylon mesh membrane, and (c) stainless steel screws. (**B**) An assembled cell with stainless steel set screw plug for the filling hole. (**C**) Cell in dissolution reaction vessel: a 25 mL centrifuge tube with a septa cap. (**D**) Aluminum heat block with stirrer: (a) aluminum body, (b) silicon band heater around the perimeter, (c) aluminum base containing a 6 position magnetic stirrer.

Liquid handling robot: A liquid handling robot (**Figure S21A**) was built from a stepper motor driven 3-axis CNC router gantry (Vevor, 6040Z) where the Z (vertical) axis was fitted with a custom syringe pump (**Figure S21B**), machined from 6061-T6 aluminum, that employed a hybrid stepper linear actuator (Hydon, 28H47-2.1-915) to operate a 1 mL glass syringe with a septa piercing needle (Hamilton, Gastight 81330). The horizontal X and Y axis were used for positioning the syringe to acquire samples from the dissolution cells in the heat block described above, and to dispense samples into 0.3 mL septa capped sample vials (Microsolv, 9532S-0PV-K) at programmed time points. Motion control of the X, Y, Z and syringe (S) axes was achieved with a 4 axis stepper motor control board (All Motion, EZ4AXIS23WV) that accepted commands from a custom control application written in DAQ Factory (Azeotech) software, through an RS485 serial communication interface. After dissolution, samples were collected and the robot was used to transfer portions of the samples from the 0.3 mL septa capped vials to 96 well plates for analysis as described below.

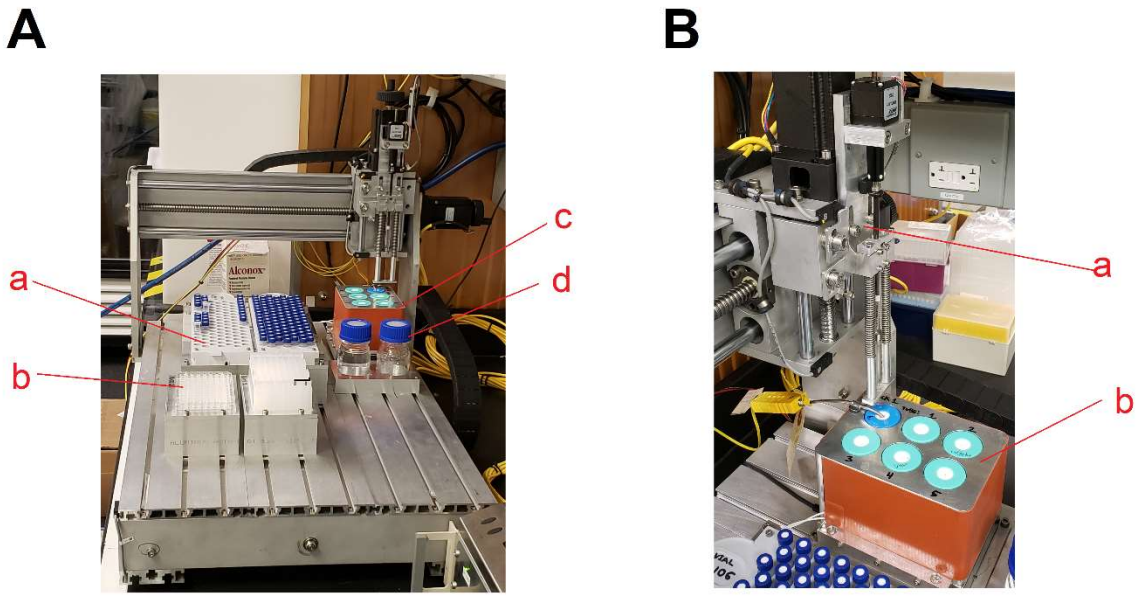


Figure S21. Liquid handling robot used for microsphere dissolution testing. (**A**) Photograph of the robot showing: (a) racks containing 0.3 mL septa capped sample vials, (b) racks containing microtiter plates for collection of analytical samples, (c) heat block with dissolution reaction vessels, (d) septa capped GL45 bottles of water for syringe washing. (**B**) Close up photograph of the syringe pump containing a 1 cc glass syringe (a), and the heat block containing the dissolution reaction vessels (b).

Dissolution Reactions: In duplicate**,** amino-MS were separated from excess buffer by pelleting at 20,000 Gs for 5 minutes in 1.5 mL microcentrifuge tubes, followed by removal of the supernatant. A 0.50 g sample of the pelleted microspheres was transferred to a dissolution cell using a capillary piston pipette (Gilson Microman, M100E). The cell was then placed into the dissolution reaction vessel described above, containing 20.0 g of borate buffer (100 mM sodium tetraborate, pH 9.4 at 37 ºC) preheated to 37 ºC. The vessel was stirred at 300 RPM then 0.250 mL samples were collected at: 0, 2, 4, 6, 8, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 28, and 30 h, and transferred to 0.300 mL septa top sample vials (Microsolv, 9532S-0PV-K) by the liquid handling robot.

Sample Analysis: A portion of each sample (0.100 mL) was transferred to a 96 well dilution plate (2.5 mL/well capacity, VWR, 37001-518) containing water (1.900 mL/well) to give diluted supernatant samples. The diluted samples were then acidified by transferring 0.400 mL to a second 96 well dilution plate containing perchloric acid (0.800 mL, 0.5 M). Next, samples of the acidified solutions (0.200 mL) were transferred to a 96 well clear bottom plate (Corning, 3635) and treated with 0.075 mL of a solution containing 3.33% $BaCl_2$, 0.03% I_2 and 0.06 % KI w/v. After incubation for 5 minutes at room temperature, the absorbance of each resulting analytical reaction was measured at 535 nm using a plate reader (Molecular Devices, Spectramax i3). A linear standard curve of A_{535} vs [PEG] that was generated by this same procedure with a solution of 8 KDa linear PEG in water (0.00375-0.030 mg/mL) in place of the diluted supernatant samples. The absorbance of the initial sample (t_0) was used for background subtraction.

Determination of t_{RG}: The time to reverse gelation was determined from a plot of solubilized PEG (mg) vs. time. For each time point the amount of solubilized PEG was calculated by: first using **Equation S2** to calculate corrected A_{535} values ($A_{C,n}$) that factor out the reduction in reaction volume and removal of PEG from previous samples. The concentration of PEG in each sample was then calculated from $A_{C,n}$ using the linear standard curve, and then converted to mg of PEG released using **Equation S3**. The resulting plot was then fitted to a sigmoidal function (**Equation S4**) within Graphpad software (Prism, Version 8). The t_{RG} of the sample was defined as the log(EC_{90}) value calculated from the fit using **Equation S5** within Graphpad.

Equation S2 $$A_{\mathrm{C,n}} = \frac{A_{\mathrm{M,n}} * V_{\mathrm{n}}}{V_i} + \frac{V_{\mathrm{s}}}{V_i} * \sum_{\mathrm{n=1}}^{\mathrm{n}} \left[\frac{A_{\mathrm{M,n-1}} * V_{\mathrm{n-1}}}{V_i}\right]$$

Where:

$A_{C,n}$ = corrected A_{535} value for the nth sample
$A_{M,n}$ = the measured absorbance of the nth sample
V_n = the total volume (buffer + amino-MS slurry) in the dissolution reaction prior to removal of the nth sample
V_i = the initial volume of the dissolution reaction (buffer + amino-MS slurry)
V_s = the sample volume
$A_{M,n-1}$ = 0, for n = 1

Equation S3 $PEG = C * DF * V_i$

Where:

PEG = the amount of PEG released (mg)
C = concentration of PEG (mg/mL) in the diluted supernatant samples determined from $A_{C,n}$ and the PEG standard curve.
DF = for the dilution of samples into water (20)
V_i = the initial volume of the dissolution reaction (buffer + amino-MS slurry) in mL

Equation S4 $PEG = B + [(T - B)/(1 + 10^{(\log(EC_{50}) - t) * H})]$

Where:

PEG = the amount of PEG released (mg)
B = the lower plateau
T = the upper plateau
EC_{50} = the half maximal amount
t = time in hours
H = the hill slope

Equation S5 $\log(EC_F) = \log(EC_{50}) + \frac{1}{H}\log\left(\frac{F}{100-F}\right)$

Where:

$\log(EC_F)$ = the time at which the amount of PEG is 90% of the maximal amount (F = 90)
F = % of maximum amount (90)
H = the hill slope

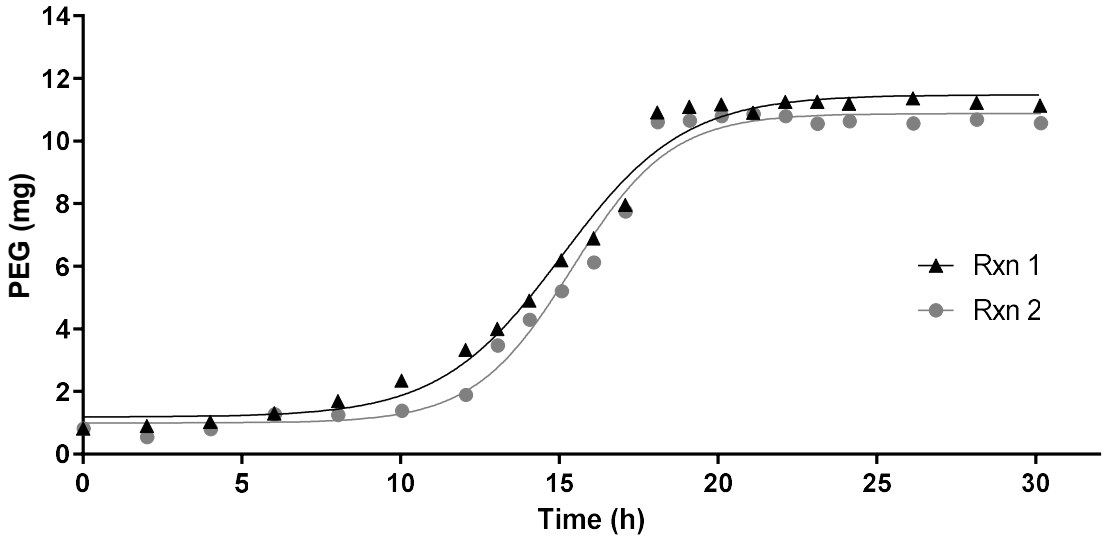


Figure S22. Dissolution curves for amino-MS showing sigmoidal fits used to determine the dissolution time (t_{RG}): Rxn 1 = 19.3 h, Rxn 2 = 18.8 h.

