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RESEARCH ARTICLE

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Autoclave sterilization of tetra-polyethylene glycol hydrogel biomaterials with β-eliminative crosslinks

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Sterilization of degradable polymeric biomaterials intended for injection presents a formidable challenge. Often, either the polymer backbone or labile crosslinks controlling degradation are adversely affected by commonly used sterilization methods. The purpose of this work was to develop an approach to sterilize tetra-polyethylene glycol hydrogel microspheres (MSs) with β-eliminative crosslinks that are destined to be carriers for drug delivery. The approach taken was to acidify the medium to compensate for the base-catalyzed cleavage of linkers at high temperatures. We determined that rates of linker cleavage at pH 4 or below were sufficiently slow as to allow autoclaving and showed that precursor amine-derivatized MSs could withstand autoclaving at pH 4 for at least four cycles of 20 minutes each at 121°C. Thus, amine-MSs need not be prepared aseptically, but instead can be prepared in a low bioburden environment, and then sterilized by autoclaving before drug attachment.

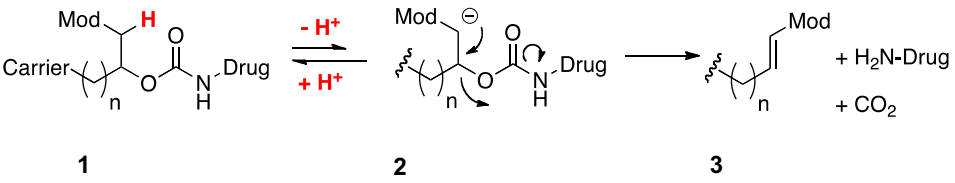
KEYWORDS

biodegradable hydrogel, drug delivery, half-life extension, microspheres, tetra-polyethylene glycol

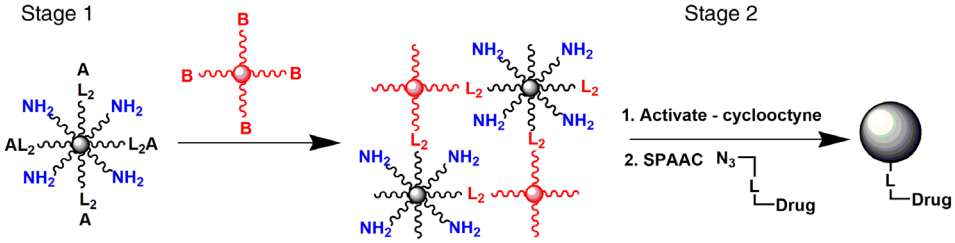
1 | INTRODUCTION

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 that slowly cleaves by β-elimination to release the native drug (Scheme 1).[1] The cleavage rate of the linker is controlled by the nature of an electron-withdrawing "modulator" (Mod) attached to a carbon 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 years when stored at low pH and temperature. One carrier we use is a large-pore tetra-polyethylene glycol (PEG) hydrogel polymer.[2-4] These hydrogels—fabricated as uniform 50-μm microspheres (MSs)—are injected subcutaneously (SC) or locally through a small-bore needle where they serve as a depot to slowly release the drug. Importantly, a slower cleaving β-eliminative linker is incorporated in crosslinks of these polymers, so gel degradation occurs after drug release.[3,5]

Fabrication of tetra-PEG hydrogel MS-drug conjugates is achieved in two stages (Scheme 2).[3,4] Stage 1 involves preparation of amine-derivatized MSs. Here, equimolar amounts of an 8-arm PEG containing 4 amine- and 4 azido-linker end groups and a 4-arm PEG containing cyclooctyne end groups are mixed in a droplet forming device. The azide (A) and cyclooctyne (B) end groups of the PEG prepolymers react within the droplets by strain-promoted alkyne-azide cycloadditions (SPAAC) to form 1,2,3-triazoles and provide homogeneous amine-derivatized tetra-PEG



SCHEME 1 β-eliminative linker cleavage



SCHEME 2 Synthesis of tetra-polyethylene glycol (PEG) microsphere drug substance

hydrogel MSs. The second stage involves attachment of the drug to the amine-MSs in a reactor downstream of the droplet maker.

