Received: 7 April 2020 | Revised: 18 May 2020 | Accepted: 18 May 2020

DOI: 10.1002/eng2.12213

RESEARCH ARTICLE

WILEY

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

Jeff Henise | **Brian Yao** | **Brian R. Hearn** | **Eric L. Schneider** | **Gary W. Ashley** | **Daniel V. Santi**

Process Development, ProLynx, San Francisco, California, USA

Correspondence
Daniel V. Santi, Process Development, ProLynx, 455 Mission Bay Blvd. South, San Francisco, California, 94158, USA.
Email: daniel.v.santi@gmail.com

Peer Review
The peer review history for this article is available at https://publons.com/publon/10.1002/eng2.12213.

Abstract

The purpose of this work was to develop equipment and procedures for large-scale aseptic production of injectable microsphere (MS) drug conjugates. The two major challenges were (a) to prepare sufficient amounts of MSs for clinical trials, and (b) to prepare the MS-drug product under aseptic conditions. The approach was to prepare the MS-drug conjugate in two stages. Stage 1 was the preparation of monodisperse tetra-PEG amine derivatized MSs (amino-MS) from two soluble PEG prepolymers under low to no bioburden conditions. To accomplish this, custom-engineered equipment compatible with both aqueous and organic solvents was fabricated for parallel microfluidic preparation of amino-MS. The system was capable of preparing up to ~2 L of high quality 50 μm diameter amino-MS per day. Stage 2 was the sterilization of the starting amino-MS and aseptic production of the MS-drug conjugate. The amino-MS were first sterilized by autoclaving then transferred to a custom-engineered autoclave-sterilized washer-reactor. This apparatus allowed for activation of the amino-MS and attachment of a linker-drug under aseptic conditions to give the sterile MS-drug conjugate drug substance. The final drug product was produced by addition of excipients to form a homogeneous suspension. The entire process is exemplified by an engineering production run of a sterile MS-peptide drug product.

KEYWORDS

aseptic manufacturing, controlled release, drug delivery system, microspheres, microfluidics, process development

1 | INTRODUCTION

The use of injectable polymeric microspheres (MS) as sustained release drug depots has been a topic of high interest. Most MS drug delivery systems encapsulate drugs noncovalently within a polymer cross-linked with ester bonds such

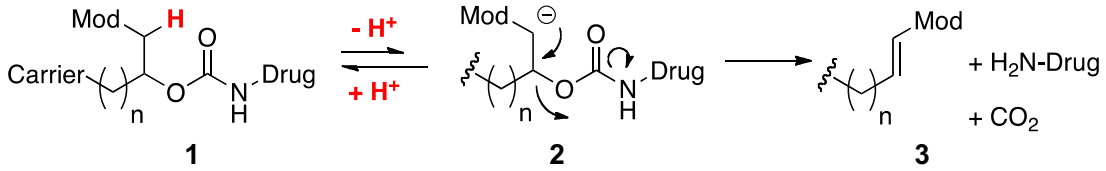
that pore size prevents drug diffusion and release. Drug release occurs by diffusion as the ester cross-links hydrolyze and pore sizes enlarge.[1-3] However, such systems have limitations that may make them unsuitable for many uses. As examples, the concomitant encapsulation-polymerization process often requires solvents that are detrimental to peptide or protein drugs; (b) irregular polymer compositions often result in a high "initial burst" release of the encapsulated drug, and heterogeneous drug release kinetics, and (c) polymer degradation via ester hydrolysis limits possible sterilization methods and generates carboxylic acids that can acidify the polymer interior and denature or modify acid-sensitive drugs.

We have developed a general approach for half-life extension of therapeutics in which a drug is covalently tethered to a long-lived carrier by a linker. This linker slowly cleaves through a hydroxide-catalyzed β-elimination reaction to release the native drug (Scheme 1).[4] The cleavage rate of the linker is controlled by the nature of an electron-withdrawing "modulator" (Mod) attached to a carbon atom containing an acidic C—H bond. After rate-determining proton removal, the intermediate rapidly collapses to provide the free drug. These linkers are not affected by enzymes and are stable for many years when stored at low pH and temperature.[5]

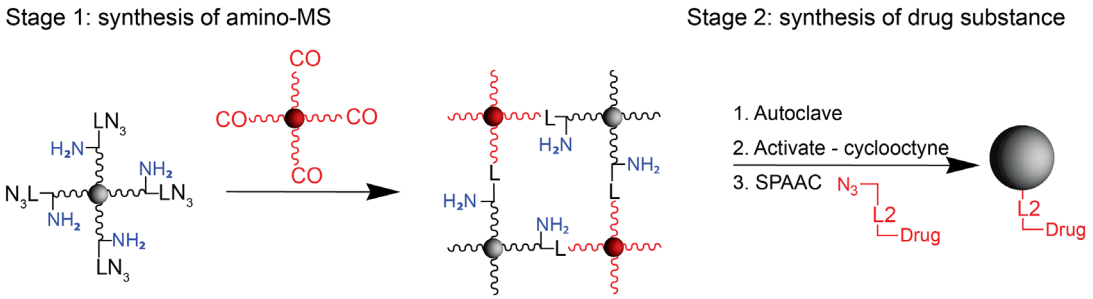
One carrier used to achieve half-lives of week and longer is a mesoporous tetra-PEG (polyethylene glycol) hydrogel polymer.[6-8] Sakai et al at Tokyo University discovered, developed and extensively studied a novel hydrogel, called a "tetra-PEG" gel, that has an extremely homogeneous polymer network with minimal defects and high mechanical strength and elasticity.[9,10] The gels are multi-purpose, but have high potential utility as vehicles for drugs and encapsulated cells. A tetra-PEG gel is formed from reaction of two 4-armed PEG prepolymers that have mutually reactive end-groups on each prepolymer - often an amine and an activated ester or carbonate. After mixing, the gels can be shaped during polymerization in molds or allowed to gel in situ at injection sites for in vivo applications. Recently, microfluidic devices have been used to produce tetra-PEG gels as MSs.[11]

In our application, tetra-PEG hydrogels - fabricated as 50 μm MSs - are injected subcutaneously (SC) or locally through a small-gage needle where they serve as a depot to slowly release the drug. The size of the microparticles were chosen because they are small/elastic enough to be injected through small-bore 29- or 30 gage needles, yet large enough to avoid phagocytosis.[12] Importantly, a cleavable β-eliminative linker, with a slower cleavage rate than used for drug release, is incorporated in each crosslink of these polymers, so hydrogel dissolution occurs after drug release.[7,13]

Production of our injectable tetra-PEG hydrogel MS-drug conjugates is achieved in two stages (Scheme 2).[8,14] Stage 1 involves preparation of amine-derivatized MSs (amino-MS) by a unique droplet-based microfluidic system that enables production under conditions of low bioburden. Here, equimolar amounts of a four-arm PEG containing 4 amine and 4 azido-linker end groups (LN_3) and a four-arm PEG containing cyclooctyne end groups (CO) are mixed in a droplet forming microfluidic device. The azide and cyclooctyne end groups (EGs) of these PEG prepolymers react within the droplets by strain-promoted alkyne-azide cycloadditions (SPAAC) to form 1,2,3-triazole crosslinks and provide amino-MS.[15] Stage 2 involves autoclave sterilization of the amino-MS,[5] activation with an amine-reactive CO and coupling to an azido-linker-drug (N_3-L2-drug) by SPAAC to form the MS-drug conjugate drug substance, and finally formulation and transfer of the drug substance to vials or syringes to provide the drug product.