2.3.4 Particle Size

Particle size was measured by microscopy and image analysis. To inhibit aggregation and form a fluid suspension, amino-MS slurry (0.1 g) was diluted with a solution of *N,N*-dimethylformamide (DMF) in water (0.9 mL, 50% v/v). Images of the resulting amino-MS suspension were collected with a white light microscope at 5x magnification (Nikon TMS, SN: 51436) with a 5x objective (Nikon E 4/0.10, 160/- NA) and a monochromatic CCD camera (Unibrain, Fire-I 580b). Images of 50% DMF alone were recorded for background. From three images, the diameter of each particle was measured using an image analysis software (Image J v1.52a). The software was calibrated to convert pixels to µm (1.98 µm pixel-1) by measurement of an image of a microscope stage micrometer (Electron Microscopy Sciences, 60210-3PG). The mean particle size and standard deviation are reported (67 ± 7.5 µm). Images and results from a representative analysis are shown in **Figure S23** below.

Figure S23. Determination of amino-MS particle size by image analysis. Images of amino-MS with quantitation lines across their equators are shown as well as background images and an image of a micrometer used to calibrate the image analysis software.

2.3.5 pH

pH was measured with a B10P Symphony pH meter (VWR), equipped with an Orion ROSS Ultra pH electrode (PN: 8103BNUWP). On the day of use, the instrument was calibrated against three standard buffers: pH 4.0 (Ricca Chem, RC1500-16), 7.0 (Ricca Chem, RC151-16), and 10.0 (Ricca Chem, RC1601-16). The temperature of the standards and samples being measured was recorded with a calibrated digital thermometer (Traceable model 4049, ISO17025 calibrated). Using this method, the amino-MS slurry pH was found to be 4.1 at 22 °C.

2.3.6 Residual alkyl azide

Residual alkyl azide resulting from free Prepolymer A azide end groups was measured spectrophotometrically by the loss of absorbance at 308 nm that occurs when the azide reacts with the cyclooctyne DBCO as described for Prepolymer A (**Scheme S4**). In triplicate, two samples of MS slurry (0.500 mL) in acetate buffer were dissolved by treating with NaOH (1.0 N, 0.025 mL) for 2 h at 18-25 °C in 1.5 mL microcentrifuge tubes. The resulting solutions were neutralized by treating with HCl (1.0 N, 0.025 mL) and pH 7.6 HEPES buffer (2 M, pH 7.6, 0.025 mL) to give *amino-MS digest*.

Reaction A: To mask free azides, one sample of *amino-MS digest* was treated with a solution of (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (BCN or bi cyclooctyne) in DMSO (10 mM, 0.025 mL) for 2 h at 18-25 °C.

Reaction B: The other sample of *amino-MS digest* was treated with DMSO (0.025 mL). **Analysis***:* Reactions A and B were treated with a solution of DBCO-acid in DMSO (10 mM, 0.025 mL). After 4-5 h at 18-25 °C, the absorbance of the samples was measured at 308 nm in 0.2 cm path length cuvettes. The raw data from an example analysis and the equations used for calculation of the result (57 ± 14 uM) are presented in **Figure S24** below.

A

	A_{308_RA}	A_{308_RB}
Sample 1	1.360	1.253
Sample 2	1.401	1.301
Sample 3	1.403	1.240
Average	**1.39**	**1.26**
StDev	**0.02**	**0.03**

B

$$C_{\text{alkyl azide}}\ [\text{M}] = \frac{A_{308\_RA} - A_{308\_RB}}{\varepsilon_{\text{DBCO,308nm}}[\text{M}^{-1}\text{cm}^{-1}] * PL[\text{cm}]} * DF$$

C

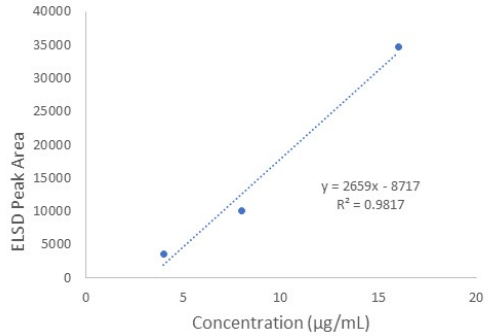
$$StDev[\text{M}] = \frac{\sqrt{\left(StDev_{\text{A308,RA}}\right)^2 + \left(StDev_{\text{A308,RB}}\right)^2}}{\varepsilon_{\text{DBCO,308nm}}[\text{M}^{-1}\text{cm}^{-1}] * PL[\text{cm}]} * DF$$

Figure S24. Determination of residual azide in amino-MS slurry. (**A**) Table of raw data from analytical titration reactions. (**B**) The equation used for calculation of alkyl-azide concentration where: A_{308_RA} is the average absorbance value from *reactions A*, A_{308_RB} is the average absorbance value for *reactions B*, *DF* is the overall dilution factor for dilution for amino-MS slurry into the final reaction (for the procedure above *DF* = 1.25), $\varepsilon_{308\ nm}$ = 13590 $M^{-1}cm^{-1}$ is the extinction coefficient of DBCO-acid, and *PL* = 0.2 cm is the path length. (**C**) The equation used for calculation of standard deviation (*StDev*) in end group concentration where: $StDev_{A380,RA}$ is the StDev in absorbance values for *reactions A*, and $StDev_{A380,RB}$ is the StDev in absorbance values for *reactions B*.

2.3.7 Residual PGPR (Polyglycerol polyricinoleate)

Residual polyglycerol polyricinoleate (PGPR) was determined by saponification of amino-MS slurry in alcoholic KOH to free ricinoleic acid followed by quantification by reverse phase HPLC with UV detection. Amino-MS slurry (0.250 mL) was dried in a speed vac for 1-2 h. The residue was dissolved in a solution of potassium hydroxide in ethanol (0.5 N, 0.250 mL) with heating to 60 °C for 1.5 h to give the *MS digest*. The resulting solution was treated with hydrochloric acid (0.5 N, 0.250 mL) and centrifuged (20,000 G for 5 min). A sample of the supernatant (0.050 mL) was then analyzed for ricinoleic acid by C18-HPLC using an ELSD detector (**Section 1.1**). The amount of ricinoleic acid in the *MS digest* was determined using a linear standard curve generated by this same procedure with a solution of ricinoleic acid in ethanol (4-16 µg/mL) in place of the supernatant sample. The standard curve, table of results from a representative analysis and equation used for calculating the amount of ricinoleic acid are presented in **Figure S25** below. In the data presented, the amount of ricinoleic acid in the samples was lower than the lowest amount of standard quantified. This corresponded to < 1.6 PPM of ricinoleic in the amino-MS slurry. Using the same procedure to determine the ricinoleic acid content of pure PGPR we found a content of 58% w/w ricinoleic acid. This would equate to < 2.8 PPM of PGPR present in the amino-MS slurry analyzed.

A



B

		Retention Time (min)	Peak Area
Standards (0.010 mL injection)	4 ug/mL	6.113	3596
	8 ug/mL	6.101	10039
	16 ug/mL	6.110	34665
Samples (0.050 mL injection)	A	6.108	679
	B	6.120	359
	C	6.151	348

C

$$C\left[\frac{\mu g\ \text{ricinoleic acid}}{\text{mL slurry}}\right] = \frac{Area_{\text{ELSD}} - b}{m} * DF * IF$$

Figure S25. Determination of residual PGPR as ricinoleic acid in amino-MS slurry digests. (**A**) Ricinoleic acid standard curve. (**B**) Table of raw data values from a representative analysis showing detection of ricinoleic acid in amino-MS digests below the lowest amount of standard quantified. This corresponds to <1.6 ppm of ricinoleic acid the amino-MS slurry. (**C**) Equation used to calculate ricinoleic acid content in where: $Area_{ELSD}$ is the ELSD peak area of a single sample, *b* is the y-intercept of the linear standard curve, *m* is the slope of the linear standard curve, *DF* = 2 is the dilution factor for dilution of amino-MS slurry into ethanolic KOH and HCl, and *IF* = 0.2 is the injection factor accounting for injection of 5 times more sample (0.050 mL) than standard (0.010 mL).

2.3.8 Residual Abil EM90

Residual Abil EM90 surfactant was determined by total silicon ICP-MS analysis conducted by Ampac Analytical (1100 Windfield Way, El Dorado Hills, CA 95742). Amino-MS slurry samples were digested in a mixture of nitric acid and H_2O_2 at 75 °C then total silicon was determined by ICP MS and found to be 58.7 ppm. The surfactant Abil EM90 was analyzed as above and found to have a total silicon content of 23,403 ppm and a density of 917 g/L. This equated to a silicon content of 0.026 (mg silicon/mg of Abil EM90), and therefore 2300 ppm of Abil EM90 in the amino-MS slurry, assuming the total silicon content of the amino-MS was only due to Abil EM90. The instrument parameters for ICP MS analysis are presented in **Figure S26** below.

Table 2: Calibration Range

Element	Linear Range (ng/mL)	Sample Concentration Equivalent (ppm)
Si	50 - 400	50 - 400

Table 3: Instrument Setup

Component	Setting
Instrument	Thermo iCAP RQ
Autosampler	Cetac ASX 560
Spray Chamber	Peltier-cooled Quartz Cyclonic Spray Chamber
Nebulizer	PFA or Quartz/Glass Concentric Nebulizer
Torch	Quartz Torch 2.5 mm i.d.
Sample Tubing	Yellow/Orange 0.508 mm i.d.
Internal Standard Tubing	Yellow/Orange 0.508 mm i.d.

Table 4: Analytical Parameters

Parameter	Setting
Power	1550 W
Cool Gas Flow	14 L/min
Aux. Flow	0.8 L/min
Neb. Flow	1.114 L/min
Sample Uptake	45 s
Sample Wash	60 s
Detection mode	Kinetic Energy Discrimination (KED)
Collision Gas	4.3 mL/min
Resolution	High

Figure S26. Instrument parameters used for ICP-MS determination for total silicon in amino-MS digests.

2.3.9 Residual solvents

A USP <467> compliant head space GC analysis method for residual solvents will be developed for amino-MS slurry. The solvents that will be tested for and suggested limits based on ICH guidelines are given in **Table S2**.

2.3.10 Elemental impurities

A USP <232> compliant ICP MS analysis method for elemental impurities will be developed for amino-MS slurry. The trace metals that will be tested for and suggested limits based on ICH guidelines are given in **Table S2**.

2.3.11 Bioburden

A USP <61> compliant bioburden analysis had been validated and conducted by Pacific Biolabs (551 Linus Pauling Drive, Hercules, CA 94547) and found <10 CFU/ g or amino-MS slurry for both total aerobic microbial count, and combined yeast - mold.

2.3.12 Endotoxin

Endotoxin testing was performed on a USP <85> compliant Endosafe Portable Test System (Charles River, PTS100) using Endosafe PTS cartridges with a 0.01 EU/mL sensitivity (PTS2001F). Calibration of the instrument is performed yearly. Performed in triplicate, 50 µL samples of amino-MS slurry were transferred into tared 1.5 mL microcentrifuge tubes using a positive displacement pipette. The amino-MS were diluted with 19 volumes of endotoxin free water (19 µL H_2O per 1 mg slurry). After 30 minutes, the samples were centrifuged at 10,000 x g for 3 minutes in a table top centrifuge and the supernatant was tested following the Endosafe PTS protocol. Final endotoxin content for three replicate determinations was 0.34 ± 0.9 EU/mL of amino-MS slurry.