The challenges to overcome to achieve practical use of the system for manufacturing MS-drug conjugates are: (a) to develop a method of producing sufficient amounts of the amine-MSs and b) to prepare the drug substance aseptically in accord with cGMP manufacturing requirements. We are surmounting the first challenge by developing a multichannel microfluidics apparatus functionally similar to the Dolomite high-throughput Telos system; clearly, other droplet forming approaches could also be used for this purpose. However, as recently reviewed,[6-8] aseptic manufacturing of microparticles is a formidable task. To overcome this challenge, we initially planned to couple the Stage 1 amine-MS formation and Stage 2 drug attachment steps in a single sterilized, sealed apparatus. Here, the sterile-filtered PEG prepolymers would be polymerized as amine-derivatized MSs and the drug would then be attached in strictly controlled and contained sterile conditions. However, maintaining aseptic conditions and transferring the technology to a cGMP manufacturing facility present a significant challenge, and we sought an abbreviated, more efficient approache to solve the aseptic manufacturing issues.

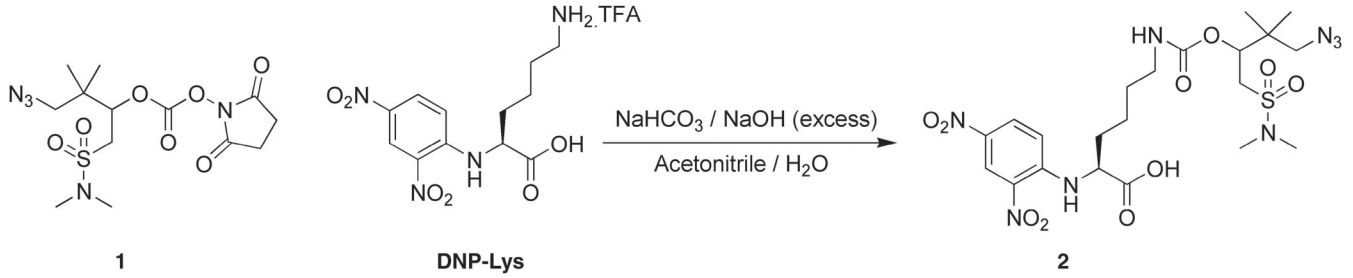
Sterilization methods for PEG hydrogels—as used here—require avoidance of reactions at the polyethylene backbone as well as the labile crosslink connections that are needed for biodegradation.[9-12] The polyether backbone of PEG hydrogels is stable to most chemical and heat sterilization methods but is susceptible toward free radicals from peroxides and gamma radiation. However, ester-containing crosslinks used in most PEG gels are often stable to gamma radiation, but susceptible to hydrolysis that occurs with chemical and heat sterilization.

In the present work, we describe an approach to uncouple the two stages of drug-substance manufacturing. We show that the PEG amine-MSs are stable to and can be simply and economically sterilized by autoclaving in slightly acidic media. Using this approach, the amine-MSs can be prepared in a low-bio burden but nonaseptic proces. They can then be transferred to a cGMP manufacturing facility for autoclave-sterilization and subsequent aseptic attachment to the drug in a simple, sealed reactor.

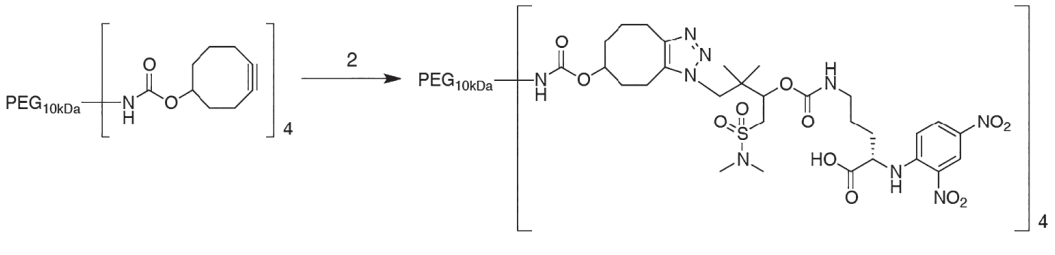
2 | MATERIALS AND METHODS

2.1 | Synthesis of N_ε-(2,4-dinitrophenyl)-N_ε-[(4-azido-3,3-dimethyl-1-(*N*,*N*-dimethylaminosulfonyl)-2-butoxycarbonyl]-L-lysine (2)

Is performed (Scheme 3) according to general reported methods.[1] A solution of linker-HSC (**1**) (40 mg, 100 µmol) in 2 mL of acetonitrile was added to a stirred mixture of N_ε-(2,4-dinitrophenyl)-L-lysine trifluoroacetate salt (DNP-Lys) (50 mg, 120 µmol) in 0.2 mL of 1 N NaOH, 0.4 mL of 1 M $NaHCO_3$, and 1.4 mL of water. After 10 minutes, the mixture was acidified with HCl and extracted with EtOAc. The extract was washed with water and brine, dried over $MgSO_4$, filtered, and evaporated to yield linker-(DNP)Lys **2** (59 mg, 100 µmol, 100%). HPLC gave a single peak (96%); LC-MS $[M + H]^+$ *m/z* 589.1 (calc. for $C_{21}H_{32}N_8O_{10}S$ *m/z* 589.2).