SCHEME 1 *β*-Elimination mechanism of linker cleavage



SCHEME 2 Synthesis of a tetra-PEG microsphere drug conjugate

In the present work, we describe for Stage 1, (a) the design, assembly and operation of equipment necessary to prepare amino-MS and a derived MS-drug conjugate; (b) syntheses and analyzes of the prepolymer precursors to the amino-MS; (c) droplet-based microfluidic preparation and analysis of amino-MS; then, in Stage 2, (d) preparation of an azido-linker-peptide; and (e) aseptic synthesis and analysis of a MS-drug conjugate. These combined processes are exemplified by aseptic synthesis of a covalent MS-peptide conjugate intended for SC administration.

2 | RESULTS AND DISCUSSION

2.1 | Preparation of amino-MS (Stage 1)

2.1.1 | Reagent syntheses

The reagents required for the preparation of amino-MS precursors include an amine-reactive cyclooctyne (5-HCO-HSC), high-quality four-arm amino-PEG (PEG-$[NH_2]_4$), and commercially available small-molecule starting materials. The current example uses 10 kDa four-arm PEG-$(NH_2)_4$, but methods are identical for 20 kDa and other sizes of four-arm PEGs. These are converted by conventional organic synthesis to Prepolymers A and B (Scheme 3) which are the immediate precursors to the amino-MS. Details of syntheses are provided in the Section 1.

*5-Hydroxycyclooctyne succinimidyl carbonate (5-HCO-HSC, **9**)*

5-Hydroxycyclooctyne (5-HCO) was prepared by modification of a reported procedure[16] suitable for preparation of large amounts of the activated precursor. In the modified procedure, Jones Reagent was substituted by NaOCl/TEMPO to avoid possible introduction of chromium into the drug substance, and individual procedures were optimized to obtain a ~25% overall yield in large-scale preparations. 5-HCO was converted to 5-HCO-HSC **9** as previously described.[17]

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

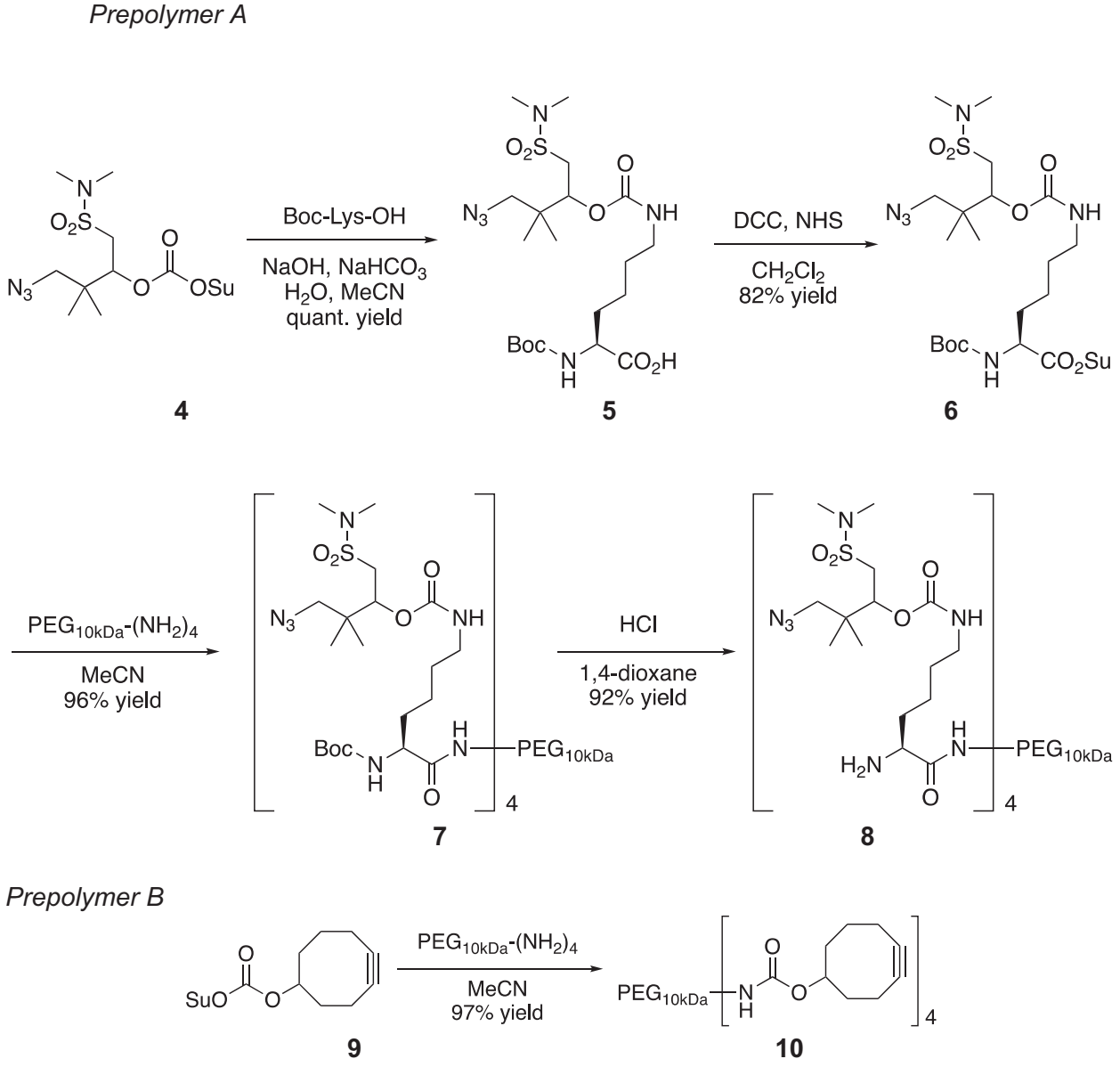
The number of amine groups and the % amine EGs (EG) in the PEG_{10kDa}-$(NH_2)_4$ starting material were determined by high performance liquid chromatography (HPLC) analysis after derivatization with 9-fluorenylmethyl *N*-succinimidyl carbonate (Fmoc-OSu) or a related reagent (see Prepolymer A and Prepolymer B below). An HPLC standard containing all possible Fmoc substitutions was prepared by reaction of excess PEG_{10kDa}-$(NH_2)_4$ with Fmoc-OSu. Concurrently, HPLC analysis of a reaction containing limiting PEG_{10kDa}-$(NH_2)_4$ with Fmoc-OSu was used to determine PEG species that contain n = 1, 2, 3 and 4 amine EG. The fraction of each Fmoc-substituted PEG was determined by its peak area/total peak area, and total reactive EG were determined as $EG = n_4 + 0.75*n_3 + 0.5*n_2 + 0.25*n_1$. In a typical example, commercially available PEG_{10kDa}-$(NH_2)_4$ had 92.2% of n = 4, 7.3% of n = 3% and 0% of n = 2 and n = 1 giving total EG = 97.7%. In the present work, an acceptable EG content for PEG_{10kDa}-$(NH_2)_4$ was 97 ± 2.5% which was shown to correlate to an acceptable ±5% variance in the time to reverse gelation (t_{RG}) of the derived amino-MS.