3. ASEPTIC SYNTHESIS OF MICROSPHERE DRUG CONJUGATE (STAGE II)

3.1 Starting material syntheses

The following procedure details the preparation and analyses of N_3-linker-[Gln^{28}]exenatide used for conjugation to cyclooctyne derivatized amino-MS. The synthesis of the cyclooctyne N-hydroxy succinimidyl carbonate (5-HCO-HSC) used for derivatizing the amino-MS in preparation for N_3-linker-[Gln^{28}]exenatide conjugation is described here in **Section 1.2**.

3.1.1 Synthesis of N_3-linker-[Gln^{28}]exenatide

N^{α}-(7-Azido-1-cyano-2-heptyloxycarbonyl)-[Gln^{28}]-exenatide. Peptide resin was prepared by PolyPeptide using conventional SPPS Fmoc methodologies with minor modifications on Rink amide MBHA resin (175 g, 0.48 mmol/g after loading of first amino acid, 87 mmol). Amino acids (2.5-3.0 eq) were coupled to the resin for 2.5-18 h using DIC and OxymaPure, and reaction completion was assessed using a ninhydrin, chloranil (for acylation onto Pro), or TNBS test. Recouplings were performed as necessary on the basis of the colorimetric tests. End capping with Ac_2O and DIPEA was performed after each completed coupling step. After assembly of the fully protected peptide, the N-terminal Fmoc (His^1) was removed to reveal the free α-amine. The protected peptidyl resin (free α-amine; 537 g, 35.7 mmol) was washed with DMF (4x 1 bed volume-BV) and then NMP (4x 1 BV). The washed resin was treated with a 60 mM solution of *O*-{4-azido-3,3-dimethyl-1-[(*N,N*-dimethyl)aminosulfonyl]-2-butyl}-*O'*-succinimidyl carbonate (20.8 g, 1.8 eq) and NMM (5.75 mL, 1.5 eq) in NMP. After mixing overnight (23 h), the reaction was confirmed complete by chloranil and TNBS tests. The coupling solution was removed, and the resin was washed successively with DMF (5x 1 BV), methanol (5x 1 BV), and MTBE (5x 1 BV). The resin was dried under vacuum overnight to provide dry linker-peptide-resin. The linker-peptide was cleaved with ice-cold (0 °C) TFA:TIPS:DTE:H_2O (90:6:3:1). The cleavage cocktail (10 mL/g of dried linker-peptide-resin) was added slowly over 3-5 min, and the cleavage reaction was warmed to 18-20 °C. In a separate container, MTBE (4x volume of the cleavage cocktail) was cooled to -25 °C. After stirring the cleavage reaction for 80 min at 18-20 °C, cold (-25 °C) MTBE (~4x volume of the cleavage cocktail) was added with vigorous stirring. After stirring for 5 min, stirring was stopped, and the precipitate was allowed to settle for 10 min. The crude peptide was filtered through a Buchner funnel and washed with MTBE (3 washes at 1x volume of cleavage cocktail). Residual volatiles were removed from the crude solids (peptide and spent resin) under vacuum, and the crude solids were suspended in 5:3:2 H_2O:AcOH:MeCN (~60 g/L). After 2 h (to decarboxylate Trp carbamic acid), the acetic acid suspension was diluted 1:1 with H_2O, filtered, and purified by a two-step RP-HPLC (phenyl-hexyl column). First eluting with a linear gradient of 20%-45% MeOH in 2% AcOH (aq) over 100 min. Clean product-containing fractions (>95% pure by analytical C18 HPLC) were combined and repurified on the same column, eluting with 15%-55% MeCN in 0.1% TFA (aq) over 60 min. Clean fractions from the second HPLC step were pooled and lyophilized to provide the title compound (30.5 g, 6.9 mmol) as a white solid. C18 HPLC purity was determined at 210 nm using a linear gradient of 33%-43% of 0.1% TFA/MeCN in 0.1% TFA/H_2O at 1 mL/min: 99.3%, RV = 23.78 mL.
LC-MS (*m/z*): calc 1470.39, obsd 1470.01 $[M+3H]^{3+}$; calc 1103.04, obsd 1103.23 $[M+4H]^{4+}$.

3.2 Preparation of buffers, solvents, and equipment

The following procedure details the preparation of equipment used for the aseptic synthesis of a microsphere drug conjugate. Synthesis was conducted in a single washer reactor of the same type used in the purification of amino-MS (**Figure S11**, **WR**).

3.2.1 Solvent, buffer, and reagent delivery tanks

For delivery of buffers, solvents, and reagent solutions to the washer reactor 20 L stainless steel tanks with an FEP encapsulated Viton O-ring sealed lid (Alloy Products Corp. B501-4795-00-F-R) were used in two configurations, A and B (**Figure S27**). Both configurations contained a valve for venting (Swage Lok, SS-43GS4-1466), a quick connect nitrogen inlet (Swage Lok, SS-QC4-B-4PM), and a dip tube with either a 2-way valve for delivery of liquid from the tank (**Figure S27A**, Swage Lok, SS-QC4-B-4PM), or a 3-way valve for either delivery of reagent solutions to the washer reactor or delivery of liquid from the tank (**Figure S27B**, Swage Lok, SS-QC4-B-4PM).

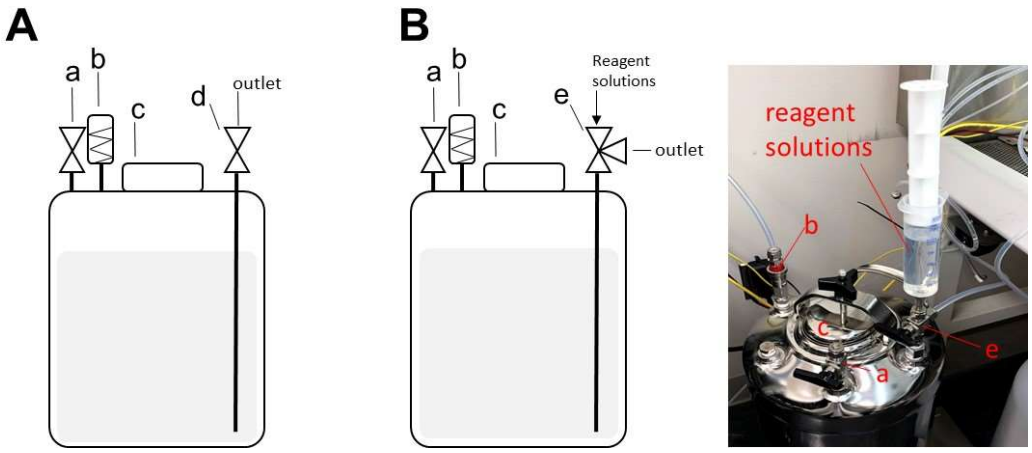


Figure S27. Stainless steel tanks (20 L) used for delivery of buffers, solvents and reagent solutions to the washer reactor. All tanks were fitted with: a valve for venting (a), a quick connect nitrogen inlet (b), a lid with an FEP encapsulated Viton O-ring seal (c). The unique configurations also contained a dip tube terminated with either (**A**) a 2-way valve for delivery of tank liquids to the outlet (d) or (**B**) a 3-way switching valve (e) for delivering tank liquids or reagent solutions to the outlet. The syringe shown in the photo was used to deliver reagent solutions (HCO-HSE, TEA, $(AcO)_2O$, and N_3-Linker-drug) to the washer rector.

3.2.1.1 Water tank

A stainless steel tank (20 L) configured as in **Figure S27A** was filled with 18 L of Milli-Q (Millipore A10) purified water that had been tested for endotoxin (<0.02 EU/mL) using a (Charles River, Endosafe PTS) test device.

3.2.1.2 Isotonic acetate-tween (IAT) formulation buffer tank

IAT buffer (18 L), composed of 63 mM sodium acetate, 37 mM acetic acid, pH 5.0, 111 mM NaCl, and 0.05% (w/v) polysorbate 20 (JT Baker, 4116-04), was prepared by weighing the following reagents into a 20 L stainless steel tank configured as in **Figure S27A** using a 110 lb

capacity balance (ULINE H5822): 1874 g of 10x IAT buffer (0.63 M NaOAc, 0.37 M acetic acid, pH 5.0, 1.11 M NaCl, 0.5% polysorbate 20), and 16,135 g of water. After swirling to mix, the pH and temperature of the buffer were measured as pH = 5.1 and t = 25.2 °C, respectively.

3.2.1.3 Acetonitrile tank

A stainless steel tank (20 L) configured as shown in **Figure S27B** was filled with 18 L of acetonitrile (BD, BDH83639.500).

3.2.1.4 IPA-citrate peptide loading buffer

IPA-citrate peptide loading buffer (18 L) composed of 100 mM citrate in 50% IPA, pH 3.5 was prepared by weighing the following reagents into a 20 L tank configured as shown in **Figure S27B** using a 110 lb capacity balance (ULINE H5822): 1961 g of 10x citrate buffer (0.16 M sodium citrate, 0.85 M citric acid pH (2.4), 7039 g of water, and 7072 g of isopropanol (BD BDH2032). After swirling to mix, the pH (3.5) and temperature (26.7 °C) of the buffer were measured.

3.2.2 Cleaning

Prior to use, process contacting equipment: the solvent delivery tanks, GL45 glass bottles, and plumbing used to connect these components, were cleaned with the following procedure. Equipment was rinsed twice with USP grade 99% isopropanol (BD, BDH2032), then with 1% Alconox (Alconox, 1104), then with 2% CIP100 (Steris, 1D10-08), then three times with Milli-Q (Millipore A10) purified water tested for endotoxin (<0.02 Eu/mL) using an Endosafe PTS (Charles River) test device. For loose plumbing and tanks, cleaning reagents were delivered from polypropylene squirt bottles (VWR, 10111) and surfaces were scrubbed with a polyester brush.

For cleaning in place (CIP) of the washer-reactor, the procedure described in **Section 2.2.2** was used.