SCHEME 3 Synthesis of N_ε-(2,4-dinitrophenyl)-N_ε-[(4-azido-3,3-dimethyl-1-(*N*,*N*-dimethylaminosulfonyl)-2-butoxycarbonyl]-L-lysine (**2**)



SCHEME 4 Synthesis of tetra-{[(11-[3,3-dimethyl-1-(*N*,*N*-dimethylaminosulfonyl)-2-(N_ε-[N_ε-(2,4-dinitrophenyl)-L-lysyl]carbonyloxy) but-4-yl])-9,10,11-triaza[6.3.0]undec-10-en-4-oxy]carbonylamino}-PEG_{10kDa} (**4**)

2.2 | Synthesis of tetra-{[(11-[3,3-dimethyl-1-(*N*,*N*-dimethylaminosulfonyl)-2-(N_ε-[N_ε-(2,4-dinitrophenyl)-L-lysyl]carbonyloxy)but-4-yl])-9,10,11-triaza[6.3.0] undec-10-en-4-oxy]carbonylamino}-PEG_{10kDa} (4)

The 10-kDa 4-armed tetra[(cyclooct-4-ynyloxycarbonyl)amino]-PEG ($PEG_{10kDa}(NH\text{-}5HCO)_4$ **3** (Scheme 4) was prepared from $PEG_{10kDa}(NH_2)_4$ and O-Cyclooct-4-yn-1-yl-O′-succinimidyl carbonate (5-HCO-HSC) as described for the analogous $PEG_{40kDa}(NH\text{-}5HCO)_4$.[13] A solution of **2** (11.2 mg, 19 μmol, 1.3 Eq) in 0.2 mL of acetonitrile was mixed with a solution of **3** (56.6 mM in cyclooctyne, 0.265 mL, 15 μmol cyclooctyne, 1.0 Eq) in 20 mM NaOAc, pH 5, and kept at 50°C for 12 hours. The solution was placed in a dialysis bag (SpectraPor 2, 12 to 14 kDa cutoff) and dialyzed against MeOH to remove excess **2**, then concentrated to dryness to provide the 4-arm PEG conjugate **4** (43 mg, 90%) which was dissolved in 1.0 mL of water to provide a stock solution. HPLC gave a major peak (85%); free DNP-Lys was <0.1%.

2.3 | Autoclave stability of PEG-linker-lysine

The PEG-linker-lysine **4** stock solution (0.1 mL) was diluted with 1.0 mL of buffer in a 2-mL screw-cap HPLC sample vial. Buffers used and pH at 25°C were: 0.125 M HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid), pH 7.6; 0.1 M citrate, pH 5.0 and pH 4.0, and 0.1-M glycine, pH 2.0. The vials were sealed and subjected to one or more standard autoclave cycles (below), and then allowed to cool to ambient temperature. Samples were analyzed by HPLC by injecting 10 μL onto a C_{18} column (Phenomenex Jupiter, 300 A, 5 μm, 4.6 x 150 mm), eluting with a linear gradient from 0% to 100% MeCN/water/0.1% trifluoroacetic acid over 10 minutes and analyzing at 350 nm. Formation of free DNP-lysine was quantitated as % reaction = (area DNP-lysine)/[(area DNP-lysine) + (area conjugate)] × 100.

2.4 | Preparation of amino-MSs

Similar to a described procedure,[14] Prepolymer A was prepared by reaction of linker HSC **1** with BOC-Lys, and the product was converted into its HSE and coupled to 4-arm $PEG_{20kDa}(NH_2)_4$. Prepolymer B (4-arm PEG_{20kda}-NH[HCO]$_4$; (**3**) was prepared by coupling 5-HCO-HSC with 4-arm $PEG_{20kDa}(NH_2)_4$ as described for $PEG_{40kDa}(NH\text{-}5HCO)_4$.[13] Amino-MSs were prepared from 2.5-mM solutions of Prepolymer A and Prepolymer B using a reported microfluidic method[4] with the

following modifications. After microfluidic production of the MSs, the particle slurry (500 mL) was washed with 6 × 1 L heptane, 6 × 1 L 95% EtOH, 2 × 1 L water, and then 8 × 1 L of autoclave buffer.