Prepolymer A

For Prepolymer A, excess Boc-Lys-OH was first reacted with the azido-linker succinimidyl carbonate (N_3-L-HSC, **4**) to provide Boc-Lys(N_3-L)-OH **5** in quantitative yield. Then, **5** was reacted with *N*-hydroxysuccinimide and *N,N′*-dicyclohexylcarbodiimide (DCC) to give, after silica gel purification, pure Boc-Lys(N_3-L)-OSu **6** in 82% yield. Finally, excess **6** was coupled to PEG_{10kDa}-$(NH_2)_4$. As analyzed by C18 HPLC with evaporative light-scattering detection (ELSD), the four-arm amino-PEG starting material converted to a single tetra-substituted (n = 4 EG) product over time via three slower-eluting intermediate peaks, n = 1, 2, and 3 EG. The product was reacted in situ with Ac_2O to cap any trace amines that might remain and purified by precipitation with methyl tert-butyl ether (MTBE). Using an analogous analytical method as described for PEG_{10kDa}-$(NH_2)_4$ (above), HPLC indicated an amine substitution of 93.3% n = 4, 6.2% n = 3, and a total EG functionalization of 98.0%. The Boc protecting groups of the [Boc-Lys(N_3-L)]$_4$-PEG_{10lkDa} **7** were removed with HCl/dioxane using C18 HPLC-ELSD to monitor the reaction progress. Product was obtained in 92% yield. The complete four-step sequence generated Prepolymer A **8** in 72% overall yield.

Prepolymer B

Reaction of PEG_{10kDa}-$(NH)_4$ with excess 5-HCO-HSC **9** in acetonitrile (MeCN) gave a single tetra-substituted product (n = 4 EG) over time via three slower-eluting intermediate peaks, n = 1, 2 and 3 EG, by C18 HPLC with ELSD. The product



SCHEME 3 Synthesis of prepolymer A and B

was capped with Ac_2O to cover any trace amines that may remain and precipitated with MTBE to give a 97% yield of Prepolymer B **10**. EG analysis as described for PEG_{10kDa}-$(NH_2)_4$ (above), gave 95.8% n = 4% and 3.1% n = 3 substitution and a 98.1% yield of total 5-HCO EG.

2.1.2 | Amino-MS preparation by microfluidics

The amino-MS forming reaction (Scheme 2, **stage 1**) is performed in a microfluidic flow focusing device that mixes aqueous solutions of Prepolymers A and B and produces ~50 μm diameter droplets dispersed in an immiscible continuous phase composed of Abil EM90 and polyglycerol polyricinoleate in decane.[13] This combination of surfactants has been shown to prevent coalescence of PEG containing micro droplets produced in similar microfluidic devices.[18] The azide and CO EGs of the prepolymers then react by SPAAC to form alkyl-triazole crosslinks and stable amino-MS gels. Each crosslink within the polymer contains a free amino group originating from the Lys residues of Prepolymer A, which are destined to become connection sites for the linker-drug conjugate.

The Dolomite Telos drop-forming microfluidic chips used here contain seven flow focusing drop formers each and are capable of producing ~8 mL/h of water-swollen amino-MS each (Figure 1A,B) (https://www.dolomite-microfluidics.com/).[19] As shown, Prepolymers A and B are pumped into the inner channels and mix at the *Y*-junction. The continuous phase is pumped into the outer channels and interrupts the flow of the prepolymers to form uniform droplets.

To enable sufficient production of amino-MS to support clinical trials, we fabricated a microfluidic chip-bank (Figure 1C) that serves as a distribution manifold capable of holding up to ten 7-channel chips and manufacturing 40 mL of emulsified droplets or ~80 mL swollen amino-MS per hour. The chip-bank is similar in function to the Dolomite Telos high-throughput system (https://www.dolomite-microfluidics.com/), but contains polyether ether ketone, perfluoroelastomer and 316 stainless steel as the product contacting surfaces, which are choice-materials for current good manufacturing practice (cGMP) compliant equipment[20] and suitable for steam sterilization. Our chip-bank also allows for monitoring of drop formation of each channel with an automated microscope for quality control. The bank was designed such that when the screws securing the chip-retaining cassettes were fitted with appropriate torque it could be autoclaved, then tightened to form the fluidic seal to the chips after cooling in a clean environment.

Multiplexing aseptic microfluidic apparatus

We developed a hermetically-sealed manufacturing system for large scale production of amino-MS. Multiplexing aseptic microfluidic apparatus (MAMA) was originally designed to be cGMP compliant and produce both the amino-MS and the MS-drug conjugate under aseptic conditions.[21] With the recent finding that the amino-MS could be sterilized,[5] the need for aseptic processing became limited to Stage 2: production of the MS-drug conjugate. The current process involves production of amino-MS with low-bioburden[22] in MAMA, followed by autoclave sterilization, then aseptic conversion to the MS-drug conjugate in a separate autoclave sterilized washer-reactor. Although there is no current need to produce the amino-MS aseptically, the system provides exceptional control of bioburden and could be validated for aseptic cGMP compliance if desirable. Further details of this system and its use are provided in Section 2.

Figure 2 shows a diagram of MAMA illustrating functional assemblies as modules A to G. Module A contains the feed tanks and in-line sterilizing-filters for prepolymer solutions and the continuous phase. Module B contains pressure regulators and flow sensors. Module C is the computer-based supervisory controller and data acquisition instrument and proportional integral derivative (PID) liquid flow controller. Module D contains the chip-bank with a robotic microscope, and an emulsion collection manifold. Module E contains two washer-reactor vessels with sterile inlet filters for the washing reagents, hermetically-sealed magnetically-coupled stirrers and Dutch-weave sieves in their bases for particle size fractionation. Module F has several washing reagent tanks. Module G is the amino-MS collection and transfer container.

A unique feature of the MAMA system is the compatibility of materials with both aqueous and organic solvents. Materials in contact with the fluidic process include United States Pharmacopeia (USP) Class VI[23] compliant 0.2 μm membrane filters, elastomeric seals and tubing, as well as glass, and 316 stainless steel.

Preparation of the MAMA system for amino-MS production

For low bioburden production of amino-MS, the MAMA system is first cleaned and disinfected by a clean-in-place (CIP) procedure using sterile-filtered liquids, during which the apparatus is closed to prevent contamination by airborne

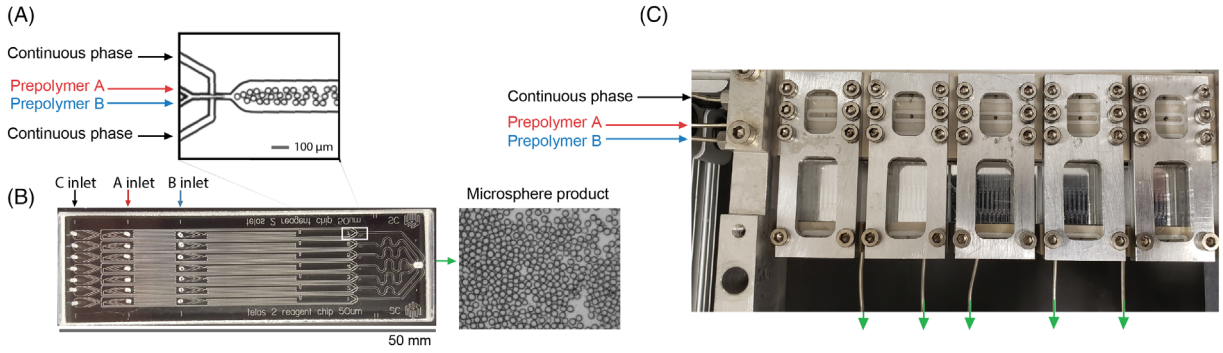


FIGURE 1 Microfluidic emulsion synthesis of tetra-PEG hydrogel amino-MS. A, Micrograph of a microfluidic flow focusing device producing amino-MS from an aqueous mixture of Prepolymers A and B. B, Dolomite Telos glass microfluidic chip containing seven drop forming channels and micrograph of amino-MS suspended in the continuous phase. C, Chip-bank manifold shown with five microfluidic chips