3.2.3 Preparation of the washer-reactor for sterilization

After cleaning, the washer reactor was prepared for autoclave sterilization in the configuration shown in **Figure S28.** Critical fasteners were tightened to the following torque specs: 1/4” Swagelok compression fittings (**B**-a) 70 in·lbs, 1/4-20 titanium socket head cap screws (**B**-b) 70 in·lbs, and ¼-20 barrel nuts on clamping studs (**B**-c) 60 in·oz. To ensure proper steam contact, the valves on the upper bulkhead (**B**-d) were opened while the valves on the lower bulkhead (**B**-e) were closed. The NPT fittings on the 0.2 µm sterilizing inlet filters (PES (**B**-f), Saint Gobain, JKPS0201N1N-NO and UE (**B**-g) Saint Gobain, JKPUE0201N1N-NO-E) and vent filter (PTFE (**B**-j), Pall, 4400) were wrapped with Teflon tape (Saint Gobain, Taega Seal) and attached loosely hand-tight to prevent compression setting during autoclaving. A type K thermocouple (and cable) with 316 stainless steel sheath (**B**-h) for immersion into the reactor was installed through one of the ports in the upper bulkhead. The lower bulkhead contained a 316 stainless steel Dutch weave sieve cloth (**B**-i, mesh: 200 x 1400, wire diameters: 0.0028”/0.0016”). All flanges in the upper and lower bulkheads and the magnetic stir drive were sealed with FFKM O-rings (Markez compound Z1305). Finally, the assembly was wrapped with

a steam permeable cloth (**Figure S28C**, Kim Tech, Kimguard KC100) secured in place with autoclave tape (VWR, 10127-464).

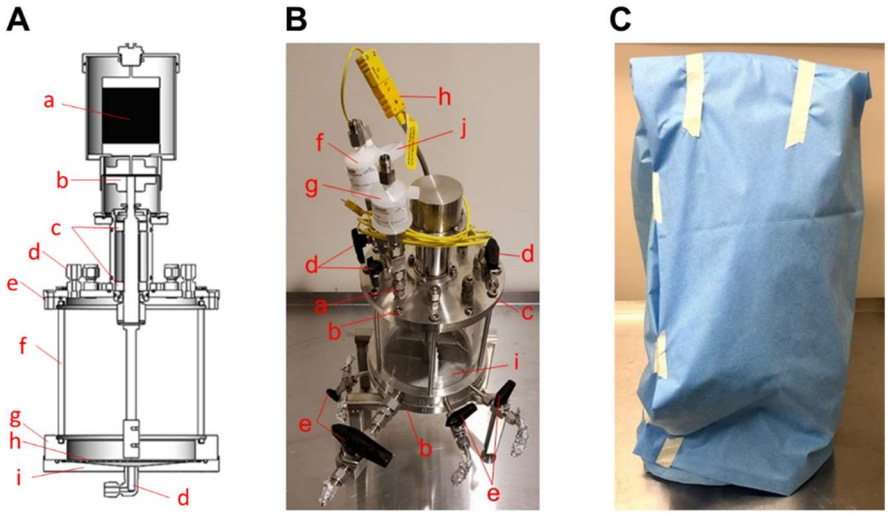


Figure S28. Description of the washer reactor and preparation for autoclave sterilization. (**A**) Cross sectional drawing of the washer reactor showing major components: (a) stir motor inside of a fluid resistant stainless steel housing, (b) reactor side of magnetic drive coupling in a hermetically sealed housing, (c) stir shaft support bearings in stainless steel housing, (d) welded in ¼" Swagelok compression fittings, (e) stainless steel upper bulkhead, (f) glass cylinder, (g) stainless steel lower bulkhead, (h) stainless steel sieve cloth and perforated support plate, (i) stainless steel floor plate. (**B**) Photograph of the washer reactor in the autoclave ready state showing: (a) Swagelok compression fittings, (b) socket head cap screws, (c) barrel nuts on clamping studs, (d) Swagelok 40G series valves in upper bulkhead, (e) Swagelok 40G series valves in lower bulkhead, (f) PES sterilizing inlet filter, (g) UE sterilizing inlet filter, (h) type K thermocouple and cable, (i) Dutch weave sieve cloth, (j) PTFE vent filter (j). (**C**) The washer-reactor assembly shown in part A wrapped in steam permeable cloth.

3.2.4 Preparation of tools, plumbing, and supplies for sterilization

The following items were prepared for autoclave sterilization by placing them into two sequential (bag within a bag) adhesive sealing autoclave sterilization pouches (Proper, Chex-All III): a tool kit consisting of stainless steel wrenches required for tightening fasteners on the washer-reactor, dip tube transfer assemblies for GL45 bottles used for wetting of sterilizing filters during integrity tests and transfer of amino-MS into the washer-reactor, liquid waste outlet tubes for the washer-reactor, dosing vials (Daikyo Crystal Zenith,19550210) in plastic racks (Wheaton, 868804), dosing vial caps (West Pharmaceutical Services, 54131770), septa (West Pharmaceutical Services, 19700302) and cap crimping tool (Kebby Industries, 13002-00-C04A).

3.2.5 Autoclave sterilization of equipment and supplies

Autoclave sterilization of the washer reactor, tools, plumbing and supplies was performed using a Sterivap model 669 autoclave (BMT MMM group). Prior to use, the autoclave was required to pass a Bowie Dick style air removal test (Steris, DART). The autoclave cycle for sterilization consisted of a) four consecutive evacuations to 2.9 PSIA, b) sterilization at 121 °C

with a 20 min hold time, c) two consecutives drying evacuations to 1.0 PSIA, and d) cooling to ~70 °C.

3.2.6 Biosafety cabinet for aseptic operations

Aseptic operations were conducted in a Class II Type A2 biosafety cabinet (NuAire, NU-440-600). Prior to use, the cabinet was wiped down with a solution of 10% household bleach (Chlorox, diluted 10 fold with water) followed by 70% ethanol. The cabinet was then irradiated with a germicidal UV lamp (NuAire, NU-959-400) for 1 hr. The autoclave sterilized washer-reactor, equipment, and supplies were introduced to the cabinet after spraying the exterior surface of their containment bags with 70% ethanol.

3.2.7 Post Sterilization / Pre use washer reactor leak test

After cooling to room temperature in the biosafety cabinet, the sterilizing inlet and vent filters were securely tightened along with the barrel nuts on the clamping studs (**Figure S28**), then all valves were closed. The washer reactor was then attached to a pressure test instrument through the vent filter (**Figure S29**). The instrument used a high resolution proportional pressure regulator (Proportion Air, MPV2PBNEE2P150PSGAXL) to apply a test pressure (20 PSIG) to the reactor. During pressurization, the reactor pressure was measured with a high resolution pressure transducer (Proportion Air, DSTEX06ZP150 PSGB) and the flow of nitrogen to the reactor was measured with a 0-50 ccm precision flow meter (Sierra Instruments, M100-L-DD-2-OV1-PV2-V3). The sensors were connected to a computer through an analog DAQ card (Lab Jack, U6 Pro) and data was recorded using a custom application written in DAQ Factory (Azeotech). The reactor was defined as leak free based on the following criteria: the time to reach equilibrium pressure must be <10 min, the pressure in the reactor at equilibrium must be >19.9 PSIG for an applied pressure of 20.0 PSIG, and the flow of nitrogen to the reactor must be <2.0 ccm at equilibrium pressure.

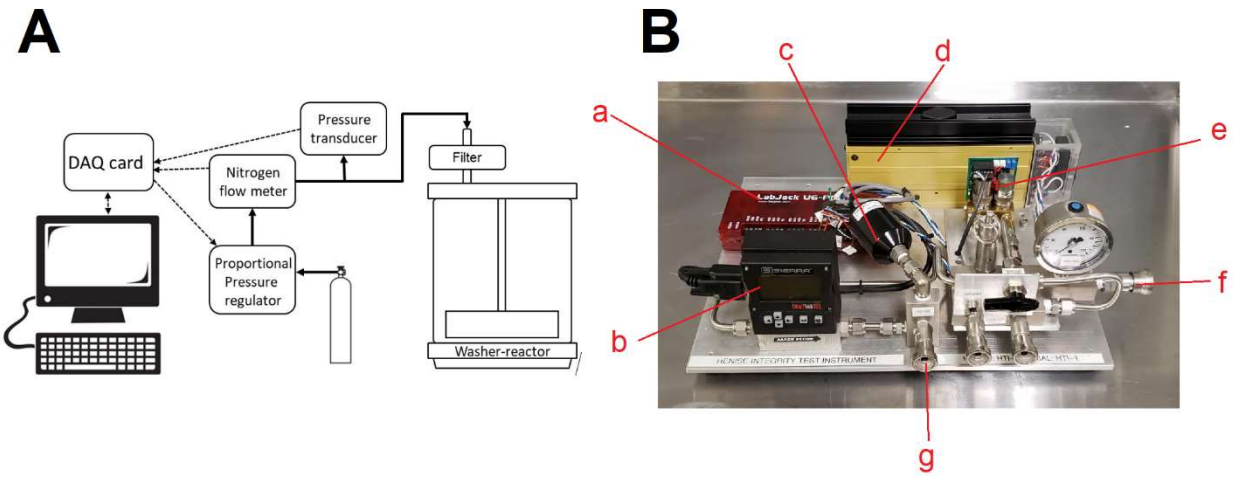


Figure S29. Instrument used for leak testing of the washer-reactor and integrity testing of sterilizing filters. (**A**) Diagram of instrument components. The flow of nitrogen in shown with solid black arrows and the flow of data is shown with dotted arrows. (**B**) Photo of the instrument showing: (a) DAQ card, (b) nitrogen flow meter, (c) pressure transducer, (d) AC-DC power supply, (e) proportional pressure regulator, (f) high pressure inlet from nitrogen tank, (g) regulated pressure outlet.

3.2.8 Post sterilization / Pre use Sterilizing filter integrity tests

The integrity of each 0.2 µm sterilizing inlet filter was measured with an automated version of the bubble point test. First, the filter was wetted with an appropriate solvent: isopropanol for the PTFE vent filter, or 60% isopropanol in water for the PES and UE inlet filters. Wetting was achieved by flowing 100 mL of the fluid through the filter by dip tube (1/8" OD x 1/16" ID FEP tube) transfer from a GL45 glass bottle at 20 PSIG with nitrogen. After wetting, the test instrument shown in **Figure S29** was attached. The instrument applied a series of nitrogen test pressures to the filter and measured the flow of nitrogen at each pressure after equilibrium was achieved. The bubble point was determined from a plot of nitrogen flow vs test pressure (**Figure S30**) by performing linear regression on points where flow was >3 ccm and solving the resulting equation for the X intercept. The X intercept represented the pressure where flow through the filter changed from diffusive to bulk flow and was defined here as the bubble point. Filters were determined to be integral if the bubble point exceeded the following values: PTFE vent filter > 1 PSIG, PES inlet filter, >17.4 PSIG, and UE inlet filter >17.5 PSIG.