2.5 | Autoclaving amino-MSs

Samples contained ~1.5 mL of amineMS slurry in 2-mL glass HPLC vials (Thermo Sci. C4010-1 W) loosely fitted with polypropylene caps and polytetrafluoroethylene-lined silicone septa (Microsolv 9502S-10C-BM). Autoclaving was performed using a Sterivap model 669 autoclave (BMT MMM group). An autoclave cycle for sterilization consists of the following: (a) evacuation to 5.80 psia, (b) autoclaving at 121°C with a 20 minutes hold time, and (c) cooling to 97°C over ~1.5 hours. The autoclave temperature was monitored with a probe immersed into 50 mL of H_2O in a 100-mL glass GL45 media bottle.

2.6 | Determination of the time-to-reverse gelation (t_{RG})

A 0.5-mL sample of amine-MS slurry in a 1.5-mL microcentrifuge tube was washed with 3 × 1 mL of 100-mM HEPES, pH 7.6, by pelleting at 21 000 g for 5 minutes. The pellet was treated with 0.020 mL of 10-mM 5-carboxyfluorescein HSE (TCI C2479) in DMSO for 30 minutes. The MSs were washed with 3 × 1 mL water and 3 × 1 mL of 100-mM NaOAc. Microsphere dissolution curves were determined for 0.1-mL samples in 2.5 mL of 100-mM borate buffer, pH 9.4, at 37°C as reported.[4] Linear regression was performed on the region where solubilization was between 50% and 95%. The time at 100% solubilization was calculated from the equation: t_{RG} = 100-Y intercept/slope.

2.7 | Measurement of amine and PEG content of MSs

A 100-mg aliquot of amine-MS slurry was dissolved in 0.900 mL of 50-mM NaOH. The amine content of a 0.060-mL sample of the dissolved amine-MSs was measured using 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) as described.[4] For the PEG assay, in a modification of a reported procedure,[15] a 0.020-mL aliquot of the above dissolved amine-MSs was diluted with 0.980-mL H_2O and acidified with 1.00 mL of 0.5-M HClO4. After transfer of 0.200-mL aliquots to a 96-well microtiter plate, each was treated with 0.050 mL of 5% w/v mixture of $BaCl_2$ and 0.025 mL of Lugol solution (0.18% w/w I_2 and 0.35% w/w KI). After 5 minutes, the A_{535} was measured using a plate reader. The PEG content was determined from a standard curve of A_{535} vs PEG generated from 1.25 to 10 μg mL^{-1} of an 8000-MW linear PEG (Sigma, PN:1546605) standard that was precalibrated by nuclear magnetic resonance using a DMF standard.[6] The ratios of nmol amine/mg PEG of the amine-MSs were calculated using measurements of the free amine and PEG from the same solution (Equation 1). Standard deviations were calculated using a formula for propagation of error upon division[16] (Equation 2).

$$\text{nmol amine/mg PEG} = (\text{nmol amine/mg slurry})/(\text{mg PEG/mg slurry}), \tag{1}$$

$$\text{SD of (amine/PEG)} = (\text{amine/PEG}) * \{[([\text{SD amine}]/[\text{amine}])^2 + ([\text{SD PEG}]/[\text{PEG}])^2]^{1/2}\}. \tag{2}$$

2.8 | Particle size analysis

A 100-mg sample of amine-MS slurry was diluted with 0.700 mL 50% N,N-dimethylformamide (DMF)/H_2O v/v. A 0.200-mL sample of the mixture was placed on a microscope slide (VWR, 48300-026) and images were collected using a white light microscope (Nikon TMS, SN: 51436) with a 5× objective (Nikon E 4/0.10, 160/− NA) and a monochromatic charge coupled device camera (Unibrain, Fire-I 580b). From three images, the particle diameters (N = ≥150) were measured using an image analysis software (Image J v 1.52a). The software was calibrated to convert pixels into micrometers (1.98 μm $pixel^{-1}$) by measurement of an image of a microscope stage micrometer (Electron Microscopy Sciences, 60 210-3PG).