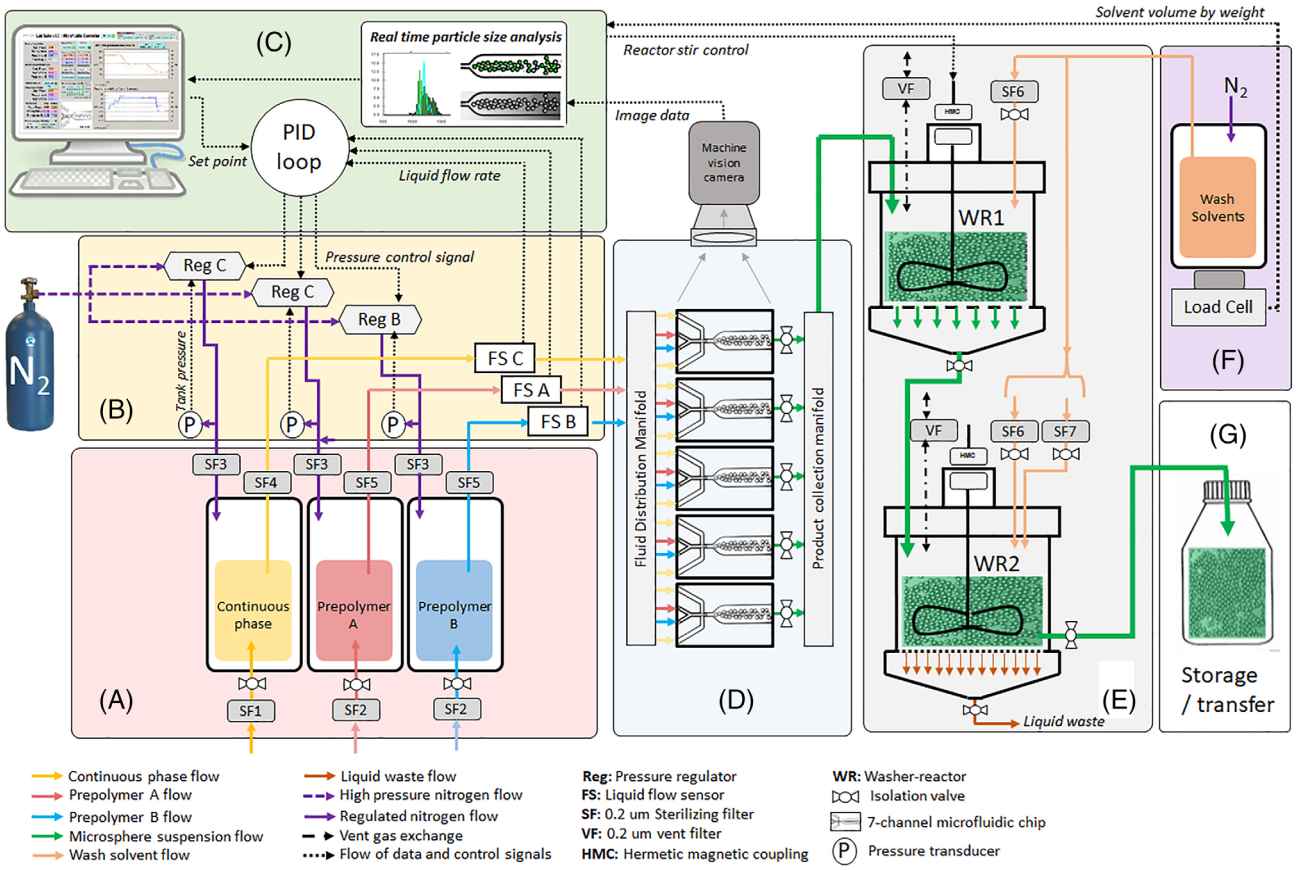


FIGURE 2 Diagram of the multiplexing microfluidic apparatus used to produce amino-MS

particles. Here, the process filters and microfluidic chips are removed, and the system is flushed with isopropanol, an alkaline detergent, water, and then dried with nitrogen. After cleaning, a pre-use calibration of the flow sensors is conducted to ensure accurate mixing of Prepolymers A and B (Figure S14). The calibration is performed by an automated method using an HPLC pump as a standard flow source and filtered water as calibration fluid; after a six-point linear calibration of each sensor, a "self-check" is used to reproduce two flows within the calibrated range. Reproducibility is generally within 0.25% of the expected value. Finally fresh process filters (Table S1) and microfluidic chips are installed and the fluidic lines connecting the individual components are connected.

MAMA operation

1. *Charging of prepolymers and continuous phase*. The feed tanks are charged with 250 mL of each prepolymer solution and 4 L of the continuous phase through 0.2 μm sterilizing filters at 20 PSI (Figure 2A). A hydrophobic polytetrafluoroethylene (PTFE) membrane is used for filtering the continuous phase, and hydrophilic polyethersulfone (PES) membranes are used for filtering the prepolymer solutions. These amounts of reagents are sufficient to produce 1 L of swollen amino-MS suspension.
2. *Emulsion synthesis and polymerization*: The prepolymers and continuous phase are pumped from the feed tanks through a second set of 0.2 μm polyvinylidene difluoride (PVDF) membrane filters (hydrophilic PVDF for prepolymers; hydrophobic PVDF for continuous phase) into the chip bank where uniform size droplets are formed (Figure 2D). Fluid flow rates are controlled by the custom-engineered nitrogen-pressure driven pump system and are computer-controlled to ±1% SD using a PID loop that adjusts the holding tank pressure to maintain flow as measured by the flow sensors (Figure 2B,C). Currently, droplets are formed with a PEG content above the equilibrium swelling concentration of the polymerized hydrogel which allows higher throughput of droplet formation and increased rate of polymerization. During operation, each of up to 70 drop-formers is imaged by an automated microscope every 5 minutes as a statistical control of particle size. If a chip fails and produces aberrant size particles, it can be excluded from the system by closing a valve and replaced. However, a failed chip

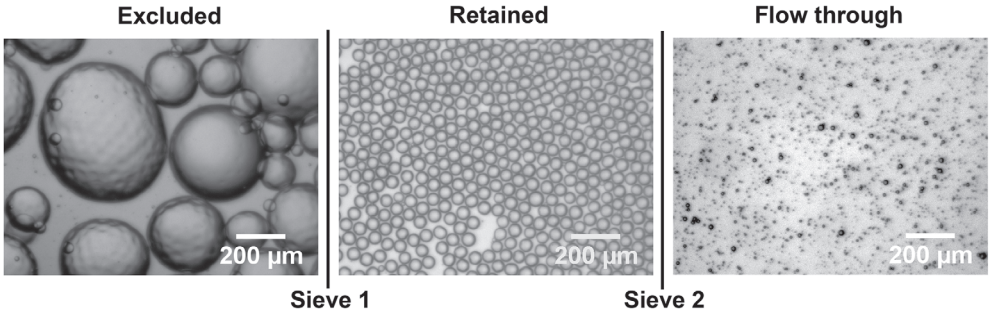


FIGURE 3 Micrographs of amino-MS after separation by sieving in washer-reactor one and washer-reactor two. The desired product (retained ~50 µm diameter particles) is ≥95% of the total batch by weight

does not require immediate attention since too-large and too-small particles are subsequently removed by sieving in the washer-reactors. As part of normal operation a population of small (<5 µm) "satellite" particles and occasional large (>200 µm) particles are produced that together comprise ≤5% of the total droplets by volume (Figure 3). Chip failure can eventually occur if a microscopic string of polymerizing hydrogel sticks to the glass and grows through the drop forming channel. This string acts like a wick causing the prepolymer solution to flow through the drop forming channel and break off into a continuous stream of large particles downstream of the channel.