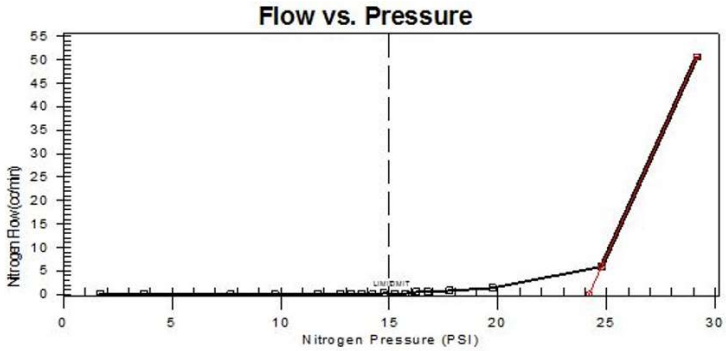
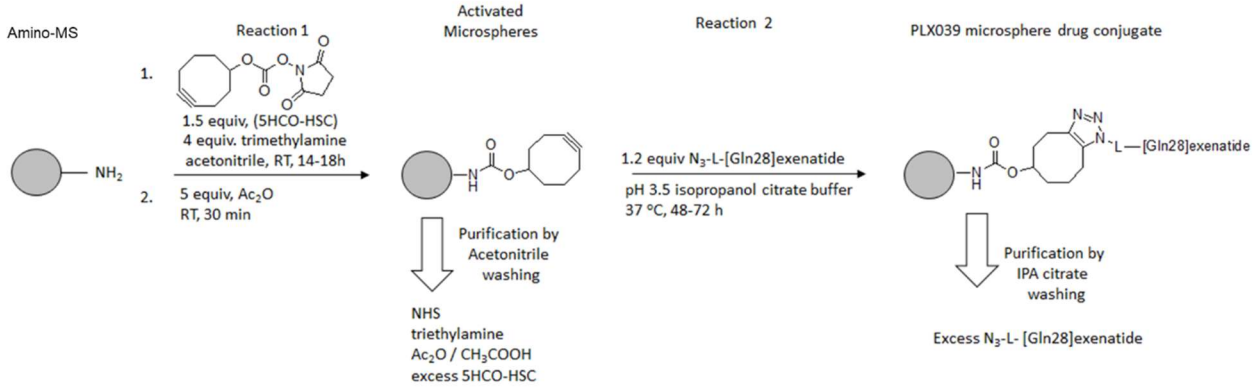


Figure S30. Example of a bubble point test plot for a 0.2 µm PTFE vent filter wetted with isopropanol.

3.3 Synthesis of the MS-drug conjugate drug substance

The following procedures detail the aseptic synthesis of the MS-drug conjugate drug substance, a microsphere drug conjugate, starting from autoclave sterilized amino-MS using the chemistry shown in **Scheme S6**.

Scheme S6. Aseptic synthesis of a microsphere drug conjugate from autoclave sterilized amino-MS.



3.3.1 Autoclave sterilization of amino-MS [6]

A 1000 mL GL45 glass bottle with a loosely fitted cap containing amino-MS (700 mL, 2.5 mM amine concentration) was placed into two sequential (bag within a bag) adhesive sealing autoclave sterilization pouches (Proper, Chex-All III). Autoclaving was performed using a Sterivap model 669 autoclave (BMT MMM group). Prior to use, the autoclave was required to pass a Bowie Dick style air removal test (Steris, DART). An autoclave cycle for sterilization consisted of a) evacuation to 5.80 PSIA, b) autoclaving at 121 °C with a 20 min hold time, and c) cooling to 97 °C over ~1.5 hours. The autoclave temperature was monitored with a probe immersed in 600 mL of water in a 1000 mL glass GL45 media bottle. After spraying the outer bag with 70% ethanol, the sterilized jar of amino-MS was allowed to cool to room temperature inside of the biosafety cabinet containing the sterilized washer reactor.

3.3.2 Transfer of amino-MS to the washer-reactor and washing

Sterile amino-MS slurry (700 mL, 2.5 mM amine, 1.8 mol amine) was transferred from the autoclaved GL45 glass bottle to the washer reactor using the dip tube transfer assembly shown in **Figure S31** using 5 PSIG of nitrogen. Washing of amino-MS was performed using the configuration shown in **Figure S31B.** A wash was accomplished by adding a buffer or solvent into the reactor from a dip tube in a delivery tank (**Section 3.2.1**) pressurized to 30 PSIG with nitrogen. Buffers and solvents were added through the 0.2 um sterilizing inlet filters in the upper bulk head of the reactor. The delivery tank was weighed during this process to determine the weight of liquid added to the reactor. After filling, the stirrer was pulsed three times at 300 RPM for 2 seconds each pulse to wash the upper surface of the reactor, then the slurry outlet ports were purged by pumping the attached 10 mL syringes (HSW, 4100-X00V0) three times each. Stirring was then set to 50 RPM for 5 minutes prior to draining the wash fluid from the reactor through the sieve and waste outlet valve. During draining, the reactor was pressurized to 3-12 PSIG with nitrogen through the vent filter as needed to maintain flow of liquid out of the reactor. The PES inlet filter was used for water, IPA-citrate buffer, and IAT buffer, and the UE inlet filter was used for acetonitrile.

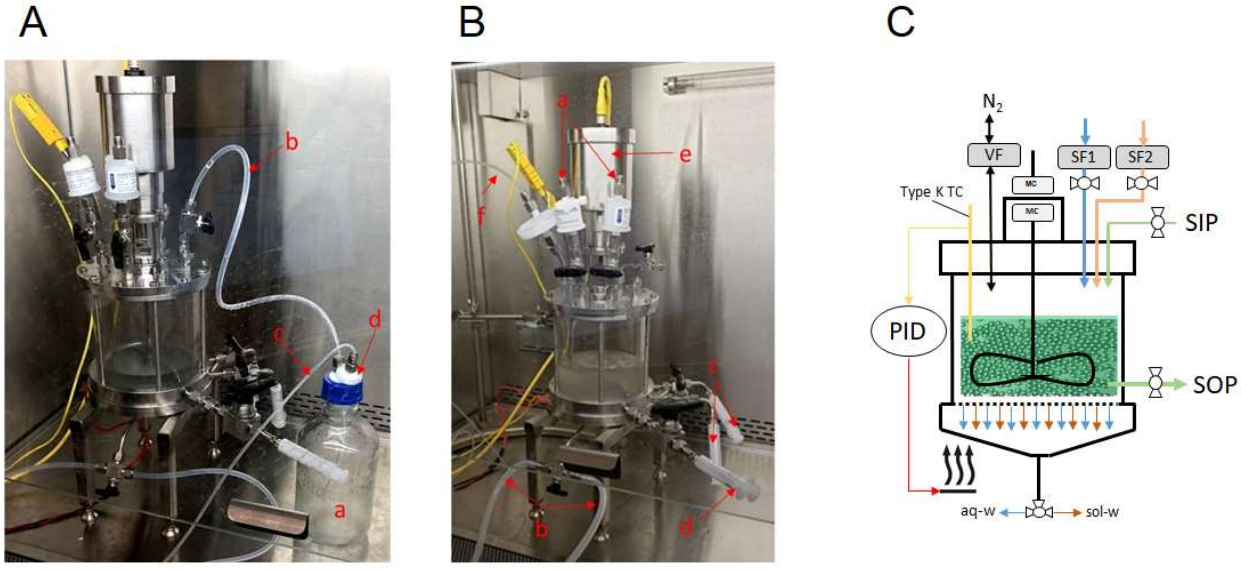


Figure S31. Set up of sterile washer reactor for loading with sterile amino-MS slurry and washing of amino-MS. (**A**) Dip tube transfer assembly for transfer of sterile amino-MS slurry into

the reactor consisting of: (a) GL45 bottle of sterile amino-MS, (b) a ¼" OD x 3/16" ID FEP dip tube, a FEP line for nitrogen pressure, and a 0.2 µm PTFE vent filter. (**B**) Washer reactor set up for washing of amino-MS consisting of: (a) buffer and solvent inlets, (b) liquid waste outlet lines, (c) sample syringes on the slurry sample ports, (d) sample syringe on the primary slurry outlet port, (e) magnetically coupled stir motor, (f) nitrogen line for pressurizing the reactor. (C) schematic representation of the reactor shown in part B showing: (vf) the vent filter, (SF1) the PES inlet filter, (SF2) the PTFE inlet filter, (mc) the magnetic stir coupling, (SIP) the slurry inlet port, (SOP) the slurry outlet port, (aq-w) the aqueous waste port, (sol-w) the solvent waste port, (PID) the PID temperature control circuit, with Type K thermocouple and heater.

3.3.3 Cyclooctyne loading reaction and capping of residual amines

Reaction 1, Scheme S6. The amino-MS slurry (700 mL, 2.5 mM amine, 1.8 mol amine) was washed four times with water (1.2 kg per wash) and four times with acetonitrile (1.1 kg per wash) using the technique described above. From the fourth acetonitrile wash, the reactor was drained from a slurry sample port (**Figure S31B-c**). Next a solution of trimethylamine (50 mL, 140 mM, 7 mmol, 4 equiv) in acetonitrile was added to the reactor through the 0.2 um UE inlet filter followed by a solution of 5-HCO-HSC (**Section 1.2**) in acetonitrile (50 mL, 52.5 mM, 2.63 mmol, 1.5 equiv). These solutions were added from a 60 mL syringe attached to the 3-way valve on the acetonitrile delivery tank (**Figure S27B**), then the valve was switched to deliver 100 g of acetonitrile from the tank to rinse the UE inlet filter. The reactor was stirred (75 RPM) at room temperature for 14 hours. A sample (1 mL) of reacted amino-MS was removed from a slurry sample port test for completeness of reaction. To test for a complete reaction, 0.2 mL of each slurry sample (unreacted and reacted) was placed into a 1.5 mL centrifuge tube and treated with a 0.04% w/v solution of picrylsulfonic acid (TNBS, Sigma, 92822). After 15 min, no color was produced in the reacted sample indicating that the amines were modified with 5-HCO-HSC (**Figure S32**). To cap any residual amines, a solution of Ac_2O in acetonitrile (50 mL, 175 mM, 8.75 mmol, 5 equiv) was added to the reactor through the 0.2 um UE inlet followed by of acetonitrile (1 kg) to rinse the filter and fill the reactor, using the same technique described for addition of the 5-HCO-HSC solution. The stirrer was pulsed three times at 250 RPM for 2 seconds each pulse to wash the top of the reactor, then the reaction was stirred for 30 minutes at 50 RPM prior to draining through the sieve at 5 PSIG. The cyclooctyne activated-MS were then washed four times with acetonitrile (1.2 kg per wash) and three times with IPA-citrate buffer (1.5 kg each, using the PES inlet filter).

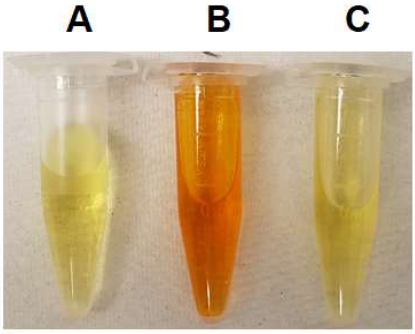


Figure S32. TNBS color test for free amines on amino-MS. (**A**) 0.04% TNBS in pH 9.4 borate. (**B**) TNBS reaction with amino-MS. (**C**) TNBS reaction with cyclooctyne activated MS.