3 | RESULTS AND DISCUSSION

Sterility is an important requirement for degradable polymeric biomaterials intended for injection, and sterilization of such materials is a crucial process to minimize the incidence of infections; for recent reviews on sterilization of

biocompatible polymers see References 7, 8, and 17. A foremost consideration in choosing a sterilization method is whether the biomaterial will sustain the procedure, and there is no single technique that can achieve sterilization of a wide variety of degradable materials without adverse effects; each system requires testing to ensure relevant properties of the material are not altered.

PEG is a common component of many polymer hydrogels, including drug and cell delivery systems. Sterilization methods for PEG hydrogels require consideration of potential reactions of the polyethylene backbone as well as the crosslinks.[9-12] The polyether backbone of PEG hydrogels is stable to most chemical and heat sterilization methods but susceptible toward free radicals from peroxides and gamma radiation. However, to allow in vivo degradation and bioresorption, most PEG hydrogels use ester-containing crosslinks that are susceptible to hydrolysis and thus prohibit exposure to acidic or basic conditions or the use of heat in the sterilization process. For example, steam sterilization of PEG-hydrogels with ester linkages resulted in sticky, aggregated particles that severely compromised the structure of the gels.[12] An approach that may provide a method to effectively sterilize PEG hydrogels containing hydrolyzable crosslinks is dense CO_2 sterilization.[12] To our knowledge, no studies have been reported on sterilization methods for biocompatible polymers that contain β-eliminative linkers related to those used here.

Previous studies in this laboratory have shown that cleavage of β-eliminative linkers proceeds by a specific-base (hydroxide) catalyzed reaction, with no contribution by buffers.[1,2] Thus, the stability of β-eliminative linkers increases 10-fold for every pH unit decrease of the medium. We reasoned that the increased cleavage rate that would occur at higher temperatures could be compensated for by lowering the pH, and that heat might provide a viable sterilization method.

The rate of β-eliminative linker cleavage as a function of temperature follows the Arrhenius equation, $k = A{\cdot}e^{-Ea/RT}$. This allows calculation of the rate of cleavage at different temperatures based on the values of the frequency factor A and activation energy E_a determined from measurement of reaction rates at different temperatures. Table 1 shows Arrhenius parameters for three β-eliminative cleavage of linkers in PEG-linker(Mod)-probe conjugates with >100-fold difference of cleavage $t_{1/2}$ values at pH 7.4; parameters for linkers with intermediate $t_{1/2}$ values can be estimated by interpolation. The 10-kDa 4-arm PEG linked to the ε-amine of DNP-Lys by a β-eliminative linker containing a *N*,*N*-dimethyl sulfonamide ($[CH_3]_2NSO_2$-) modulator showed a cleavage $t_{1/2}$ at 37°C of 100 hours, $A = 1.9 \times 10^{13}$ seconds, and $E_a = 123.5$ kJ/mol. Using these parameters, the rate of linker cleavage should increase ~28 500-fold in going from 37°C to an autoclave temperature of 121°C. However, since the cleavage rate decreases 10-fold with each unit decrease in pH, this temperature-induced rate increase can be countered by a drop in pH by log(28500) or ~4.5 units. It is thus calculated that hydrogels formed using this linker should be as stable to autoclave temperatures at pH ~3 as they are to 37°C, pH 7.4. As expected, the E_a values decrease with increasing cleavage rate of linkers, and linkers with faster cleavage will show a proportionally lower rate increase with temperature.

TABLE 1 Arrhenius parameters for β-eliminative linker cleavage

Modulator	$t_{1/2}$ hours, 37°C, pH 7.4	A s^{-1}	E_a kJ/mol
4-CF_3-C_6H_4-SO_2-[a]	14	1.9 x 10^{13}	107.0
$O(CH_2CH_2)_2N$-SO_2-[b]	400	2.5 x 10^{13}	117.0
Me_2N-SO_2-[b]	1672	7.7 x 10^{13}	123.5

[a]Aminoacetamido-fluorescein carbamate, from Reference 1.
[b]ε-DNP-Lys carbamate.