3. *Polymerization and large particle removal*. The single outlet of each chip delivers the emulsion to a manifold that converges into a single stream that is delivered to the first sieve-bottom washer-reactor one (Figure 2E, **WR1**). After the entire batch of emulsion is collected the reactor is heated to 40°C for 18 hours to polymerize the droplets by SPAAC. Then, the amino-MS suspension is stirred and drained through the ~80 µm sieve in the reactor's base at 5 PSI to remove aberrant large particles (Figure 3, **excluded**) and transfer the amino-MS slurry to washer-reactor two. Finally, washer-reactor one is rinsed with continuous phase to fully recover the remaining desired ~50 µm size amino-MS.
4. *Amino-MS washing*. While stirring, the continuous phase of the amino-MS suspension in washer-reactor two (Figure 2E, **WR2**) is drained through the ~20 µm sieve in the reactor's base that retains the desired ~50 µm amino-MS and allows passage of unwanted small spheres <15 µm diameter (Figure 3, **flow through**). Finally, particles are washed with heptane to remove decane and surfactants of the continuous phase, 95% ethanol to remove heptane, then water and finally pH 4.0100 mM acetate storage/autoclave buffer.
5. *Amino-MS storage and transport*. The amino-MS suspension in washer-reactor two is transferred by gravity into a GL-45 glass bottle (Figure 2G) via a port above the sieve in the reactor's lower bulkhead. The amino-MS suspension is stored at 4°C prior to autoclave sterilization.

Analytical release of amino-MS

After production, amino-MS are subjected to a battery of quality control assays to ensure batch-to-batch uniformity within acceptable quality specifications. As detailed in the Table S2, release criteria include appearance, amine/PEG ratio, dissolution time (t_{RG}), particle size, pH, residual reagents, surfactant and solvents, trace elements, bioburden, and endotoxin. The analytical methods for these assays are presented in Section 2.

Proposed parallel continuous amino-MS production

For large scale production of amino-MS we propose a possible strategy where several emulsion generation devices (chip-banks) can be operated in parallel to achieve continuous droplet formation (Figure 4). As described above, in-line process monitoring allows individual devices to be replaced without interrupting the process. The emulsion can be fed into parallel washer-reactors that accumulate material for polymerization. Once the first washer-reactor (Pol1) is full it could be heated to conduct polymerization while accumulation of emulsion would be shifted to the second washer-reactor (Pol2). Purification would be handled in the same manner using a parallel array of purification washer-reactors. Here, after polymerization the amino-MS suspension in Pol1 would be fed into the first purification washer-reactor (Pur1) and so on. Using three washer-reactor systems a CIP cycle would be incorporated to coordinate continuous production of amino-MS: reactor number one would be in the cleaning phase, number 2 would be accumulating material, and number three would be purifying material.

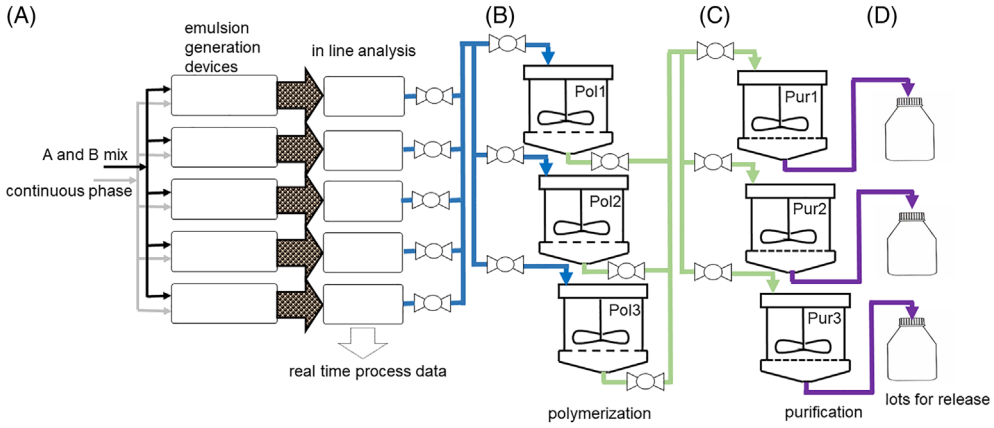
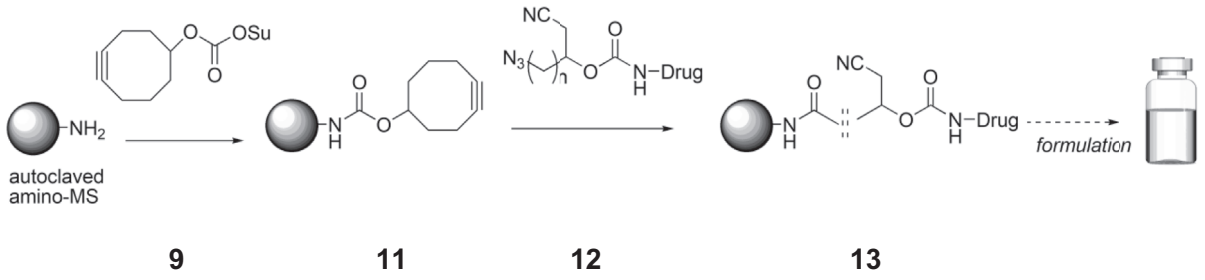


FIGURE 4 Proposed configuration of manufacturing equipment for continuous production of amino-MS. A, Parallel emulsion generation devices with in-process monitoring. B, Three parallel washer-reactors (Pol1, Pol2, and Pol3) containing coarse sieves for accumulation of droplets for polymerization into amino-MS. C, Washer-reactors containing fine sieves for accumulation and purification of amino-MS. Three parallel washer-reactors (Pur1, Pur2, and Pur3) are shown. D, Lots of purified amino-MS for analytical release



SCHEME 4 Aseptic production of a MS-drug conjugate

2.2 | Preparation of a MS-drug conjugate drug product (Stage 2)

In this aseptic process (Scheme 4), amino-MS are first sterilized by autoclaving in acidic media and then transferred to an autoclave sterilized washer-reactor for drug attachment chemistry. The amine groups are activated by attachment of a cyclooctyne group, and the resulting cyclooctyne-MS (CO-MS) **11** are coupled with an azido-linker-drug conjugate **12** by SPAAC. Finally, the MS-drug conjugate **13** is formulated as a suspension in hyaluronic acid (HA) and filled into vials or syringes to give the drug product. Detailed methods for this process are given in Section 3.

2.2.1 | Reagent synthesis

The reagents required for modification of amino-MS and preparation of the MS-drug conjugate include an amine-reactive cyclooctyne (5-HCO-HSC, described above) for activation of amino-MS, and an azido-linker-drug conjugate is coupled to the resulting CO-MS by SPAAC.