3.3.4 Peptide loading reaction

Reaction 2, Scheme S6. After the last IPA-citrate buffer wash of the cyclooctyne activated MS, the reactor was stirred at 50 RPM and drained until ~250 mL of compact slurry remained. Next a solution of N_3-Linker-[Gln^{28}]exenatide in IPA-citrate buffer (72.2 mL, 29.1 mM, 2.1 mmol, 1.2 equiv) was added to the reactor through the 0.2 um PES inlet filter followed by IPA citrate (100 g) to rinse the filter. The peptide solution was added from a 60 mL syringe attached to the 3-way valve on the IPA-citrate delivery tank (**Figure S27B**) then the valve was switched to deliver IPA-citrate buffer from the tank for the filter rinse. The reactor was then heated to 37 ± 1.3 ºC for 90 hours using a PID controller (Omega Platinum series CS8DPT-C24-EIP-A), with a Type K 316-stainless steel sheathed (12" x 0.25") thermocouple (McMaster, 3856K86) attached through a port in the reactor's upper bulkhead, and two 6" x 1" silicone band heaters (BenchMark thermal, EFH SH-1X6-10-115) with an energy density of 10 watts/in^2 fixed to the bottom of the reactor.

To assess the progress of the reaction, 1 mL samples of slurry were removed from a slurry sample port (**Figure S31B-c**) for analysis at 0, 67 and 90 h. Samples were analyzed for peptide loading ([Peptide]/[PEG]) as follows: slurry (100 mg) was washed four times with 1.0 mL of IPA-citrate by vortexing to mix and pelleting at 20,000 Gs for 5 minutes, followed by three washes 1.0 mL IAT buffer. The MS pellet from the last wash was weighed and treated with 0.900 mL of 50 mM NaOH for every 100 mg of slurry for 1 hour to dissolve. Then the resulting solution was assessed for [Peptide] (**Section 3.5.2**) by absorbance at 280 nm (ε = 5500 $M^{-1}cm^{-1}$) and for [PEG] as described in **Section 2.3.2**. The MS contain 4 loadable groups for every 20000 MW of PEG, which equates to 200 nmol of loadable group/mg PEG. The % loading was calculated as [([Peptide]/[PEG])/200]*100% and was found to be 84% at 67 hours and 96% at 90 hours. The peptide loaded MS slurry in the reactor was then washed six times with IPA-citrate buffer (1.3 kg per wash), then eight times with IAT formulation buffer (1.6 kg per wash).

3.4 Formulation and fill

The following procedures detail the aseptic formulation of the MS-drug conjugate with hyaluronic acid and filling into dosing vials.

3.4.1 Formulation with HA

The peptide loaded MS were drained from the reactor through a valve with a 3/8" bore located in the lower bulkhead of the reactor above the sieve, while stirring at 50 RPM and pressurizing the reactor to 2 PSIG. The reactor was then rinsed with 50 mL of IAT buffer. The combined slurry and rinse (300 mL total) was collected in an autoclave sterilized tared 500 mL GL45 bottle then the bottle was weighed to determine the amount of slurry (298.2 g). The peptide concentration of the MS slurry (potency) was determined as described below (**[peptide], Section 3.5.2**) and found to be 4.0 mM. The volumes of IAT buffer, V_{IAT}, and concentrated hyaluronic acid (HA) in IAT buffer, V_{HA}, to be added to the MS slurry to achieve the desired peptide (drug) concentration, $[D_{MS}]_f$, and final HA concentration, $[HA]_f$, were calculated using the **Equation S6** and **Equation S7** below.

Equation S6

$$V_{HA} = V_{MS} \cdot \frac{[D_{MS}]_0}{[D_{MS}]_f} \cdot \frac{[HA]_f}{[HA]_0}$$

Equation S7

$$V_{IAT} = V_{MS}\left[\frac{[D_{MS}]_0}{[D_{MS}]_f} \cdot \left(1 - \frac{[HA]_f}{[HA]_0}\right) - 1\right]$$

Here, V_{MS} was the volume of MS slurry (assumed to be equal to the weight of the slurry in grams) having the initial concentration of drug $[D_{MS}]_0$ and $[HA]_0$ was the HA in the concentrated solution added to obtain $[HA]_f$.

For the peptide MS-conjugate described here:

$[HA]_0$	=	10% HA w/v
$[HA]_f$	=	1.2% HA w/v
$[D_{MS}]_0$	=	4.0 mM
$[D_{MS}]_f$	=	3.5 mM
V_{MS}	=	298.2 mL
V_{HA}	=	40.9 mL
V_{IAT}	=	1.7 mL

The highly viscous solution of 10% (w/v) 61.8 kDa HA (Lifecore Biomedical, HA40K-5.) in IAT buffer (40.9 mL), and IAT buffer (1.7 mL) were sterile filtered into the MS slurry from a plastic 10 mL syringe (HSW, 4100-X00V0) attached to a 40 mm disk 0.2 µm PES sterilizing filter (Saint Gobain, D40CS0201N1N-PH-1) using a syringe pump (New Era, NE1000) and flow rate of 0.5 mL/min. After addition of the HA solution, the bottle was swirled by hand to mix (**Figure S33**).

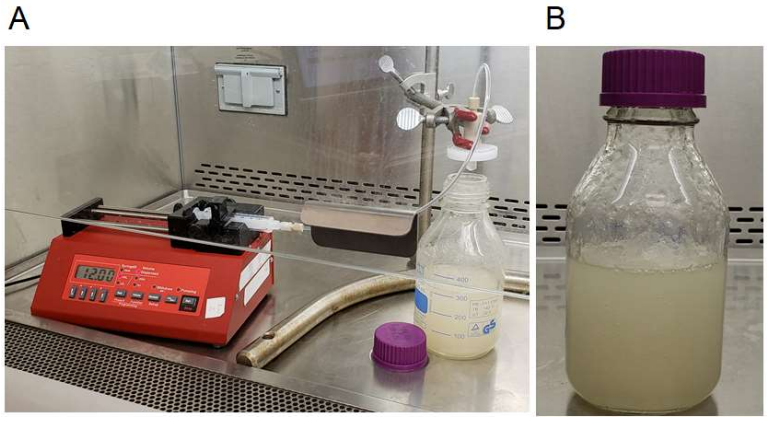


Figure S33. Formulation of the microsphere drug conjugate with HA. (**A**) Syringe pump and filter used for addition of the viscous HA solution. (**B**) Appearance of formulated microsphere drug conjugate.

3.4.2 Vial filling

The apparatus used for vial filling was described in **Figure S34**. Working in the biosafety cabinet described above, the formulated MS slurry was poured into the autoclave sterilized teflon-coated hopper of the filling apparatus. Then autoclave sterilized Daikyo Crystal Zenith 2 mL dosing vials (West Pharmaceutical Sciences, Inc, 19550210) in plastic racks (Wheaton, 868804), were filled by withdrawing 1.4 mL of slurry into a 3 mL syringe (BD, 309657) attached to the three way switching valve at the base of the hopper (**Figure S34A, Step 1**). The valve was then positioned to allow the syringe to expel the slurry into a vial (**Figure S34A, Step 2**). After filling, the vials were capped with chlorobutyl rubber vial stoppers (West Pharmaceutical

Sciences, Inc, 19700302) and Flip-Off caps (West Pharmaceutical Sciences, Inc, 54131770). The caps were crimped in place using a hand-held crimping tool (Kebby Industries, 13002-00-C04A). For the batch described here, 225 vials were produced.

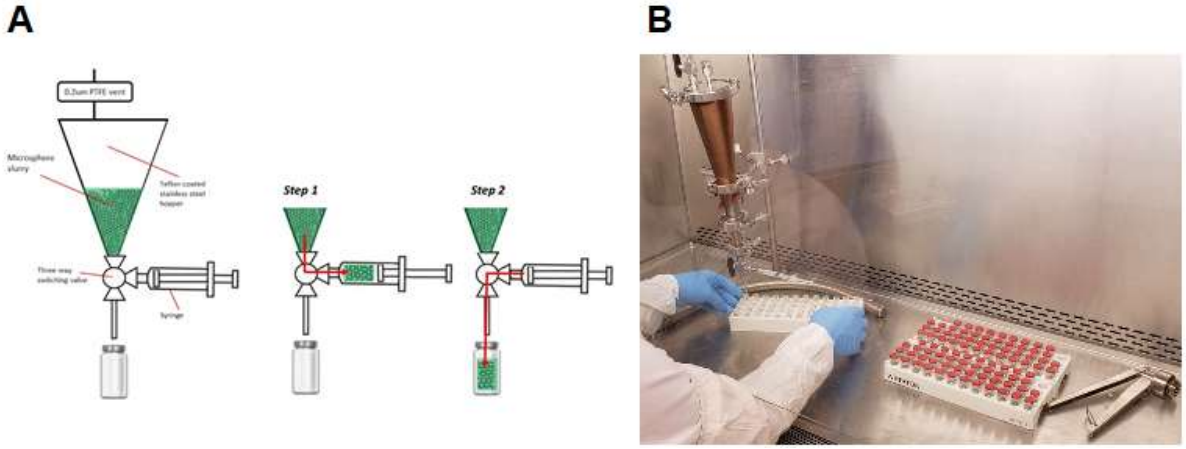


Figure S34. Apparatus used for filling of dosing vials. (**A**) Diagram of the system. (**B**) photograph of the system.

3.5 Analysis of the MS-drug conjugate – Table of results

The following procedures describe the analytical methods used for characterization of the MS-drug conjugate described here. The results of all methods are given below in **Table S3**.