TABLE 2 Estimated and measured cleavage of a β-eliminative linker with the $(CH_3)_2NSO_2$-modulator at 121°C

pH, 25°C [a]	$t_{1/2}$, 37°C, calc.	pH 121°C calc.	$t_{1/2}$, 121°C, calc.	% Product/cycle, 121°C, calc.[b]	% Product/cycle, 121°C, obsd.
7.4 [a]	70 d	6.2	0.93 h	22%	13%
6.0	4.8 y	5.7	2.9 h	7.6%	3.8%
5.0	48 y	5.0	14.7 h	1.6%	1.9%
4.0	480 y	4.0	6.1 d	0.16%	0.1%
2.0	48 000 y	2.0	1.7 y	~0	0.2%

[a]pH of buffers were measured at 25°C and estimated at 121°C using reported temperature coefficients. The buffers, pH values at 25°C, and $\Delta pH/\Delta T$ values were Hepes, pH 7.4, −0.014; phosphate, pH 6, −0.0028; phthalate, pH 5, −0.00013; glycine, pH 2, +0.00044.
[b]Estimations use the calculated pH at 121°C; they do not include the slow cooling period of a cycle and are expected to be lower than observed.

Using the aforementioned PEG-DNP-Lys conjugate with a β-eliminative linker containing the $(CH_3)_2NSO_2$- modulator, we estimated the rate of linker cleavage at acidic pH values at 121°C (Table 2) as follows. First, from the $t_{1/2}$ of 100 hours at pH 7.4, 37°C, and the known specific base catalysis of β-elimination, we calculated $t_{1/2}$ values at lower pH values. Next, from the temperature coefficients of buffers used, we calculated corresponding pH values at 121°C. Then, using the Arrhenius equation and estimated rate constants at lower temperatures, we calculated $t_{1/2}$ values at 121°C at the various relevant pH values. Finally, using these $t_{1/2}$ values, and the equation %product = 1-e^{-kt}, we estimated the percent reaction at 121°C over the 20-minute duration of an autoclave cycle.

The PEG-DNP-Lys conjugate was subjected to autoclaving for 20 minutes at 121°C, and the amount of DNP-Lys released was determined by high performance liquid chromatography (HPLC). As shown in Table 2, the experimental results are in excellent agreement with estimated values. From these data, it can be seen that 20-minute autoclaving at pH 2 and 4 results in minimal (≤0.2%) cleavage of the linkers.

We next studied the stability of the linker in the PEG-DNP-Lys conjugate at pH 4.0, 5.0, and 6.2 at 121°C over eight sequential autoclave cycles (Figure 1). The linker cleaves an average of only 0.11% per cycle at pH 4.0 and is >99% intact

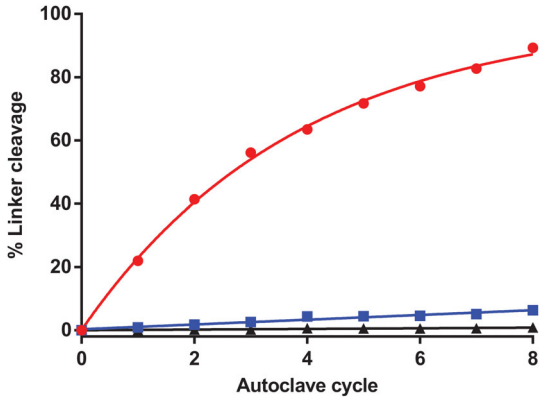


FIGURE 1 Cleavage of a β-eliminative linker with a $(CH_3)_2NSO_2$- modulator at pH 4.0, 5.0, or 7.4 at 25°C or pH 6.2 (–●–), 5.0 (–■–), and 4.0 (–▲–) at 121°C for eight consecutive autoclave cycles