Azido-linker-drug conjugate

N^{α}-(7-Azido-1-cyano-2-heptyloxycarbonyl)-[Gln^{28}]exenatide (N_3-L(CN)-[Gln^{28}]exenatide). In the present example, the drug is [Gln^{28}]exenatide, a stabilized analog of the GLP-1R agonist exenatide[14] which contains an azido-linker at the N-terminus. [Gln^{28}]exenatide was synthesized by solid phase peptide synthesis using Fmoc/tBu chemistry and reacted on-resin at the α-amine group with *O*-(7-azido-1-cyanohept-2-yl)-*O*′-succinimidyl carbonate in *N,N* dimethyl formamide containing N-methyl morpholine. Reaction of the peptide-resin with 90:5:5 TFA:H_2O:TIPS followed by preparative RP-HPLC provided good yields of the N_3-L(CN)-[Gln^{28}]exenatide **12**.

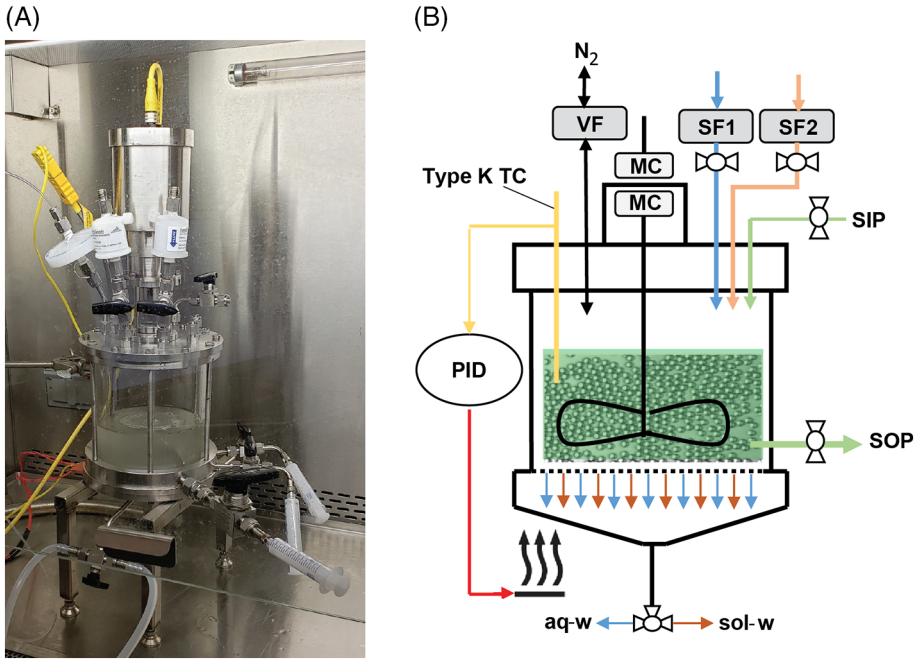


FIGURE 5 Sealed washer-reactor for production of MS-drug conjugates. A, Photo and B, schematic. Type K TC, type K thermocouple probe in stainless steel sheath; N_2, nitrogen gas 0 to 10 PSI; VF, 0.2 μm PTFE vent filter; MC, magnetic coupling; SF1, 0.2 μm polyethylene (UE) filter for MeCN; SF2, 0.2 μm PES for aqueous and alcohol solutions; SIP, MS slurry inlet port; SOP, MS slurry outlet port; Aq-w, aqueous waste outlet; Sol-w, solvent waste outlet; PID, PID controller for heater

2.2.2 | Washer-reactor

A hermetically sealed washer-reactor was fabricated for aseptic drug attachment chemistry on up to 1.5 L of amino-MS slurry (Figure 5). The unit is composed of a 150 x 136 mm glass cylinder with stainless steel upper and lower bulkheads. All process-contacting components are made of 316 stainless steel, USP Class VI compliant FFKM, silicon carbide/graphite (mechanical seal for stir shaft) or glass. The upper bulkhead contains a magnetically-coupled stirrer, and ports fitted with a temperature probe, valves and 0.2 μm sterilizing filters for introducing reagents or N_2. The lower bulkhead contains a Dutch-weave sieve that retains MS ≥15 μm in diameter, a port below the sieve for removing smaller particles and liquid waste, and ports above the sieve for sampling and removal of MS slurry. An external PID controlled heater is attached to the bottom of the reactor.

2.2.3 | Sterilization

The washer-reactor and amino-MS were sterilized by autoclaving.

Washer-reactor sterilization

The washer-reactor (Figure S28) and accessories were sterilized by autoclave. Here, reactor-valves were positioned to allow steam contact to all process contacting surfaces and the assembled reactor was wrapped with Kim Tech KC100 steam permeable cloth that was sealed with autoclave tape. After an autoclave cycle (121°C, 20 minutes), the reactor was allowed to cool to ~75°C and transferred to a clean environment. After cooling to ambient temperature, integrity tests - for example, reactor assembly leak-tightness and filter bubble points[24]-were performed.

MS sterilization

A 2 L GL45 bottle containing amino-MS in 0.1 M NaOAc, pH 4.0, was placed in a steam-permeable Tyvek bag, subjected to one autoclaving cycle (121°C, 20 minutes), and allowed to cool to 97°C prior to removal from the autoclave. The bottle was transferred to a clean environment, allowed to cool to ambient temperature, then the amino-MS were transferred to the washer-reactor through the SIP valve in the upper bulkhead using a dip-tube and 5 psi N_2.[5]

2.2.4 | Amino-MS activation and loading

The amino-MS were suspended in MeCN and reacted with an excess of 5-HCO-HSC to modify the amine groups with 5-HCO followed by reaction with Ac_2O in situ to cap trace unreacted amino groups, and then purified by washing

with MeCN. The resulting CO-MS **11** were exchanged into isopropanol (IPA)/citrate peptide loading buffer, then the azido-linker-drug **12** was attached by SPAAC. The resulting MS-drug conjugate **13** was purified from soluble reactants by extensive washing.

Cyclooctyne modification of amino-MS

The NaOAc autoclave buffer was first exchanged for water and then with MeCN. Wash solvents were introduced to the washer-reactor through the PES or UE sterilizing filters by dip tube transfer from stainless steel tanks. After filling the reactor for each wash the stirrer was pulsed three times (250 RPM, 3 seconds) to rinse the top of the reactor, and the dead ends of the slurry sample and outlet ports were purged by pumping the attached syringes. Between washes, the reactor was drained through the below-sieve port while stirring at 50 RPM. After draining the final MeCN wash from the sterilized amino-MS, 4 eq. of triethylamine - compared to amines of amino-MS - in MeCN was added through the UE inlet filter, followed by 1.5 eq. of 5-HCO-HSC in MeCN; the reactor was then stirred (50 RPM) at room temperature for 14 hours. Samples of the MS slurry were removed before and after reaction with 5-HCO-HSC to test for completeness of the reaction; here, a small sample of MS slurry was treated with 2,4,6-trinitrobenzenesulfonic acid (TNBS); absence of orange color in the modified MS as compared to the starting amino-MS indicated complete reaction of amines (Figure S32). Next, 5 eq. of Ac_2O in MeCN was added to the reactor through the UE inlet filter to insure complete capping of trace amines. The stirrer was pulsed three times to wash the top of the reactor, then stirring was continued for 30 minutes, and the reactor was drained under pressure.