Table S3. Analytical release of the formulated MS-drug conjugate.[1]

Assay Parameter	Analytical Method	Result	Acceptance Specification
Appearance	Visual inspection	Thick pale yellow translucent slurry [2]	No discoloration, volume change, or growth [3]
Potency [Peptide]	Peptide content of slurry	3.4 ± 0.1 nmol / mg of slurry [2]	3.2 – 3.6 nmol peptide / mg of slurry [3]
Identity [Peptide]/[PEG]	Peptide/PEG content	145 ± 13 nmol peptide / mg of PEG [2]	120 – 170 nmol peptide / mg of PEG [3]
Dissolution time (t_{RG})	Kinetics of dissolution at pH 9.4, 37 °C	30 ± 1.2 hours [2]	29 - 31 hours [3]
Peptide release rate	Kinetics of release, initial rate at pH 9.4, 37 °C	48 ± 0.4 nmol/ hour [2]	2.3 – 4.3 percent / hour [3]
Free peptide	HPLC	<0.1% [2]	<0.5% [3]
Particle size	Microscopy and image analysis	54 ± 4 µm mean diameter [2]	10 - 80 µm distribution [3]
pH	pH electrode	5.1 [2]	5.0 – 5.2 [3]
Injection force, breakaway	Texture analyzer	1.7 ± 0.1 kg [2]	0.5 – 2.9 kg [3]
Injection force, glide	Texture analyzer	1.7 ± 0.1 kg [2]	0.5 – 2.9 kg [3]
Dose delivered	Peptide delivered from injection forrce tests	1.8 ± 0.1 µmol [2]	1.6 – 2.0 µmol [3]
Residual solvents	USP <467>	To be determined	Acetonitrile < 200 [4] Isopropanol < 5000 ppm [4] Triethylamine < 5000 ppm [4]
Elemental Impurities	UPS <232>	To be determined	Class I As 15 ppm [5] Cd 2 ppm [5] Hg 3 ppm [5] Pb 5 ppm [5] Class IIA Co 5 ppm [5] V 10 ppm [5] Ni 20 ppm [5]
Sterility	USP <71>	Sterile [2]	Sterile [3]
Endotoxin	USP <85>	0.22 ± 0.23 EU/mL [2]	<300 EU/mL [6]

[1] Analytical methods are described below.

[2] Results from the batch of MS-drug conjugate described here, reported errors are: SD n=5 for potency, identity, injection force, dose delivered and endotoxin, range/2 n=2 for dissolution time and peptide release, and SD n>100 for particle size.

[3] Preliminary acceptance specifications are estimates of those anticipated.

[4] Residual solvent limits are set to the Permitted Daily Exposure limits according to ICH: GUIDELINE FOR RESIDUAL SOLVENTS Q3C(R6) 2016. The listed limits are for finished drugs assuming a 1 mL daily dose.

[5] Elemental impurities are set to the Permitted Daily Exposure limits for parenteral administration assuming a 1mL dose as in ICH: GUIDELINE FOR ELEMENTAL IMPURITIES Q3D 2014.

[6] Specification based on limit dose of 5 EU/kg (USP <85>) assuming a dose of 1 mL.

3.5.1 Appearance

Five vials of MS-drug conjugate were visually inspected for the following parameters: container seal (intact or compromised), condensation in vial (yes or no), slurry color, and microbial growth (yes or no). To detect particle settling, a digital caliper (Mitutoyo, 500-196-30) was used to measure the depth of the slurry and any visible supernatant. Percent settling was calculated as: [(depth of supernatant)/(depth of supernatant + depth of slurry)]*100. The results for the batch described here are given in **Figure S35**.

Figure S35. Appearance of formulated MS-drug conjugate in dosing vials showing the standard appearance: Intact seal, no condensation, pale yellow slurry, no growth, and 0% settling.

3.5.2 Potency [Peptide]

In quadruplate, 0.100 mL samples of MS-drug conjugate were treated with 0.900 mL of 50 mM NaOH for 1 hour to dissolve. Then 0.200 mL samples of the resulting solutions were transferred onto a UV-transparent 96 well microtiter plate (Corning, 3635). The A_{280} of the samples was measured using a plate reader (Molecular Devices, Spectramax i3) set to report absorbance values equivalent to a 1 cm path length. The A_{280} of a 0.100 mL sample of formulation buffer (1.2% w/v HA in IAT) diluted with 0.900 mL 50 mM NaOH was used for background subtraction. The peptide concentration of the MS slurry was calculated using ε_{280} = 5500 M^{-1} cm^{-1} and found to be 3.4 ± 0.1 mM.

3.5.3 Identity [peptide]/[PEG]

[PEG]: [PEG] content was determined after dissolving in sodium hydroxide using the procedure described in **Section 2.3.2** and found to be 23.4 mg ± 1.9 PEG/ mL slurry.

[peptide]/[PEG]: The peptide to PEG content ratio ($Ratio_{peptide/PEG}$) of the MS-drug conjugate was calculated from individual measurements of the peptide in mM (µmol amine/mL slurry) and PEG (mg PEG/mL slurry) content of the MS-drug conjugate slurry using the equation shown in **Figure S36A**. The standard deviation in the $Ratio_{peptide/PEG}$ was given by the equation shown in **Figure S36B**. The result from the batch described here was $Ratio_{peptide/PEG}$ = 145 ± 13 nmol peptide / mg PEG.

A

$$Ratio_{\text{peptide/PEG}} = \frac{Peptide\ [\mu\text{mol peptide/mL slurry}]}{PEG\ [\text{mg PEG/ mL slurry}]} * 1000[\text{nmol/ }\mu\text{mol}]$$

B

$$SD_{\text{ratio}} = Ratio_{\text{peptide/PEG}} * \left\{ \left(\frac{SD_{\text{peptide}}}{Avg_{\text{peptide}}} \right)^2 + \left(\frac{SD_{\text{PEG}}}{Avg_{\text{PEG}}} \right)^2 \right\}^{1/2}$$

Figure S36. Equations used to calculate the peptide to PEG content ratio of the MS-drug conjugate. (**A**) Equation for the $Ratio_{\text{peptide/PEG}}$ where $AVG_{peptide}$ and AVG_{PEG} were the average values from quadruple determinations of the peptide and PEG content of the MS-drug conjugate. Where peptide concentration in mM was equivalent to (µmol peptide / mL slurry). (**B**) Equation for the standard deviation (SD_{ratio}) in the $Ratio_{\text{peptide/PEG}}$ where: SD_{peptide} and SD_{PEG} were SD from quadruple determinations of the peptide and PEG content of the MS-drug conjugate slurry and AVG_{peptide}, AVG_{PEG} were the values described in part A.

3.5.4 Dissolution (t_{RG} and peptide release rate)

Dissolution Reactions: Dissolution reactions were conducted as described in **Section 2.3.3** with the following change: 0.5 ± 0.075 g of the MS-drug conjugate slurry was loaded directly into the dissolution cells rather than first pelleting at 20000 Gs.

Determination of t_{RG}: Determination of t_{RG} was conducted as described in **Section 2.3.3** with the following changes: Samples of the dissolution reactions (0.25 mL) were collected at 0, 2, 4, 6, 8, 10, 12, 16, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 32, 33, 34, 35, 36, 38, and 40 hours. For PEG determination, the samples were not diluted with water. A portion of each sample (0.020 mL) was transferred directly to a 96 well dilution plate containing perchloric acid (1.580 mL, 0.5 M). For **Equation S3** DF=1.

Determination of peptide release rate. The liquid handling robot was used to transfer 0.200 mL of the collected dissolution reaction time point samples to a UV-transparent 96 well microtiter plate (Corning, 3635) covered with an optical clear film cover (VWR, 89140-948). The A_{280} of the samples was measured using a plate reader (Molecular Devices, Spectramax i3) configured to report absorbance values equivalent of a 1 cm path length. For each time point, the A_{280} was corrected to account for the reduction in reaction volume and removal of peptide from previous samples using **Equation S2**. Then, the amount of peptide released at each time point was calculated by **Equation S8**. Linear regression was then performed on the data points from 0-24 hours to calculate the release rate as the slope (**Figure 37A).**

Equation S8 $$Peptide = \frac{A_{C,n}}{\varepsilon_{280}} * Vi * CF$$

Where:

Peptide = the amount of peptide in nmol

$A_{C,n}$ = the corrected A_{280}values from **Eq. S2**

ε_{280} = the peptide extinction coefficient at 280 nm (5500 $M^{-1}cm^{-1}$)

V_i = initial volume of the dissolution reaction (V buffer + V MS-drug conjugate slurry) in L

CF = the conversion from mol to nmol (10^9)

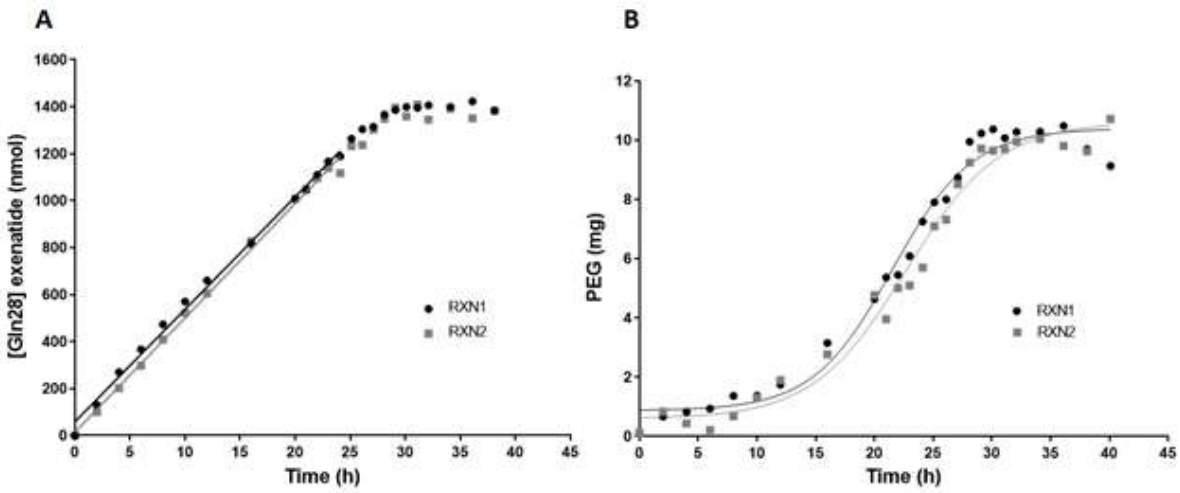
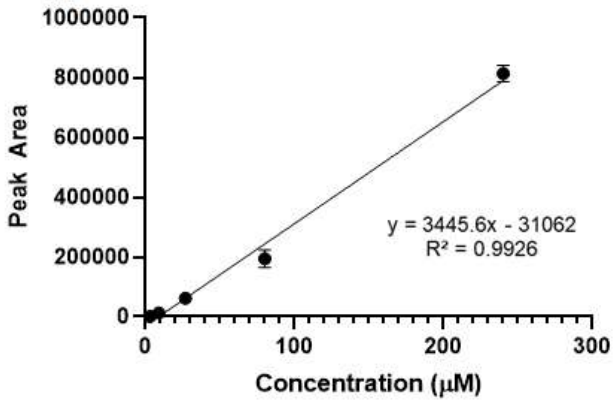


Figure S37. Dissolution curves for the MS-drug conjugate showing peptide release and microsphere dissolution. (**A**) The dissolution curve showing peptide release for two samples with their linear regression fits from 0-24 hours, where release rate is given by the slope. (**B**) The dissolution curve showing PEG release for the same two samples fitted to Sigmoidal functions that determine t_{RG}.