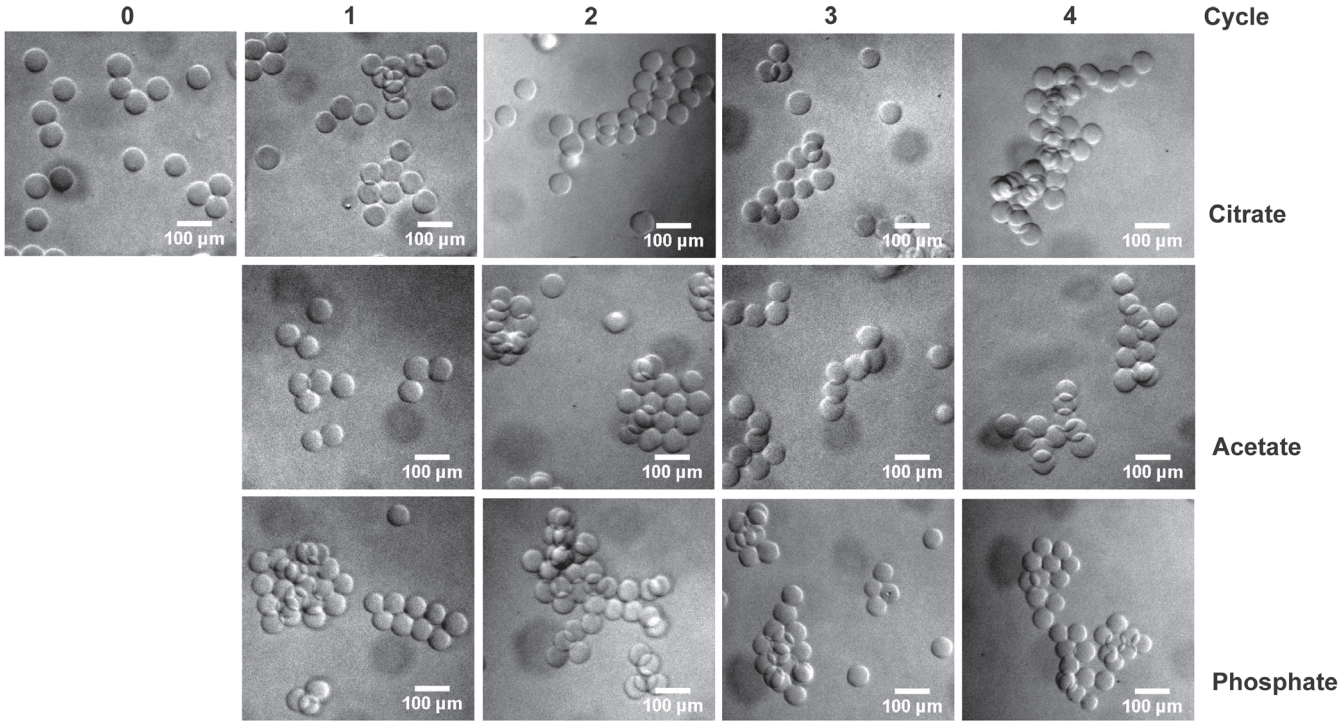


FIGURE 2 Appearance of microspheres (MSs) before and after consecutive autoclave cycles in three different buffers. The particle diameters were measured as pixel width by image analysis and converted into micrometers

TABLE 3 Properties of amine-MSs before and after autoclaving at pH 4

Buffer	No. of cycles	Appearance	Particle size (μm)[a]	nmol Amine/mg PEG[b]	t_{RG}, hour
Citrate	0	Normal	67 ± 6	110 ± 11	23
Citrate	1	Normal	67 ± 4	110 ± 30	23
	2	Normal	65 ± 4	94 ± 7	25
	3	Normal	66 ± 14	89 ± 6	27
	4	Normal	64 ± 4	83 ± 17	26
Acetate	1	Normal	69 ± 14	120 ± 20	25
	2	Normal	69 ± 15	120 ± 10	25
	3	Normal	67 ± 11	110 ± 4	25
	4	Normal	65 ± 4	110 ± 10	23
Phosphate	1	Normal	64 ± 14	120 ± 10	25
	2	Normal	64 ± 14	110 ± 120	25
	3	Normal	62 ± 13	110 ± 10	23
	4	Normal	59 ± 11	110 ± 20	26

Abbreviation: MS, microsphere.
[a]Mean ± SD, >50 microsphere measurements.
[b]Mean ± SD, four replicate measurements.

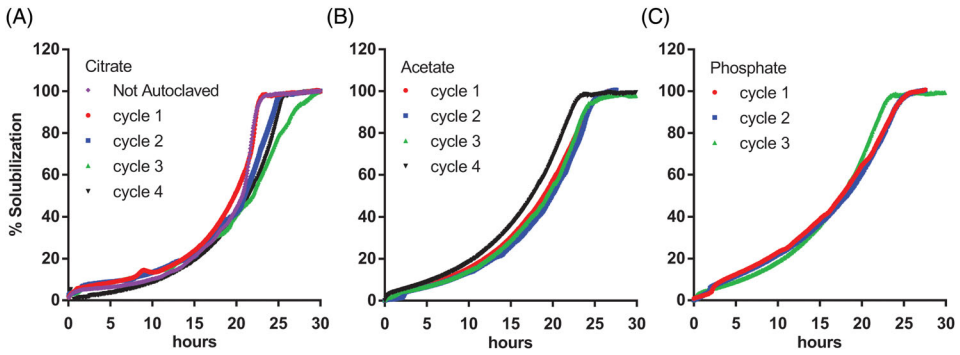


FIGURE 3 Dissolution curves for autoclaved amine-microspheres (MSs) with β-eliminative crosslinkers. t_{RG} values are reported in Table 1. amine-MSs autoclaved in (A) pH 4.0 citrate, (B) pH 4.0 acetate, and (C) pH 4.0 phosphate compared to nonautoclaved particles, showing that no significant change is dissolution behavior for the four samples studied in each buffer