Drug attachment to CO-MS

The CO-MS were washed with MeCN and then exchanged into peroxide-free IPA/citrate peptide loading buffer (50% v/v IPA, 0.1 M Na citrate, pH 3.5-3.8). As before, after filling the reactor for each wash the stirrer was pulsed three times to rinse the top of the reactor, and the slurry sample ports in the lower bulkhead were purged by pumping the attached syringes. The reactor was then drained at 5 PSI while stirring. Next, 1.2 eq. (relative to CO-groups) of the azido-linker-drug in IPA/citrate buffer was added to the reactor through the PES inlet filter followed by heating the reactor to 37°C for 90 hours to complete the SPAAC reaction. The resulting MS-drug conjugate slurry was then extensively washed with IPA/citrate buffer, and then exchanged into isotonic acetate tween formulation buffer (IAT: 10 mM NaOAc, pH 5.0, 0.05% polysorbate 20 and 111 mM NaCl).

2.2.5 | Formulation and filling

In this final step, the MS-drug conjugate drug substance was suspended in an excipient to prevent settling (Figure S33), and aseptically transferred to dispensing vials (Figure S34).

Formulation

After exploratory studies, we found that MS-drug conjugate suspensions remained stable for prolonged periods in 1.2% HA, M.W. 61.8 kDa. For example, a slurry of MS-drug conjugate in 1.2% HA buffer showed no visual evidence of settling after 7 months of standing.

The MS-drug conjugate slurry was drained from the reactor into a GL-45 glass laboratory bottle while stirring and pressurizing the reactor to 2 PSI with nitrogen. The reactor was rinsed with IAT buffer then the peptide concentration of the combined slurry and rinse was determined by NaOH-catalyzed peptide release and absorbance of the resulting solution at 280 nm ($\varepsilon = 5500\ M^{-1}\ cm^{-1}$). Using a syringe pump, appropriate amounts of 10% (w/v) 61.8 kDa HA in IAT buffer and IAT buffer were sterile-filtered (0.2 μm, PES) into the bottle containing the MS-drug conjugate slurry to give the desired drug potency and a final HA concentration of 1.2% (w/v).

The volumes of IAT, V_{IAT}, and concentrated HA, V_{HA}, added to achieve the desired drug concentration $[D_{MS}]_f$ and the final HA concentration, $[HA]_f$, were calculated using Equations (1) and (2):

$$V_{HA} = V_{MS} \cdot \frac{[D_{MS}]_0}{[D_{MS}]_f} \cdot \frac{[HA]_f}{[HA]_0} \tag{1}$$

$$V_{IAT} = V_{MS} \left[\frac{[D_{MS}]_0}{[D_{MS}]_f} \cdot \left(1 - \frac{[HA]_f}{[HA]_0} \right) - 1 \right] \tag{2}$$

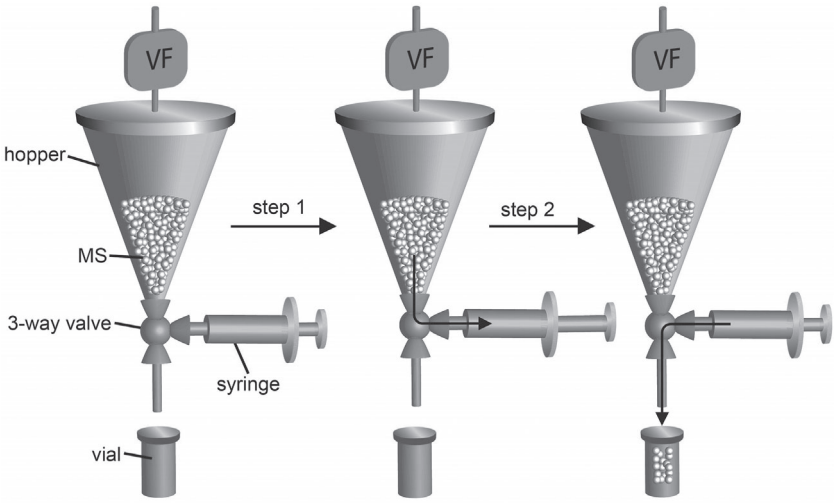


FIGURE 6 Manual filling system for MS-drug conjugates. Hopper and syringe assembly showing MS-drug conjugate slurry (MS), Teflon coated hopper with stainless steel lid, syringe, 3-way valve, vent filter, and a vial. In step 1, a given volume of MS is drawn into the syringe, and in step 2 the syringe content is expelled into a dispensing vial

Here, V_{MS} is the volume of MS-drug conjugate slurry having the initial concentration of drug $[D_{MS}]_0$ and $[HA]_0$ is the HA in the concentrated solution added to obtain $[HA]_f$. For the MS-drug conjugate described here, since $[HA]_0$ is 10% HA and $[HA]_f$ is 1.2% HA the equations become 3 and 4:

$$V_{HA} = V_{MS} \cdot \frac{[D_{MS}]_0}{[D_{MS}]_f} \cdot 0.12 \tag{3}$$

$$V_{IAT} = V_{MS} \cdot \left[\left(\frac{[D_{MS}]_0}{[D_{MS}]_f} \cdot 0.88 \right) - 1 \right] \tag{4}$$

Dosing vials

Manual filling of dosing vials was performed with an apparatus (Figure 6) designed to mimic the function of a Colanar rotary piston pump filling system (https://www.colanar.com/products/fill-systems/rotary-piston-pumps-fsd). The apparatus consists of a teflon-coated stainless steel hopper fitted with a stainless steel lid with a vent filter, a three-way switching ball valve at the base, and a 3 mL syringe attached to the valve's side port to withdraw and dispense MS slurry. In a trial run, filling of 80 vials with MS slurry in 1.2% HA and analysis of every tenth vial gave 2.6% SD in the amount MS delivered. Prior to use, the filling apparatus, trays containing 48 vials, caps and the capping tool were sterilized in steam permeable Tyvek bags by autoclaving, then transferred to a clean environment to cool. After cooling to ambient temperature, the filling apparatus was assembled and charged with formulated MS-drug conjugate. The three-way valve was first positioned to allow a measured amount of MS-drug conjugate slurry - 1.4 mL in the present case - to be withdrawn from the hopper with the attached syringe (Figure 6, **Step 1**), and then positioned to allow the expulsion of the MS-drug conjugate slurry in the syringe into a sterile vial (Figure 6, **Step 2**). The formulation does not settle or stick to the Teflon-coated hopper during filling. Potency analysis of the MS-drug conjugate by NaOH-catalyzed peptide release of the first and the last vial of each rack showed 3.4 ± 0.1 mM peptide indicating consistent filling. After filling four 48-vial racks, the vials were fitted with rubber septa and crimp-sealed with metal collars.

Syringeability and injectability

Both the ease of withdrawal of a product from a container - syringeability - and its subsequent injection into the intended administration site - injectability - are key product performance parameters of MS suspensions.[25] In model studies, syringeability of fluorescein-labeled MSs[13] in 1.2% HA was determined by the accuracy and reproducibility of withdrawing 250 μL aliquots from a sealed 2R Schott vial into a 1.0 mL syringe using a 16 G x 1.5″ needle. After each withdraw using a different syringe assembly, the MS were dissolved in 50 mM NaOH and the fluorescein content was by absorbance at 495 nm. The results from 8 consecutive withdraws from a single vial showed a fluorescein content of 70.4 ± 3.2 μM. For the formulated MS-drug conjugate described here, 0.50 mL samples were withdrawn using a 16 G x 1.5″ needle. After switching to a 27 G x 1.5″ needle, the injection breakaway and glide force of were measured with a texture analyzer and

an injection rate of 5 mm/s; the effluent of the injections was collected and the amount of peptide delivered (1.8 ± 0.1 μM) was determined by NaOH-catalyzed release and absorbance of the resulting solution at 280 nm, showing consistent syringeability and injectability.