3.5.5 Free peptide ([Gln^{28}]exenatide)

In triplicate, samples of the MS-drug conjugate (0.200 mL) were treated with of 95% ethanol (0.600 mL) in 1.5 mL microcentrifuge tubes then vortexed for 5 seconds at 2500 RPM. The samples were then centrifuged at 20,000 Gs for 5 minutes. Then, a sample of the supernatant (0.010 mL) was analyzed by reverse phase C18-HPLC (**Section 1.1**) with absorbance detection at 280 nm. The amount of free peptide was determined using a linear standard curve generated by this same procedure with a solution of [Gln^{28}]exenatide (3.7-900.0 µM, GenScript, Exenatide_N28Q) in IAT-formulation buffer containing 1.2% w/v hyaluronic acid (**Section 3.4**) in place of the MS-drug conjugate . The standard curve and table of results are presented in **Figure S38**. The amount of free peptide was below the limit of quantitation, which corresponds to < 3.7 µM of free peptide in the MS-drug conjugate or < 0.12% of total peptide.

A



B

		Retention Time	Peak Area
Standards (uM)	240.2	7.92	812811
	80.1	7.92	194160
	26.7	7.92	61405
	8.9	7.93	12264
	3.0	7.93	585
Samples	A	Not Detected	Not Detected
	B	Not Detected	Not Detected
	C	Not Detected	Not Detected

Figure S38. Determination of free peptide in the MS-drug conjugate. (**A**) Peptide standard curve. Error bars are SD from triplicate measurements. (**B**) Table of raw data showing no quantifiable free peptide (below the limit of quantitation).

3.5.6 Particle size

Particle size was determined using the method described in **Section 2.3.4** and was found to be 54 ± 4 µm**.**

3.5.7 pH

pH was determined using the method described in **Section 2.3.5** and was found to be 5.1**.**

3.5.8 Injection Force Profile and Dose Delivered

Texture analyzer: A custom texture analyzer was used to measure the injection force profile and the dose delivered from automated injections of 0.5 mL samples of the MS-drug conjugate in 1 mL syringes fitted with a 27 gauge needles. The instrument (**Figure S39**) was composed of a stepper motor driven linear actuator (Thompson linear, 2RB12H0N0320-075N999A0A02) attached to 10 kg S-beam load cell (Omega, LCM101_LCM111). The stepper motor (Oriental Motor, PKP264D28A2-R2E) was fitted with a 200 count/rev quadrature encoder for true closed loop operation. A custom application written in DAQ Factory (Azeotech) software was used to control the motor through a computer interfaced motion control board (All Motion, EZ23ENHC) and acquire data (analog signal) from the load cell through a USB DAQ card (LabJack, U6 Pro) during the injection. Prior to measurement, the load cell was calibrated against NIST traceable weights (Troemner, Class 1 weight standards 0.500, 1.000, 10 kg) to generate a linear calibration curve of the force vs. output voltage. The software used the calibration curve to convert the measured voltage to kgF during measurement.

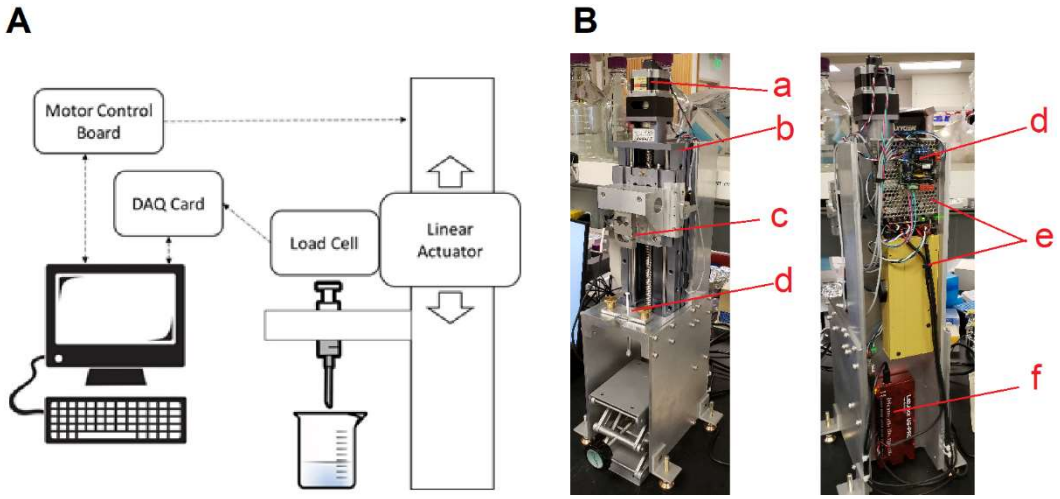


Figure S39. Texture analyzer used for injection force measurements. (**A**) A diagram of the custom texture analyzer. The large arrows indicate movement of the linear actuator and attached load cell used to perform the injection. The dotted black arrows indicate the flow of data. (**B**) Photographs of the texture analyzer showing: (a) the stepper motor with quadrature encoder, (b)

the linear actuator, (c) the load cell, (d) a syringe mounted in the instrument, (e) AC-DC power supplies for the motor controller and load cell, (f) the DAQ card.

Injection force measurements: A syringe was prepared to deliver a 0.5 mL injection of MS-drug conjugate as follows. A vial of MS-drug conjugate slurry was vortexed for 3 seconds at 2500 RPM to homogenize then a 0.5 mL sample was withdrawn using a 1 mL syringe (Henke Sass Wolfe, SoftJect 8300018745) fitted with a 16 G x 1 ½" needle (BD, Precision Glide 305198). The needle was then switched to a 27 G x1 ¼" (BD, Precision Glide 305136) needle for injection. The syringe was placed in the texture analyzer with the load cell slightly above the plunger (the position at which the load cell would touch the plunger if the syringe was at the 0.55 mL mark). The injection was then performed at a rate of 5 mm/sec. The custom DAQ Factory app generated a plot of force vs. time for the injection. From the plot (**Figure S40A**), the breakaway force and glide force were determined as follows. The breakaway force was the maximum observed force on the onset of syringe plunger motion within the first 2.3 seconds of injection. The glide force was the average (equilibrium) force recorded from 2.3-7.0 seconds. It was also common to observe 1-2 stiction events during an injection with a magnitude of 1-2 kg above the glide force. The injected material was collected into a tared 15 mL centrifuge tube that was weighed to determine the amount of material delivered. The delivered slurry was dissolved in 0.900 mL of 50 mM NaOH for every 100 mg of slurry. The resulting solution was then assessed for [peptide] as described above. The peptide dose delivered was calculated by multiplying the determined peptide concentration (nmol peptide/mg slurry) by the weight of slurry collected (mg). The breakaway force was 1.7 ± 0.1 kg, the glide force was 1.7 ± 0.1 kg, and the dose delivered was 1.8 ± 0.1 nmol of peptide.

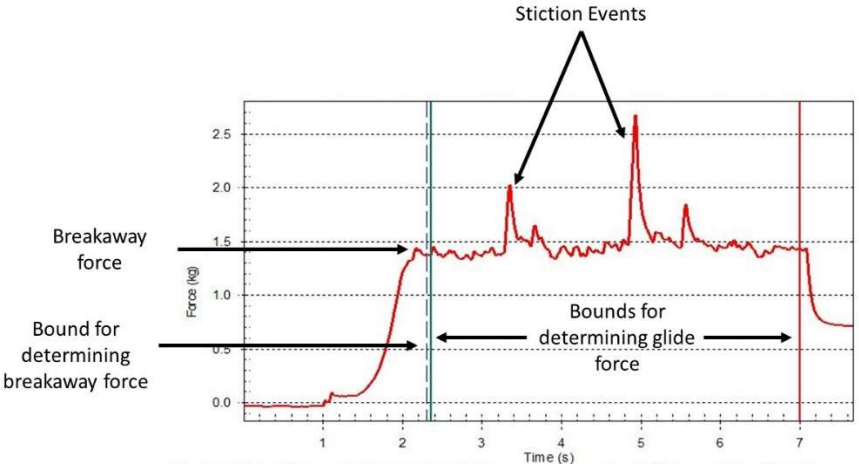


Figure S40A. A plot of force vs. time from an injection force experiment, showing breakaway force, regions used to quantify the breakaway and glide force, and stiction events.

3.5.9 Residual Solvents

A USP <467> compliant head space GC analysis method for residual solvents will be developed for the MS-drug conjugate. The solvents that will be tested for and suggested limits based on ICH guidelines are given in **Table S3**.

3.5.10 Elemental Impurities

A USP <232> compliant ICP MS analysis method for elemental impurities will be developed for the MS-drug conjugate slurry. The trace metals that will be tested for and suggested limits based on ICH guidelines are given in **Table S3.**

3.5.11 Sterility <USP 71>

A USP <71> compliant bioburden analysis has been validated and conducted by Pacific Biolabs (551 Linus Pauling Drive, Hercules, CA 94547) and found <10 CFU/ g or amino-MS slurry for both total aerobic microbial count, and combined yeast - mold.

3.5.12 Endotoxin <USP 85>

Endotoxin testing was performed on a USP <85> compliant Endosafe Portable Test System (Charles River, PTS100) using Endosafe PTS cartridges with a 0.01 EU/mL sensitivity (PTS2001F). Calibration of the instrument is performed yearly. Performed in triplicate, 0.050 mL samples of the MS-drug conjugate slurry were transferred into tared 1.5 mL microcentrifuge tubes using a positive displacement pipette. The slurry samples were diluted with 9 volumes of endotoxin free water (0.900 mL H_2O per 0.100 g slurry). After 30 minutes, the samples were centrifuged at 10,000 x g for 3 minutes in a table top centrifuge and the supernatant was tested following the Endosafe PTS protocol. Final endotoxin content for three replicate determinations was 0.22 ± 0.23 EU/mL of the MS-drug conjugate slurry.

4. REFERENCES

1 Meier, H.; Petersen, H. Synthese von 5-substituierten Cyclooctynen. *Synthesis* **1978**, *8*, 596-598.

2 Hanack, M.; Spang, W**.** Vinyl-Kationen, 32. Solvolysereaktionen von Cyclononinyl- und Cyclooctinylderivaten. *Chemische Berichte*, **1980**, *113*, 2015-2024.

3 Schneider, E. et al. Hydrogel Drug Delivery System Self-Cleaving Covalent Linkers for Once-a-Week Administration of Exenatide. *Bioconjugate Chemistry*, **2016,** *27*, 1210-1215.

4 Skoog, B. et al. Determination of polyethylene glycols 4000 and 6000 in plasma protein preparations. *Vox Sang*, **1979**, *37*(6), 345-349.

5 Alvares, R.D.; Hasabnis, A.; Prosser, R.S.; Macdonald, P.M. Quantitative detection of PEGylated biomacromolecules in biological fluids by NMR. *Anal. Chem.* **2016**, *88*(7), 3730-3738.

6 Henise, J. et al. Autoclave sterilization of tetra-polyethylene glycol hydrogel biomaterials with b-elimination crosslinks. *Engineering Reports*, **2020**, *2*(1), e12091.