Analytical release assays

The analytical release assays for the formulated MS-drug conjugate included appearance, potency ([peptide]), identity ([peptide]/[PEG]), dissolution (peptide release rate and t_{RG}), free peptide, particle size, pH, injection force, dose delivered, sterility and endotoxin. Results from these assays are provided in Table S3.

3 | SUMMARY

In summary, we developed equipment and procedures for large-scale aseptic production of injectable covalent MS-drug conjugates to support clinical development. We prepared the drug substance in two stages. Stage 1 involved the preparation of a tetra-PEG amino-MS hydrogel carrier containing free amine groups from two soluble PEG prepolymers. Here, equipment was designed and fabricated for large scale microfluidic preparation of the amino-MS with low bio-burden - capable of continuous preparation of ~2 L/day of high quality 50 ± 5 μm diameter water-swollen amino-MS. Although the MAMA system does not have the throughput that can currently be achieved with some other parallel droplet formers,[26] it is unique in that it is made of materials stable to both aqueous and organic solvents and to autoclaving, and can produce amino-MS in an aseptic environment. In Stage 2, the amino-MS from Stage 1 were first sterilized by autoclave in aqueous acidic media - a sterilization method unique to the acid-inert tetra-PEG hydrogel amino-MS used here.[5] They were then transferred to a custom-engineered hermetically sealed washer-reactor that facilitated performance of aseptic chemistry for activation of the amino-MS and attachment of a linker-drug conjugate to give the MS-drug conjugate drug substance. Finally, excipients were added to formulate homogeneous suspensions of the MS-drug conjugate to give the sterile drug product in vials. As an example of the manufacturing process, we have described manufacturing and analytical methods used for an engineering run of a large batch of sterile MS-peptide conjugate drug product. Finally we would like to point out that the equipment described here may have further utility where the first stage of the process can be translated to preparing other types of MSs from soluble prepolymers under low bioburden or aseptic conditions, whereas the second stage can be translated to chemical modification of preformed sterilized polymer MSs under aseptic conditions.

PEER REVIEW INFORMATION

Engineering Reports thanks Amin Famili, Tsutomu Ono, and other anonymous reviewers for their contribution to the peer review of this work.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

ORCID

Jeff Henise https://orcid.org/0000-0003-0975-1006

REFERENCES

1. Blasi P. Poly(lactic acid)/poly(lactic-co-glycolic acid)-based microparticles: an overview. *J Pharm Investig*. 2019;49:337-346.
2. Hennink WE, van Nostrum CF. Novel crosslinking methods to design hydrogels. *Adv Drug Deliv Rev*. 2002;54(1):13-36.
3. Hoare TR, Kohane DS. Hydrogels in drug delivery: Progress and challenges. *Polymer*. 2008;49(8):1993-2007.
4. Santi DV, Schneider EL, Reid R, Robinson L, Ashley GW. Predictable and tunable half-life extension of therapeutic agents by controlled chemical release from macromolecular conjugates. *Proc Natl Acad Sci U S A*. 2012;109(16):6211-6216.
5. Henise J, Yao B, Ashley GW, Santi DV. Autoclave sterilization of tetra-PEG hydrogel biopolymers with β-eliminative crosslinks. *Eng Rep*. 2020;2:1-9.
6. Ashley GW, Henise J, Reid R, Santi DV. Hydrogel drug delivery system with predictable and tunable drug release and degradation rates. *Proc Natl Acad Sci U S A*. 2013;110(6):2318-2323.
7. Henise J, Hearn BR, Ashley GW, Santi DV. Biodegradable tetra-PEG hydrogels as carriers for a releasable drug delivery system. *Bioconjug Chem*. 2015;26(2):270-278.

8. Schneider EL, Henise J, Reid R, Ashley GW, Santi DV. Hydrogel drug delivery system using self-cleaving covalent linkers for once-a-week Administration of Exenatide. *Bioconjug Chem*. 2016;27(5):1210-1215.
9. Sakai T. Gelation mechanism and mechanical properties of tetra-PEG gel. *React Funct Polym*. 2013;73:898-903.
10. Sakai T, Matsunaga T, Yamamoto Y, et al. Design and fabrication of a high-strength hydrogel with ideally homogeneous network structure from tetrahedron-like macromonomers. *Macromolecules*. 2008;41:5379-5384.
11. Watanabe T, Motohiro I, Ono T. Microfluidic formation of hydrogel microcapsules with a single aqueous Core by spontaneous cross-linking in aqueous two-phase system droplets. *Langmuir*. 2019;35(6):2358-2367.
12. Champion JA, Walker A, Mitragotri S. Role of particle size in phagocytosis of polymeric microspheres. *Pharm Res*. 2008;25(8):1815-1821.
13. Henise J, Fontaine SD, Hearn BR, et al. In vitro-in vivo correlation for the degradation of tetra-PEG hydrogel microspheres with tunable β-eliminative crosslink cleavage rates. *Int J Polym Sci*. 2019;2019:1-7.
14. Schneider EL, Hearn BR, Pfaff SJ, et al. A hydrogel-microsphere drug delivery system that supports once-monthly administration of a GLP-1 receptor agonist. *ACS Chem Biol*. 2017;12(8):2107-2116.
15. Dommerholt J, Rutjes F, van Delft FL. Strain-promoted 1,3-dipolar Cycloaddition of Cycloalkynes and organic Azides. *Top Curr Chem (Cham)*. 2016;374(2):16.
16. Meier H, Petersen H. Synthese von 5-substituierten Cyclooctynen. *Synthesis*. 1978;1978:596-598.
17. Fontaine SD, Hann B, Reid R, Ashley GW, Santi DV. Species-specific optimization of PEG∼SN-38 prodrug pharmacokinetics and anti-tumor effects in a triple-negative BRCA1-deficient xenograft. *Cancer Chemother Pharmacol*. 2019;84:729-734.
18. Steinhilber D, Rossow T, Wedepohl S, Paulus F, Seiffert S, Haag R. A microgel construction kit for bioorthogonal encapsulation and pH-controlled release of living cells. *Angew Chem Int Ed*. 2013;52:13538–13543.
19. Holtze C. Large-scale droplet production in microfluidic devices—an industrial perspective. *J Phys D Appl Phys*. 2013;46(11):114008.
20. FDA, Current good manufacturing practice for finished pharmaceuticals–Equipment construction 21 CFR 211.65, in: FDA (Ed.). 2019.
21. FDA, Guidance for Industry, Sterile Drug Products Produced by Aseptic Processing–Current Good Manufacturing Practice, in: FDA (Ed.). 2004.
22. Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests, USP <61>, United States Pharmacopeial Convention, 2009.
23. Biological Reactivity Tests, In Vivo, USP <88>, United States Pharmacopeial Convention, 2012.
24. Emory SF. Principles of integrity testing hydrophilic microporous membrane filters part I. *Pharm Technol*. 1989;13(9):68-77.
25. Cilurzo F, Selmin F, Minghetti P, et al. Injectability evaluation: an open issue. *AAPS PharmSciTech*. 2011;12(2):604-609.
26. Yadavali S, Jeong HH, Lee D, Issadore D. Silicon and glass very large scale microfluidic droplet integration for terascale generation of polymer microparticles. *Nat Commun*. 2018;9(1222):1-9.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Henise J, Yao B, Hearn BR, Schneider EL, Ashley GW, Santi DV. High-throughput, aseptic production of injectable Tetra-PEG hydrogel microspheres for delivery of releasable covalently bound drugs. *Engineering Reports*. 2020;e12213. https://doi.org/10.1002/eng2.12213