after eight successive cycles; at pH 5.0, linkers cleave an average of 0.77% per cycle. The buffers, pH at 25°C, and $\Delta pH/\Delta T$ values used for estimating pH at 121°C used were: HEPES, pH 7.4, −0.014; acetate, pH 5, −0.0002, and citrate, −0.0024.

Finally, we prepared amine-derivatized MSs containing the same $(CH_3)_2SO_2$-N- β-eliminative linker at each crosslink and subjected the amine-MSs to four autoclaving cycles at pH 4.0 in 100-mM acetate, phosphate, or citrate buffers. An autoclave cycle consists of 121°C for 20 minutes, followed by cooling to ~97°C for ~2.0 hours. After the last cycle, critical quality attributes of the particles were assessed, which included appearance and particle size, time-to-reverse gelation (t_{RG}), and the nmol amine/mg PEG.

As shown in Figure 2 and Table 3, the appearance and size of the MSs are not affected by autoclaving. Likewise, the time-to-reverse gelation (t_{RG}) measured from dissolution curves over 4 cycles varied by <7% SD of the mean (Table 3 and Figure 3), similar to the error obtained (≤8% SD) with four replicates of the same sample. There was no significant change in free amines of the amine-MSs in phosphate or acetate buffers for at least four autoclave cycles (Figure 4). However, in citrate buffer, there was a loss of ~7% of amines each cycle; this is attributable to the formation of citric acid anhydride during autoclaving, which forms in acidic citrate solutions and can acylate amine groups.[18]

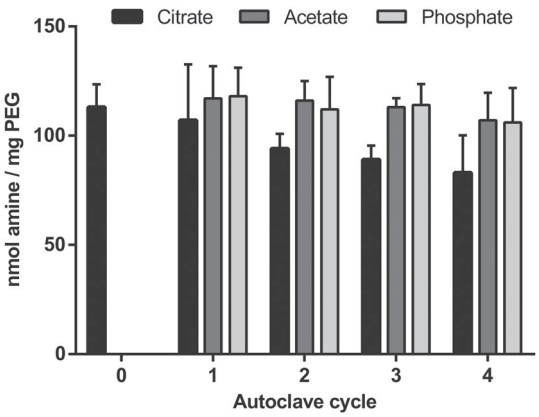


FIGURE 4 Free-amine groups on amine-MSs after successive autoclave cycles in different buffers. Data from Table 3

4 | CONCLUSIONS

We have shown that the heat lability of β-eliminative linkers can be compensated for by adjusting the pH to the acidic range. At pH 4.0, soluble PEG-containing linkers can be subjected to at least 8 cycles of autoclaving with less than 1% cleavage. At pH 4.0, amine-MSs can likewise be autoclaved for at least four cycles without change in the critical quality attributes of morphology, t_{RG}, and, providing citrate buffer is not used, free-amine groups. Hence, amine-derivatized hydrogel MSs with β-eliminative crosslinks destined as carriers for drug delivery need not be fabricated under aseptic conditions. They can be prepared in a low bioburden environment and then sterilized by autoclaving before aseptic drug attachment. It is also likely that amine-MSs attached to heat-stable small molecule drugs—but not heat-labile protein drugs—by β-eliminative linkers could be sterilized by terminal sterilization.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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SUPPORTING INFORMATION

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

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Supplementary Information

Autoclave sterilization of tetra-PEG hydrogel biopolymers with β-eliminative crosslinks

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Analysis of crosslink loss in hydrogel due to increased temperatures

Many properties of tetra-PEG hydrogels depend upon the extent of crosslinking, and thus the degree to which crosslinks are cleaved during a sterilization process. One such property is the time at which the hydrogel dissolves at a particular pH and temperature, known as the reverse gelation time (t_{rg}). The relationship between crosslinking and t_{rg} has been described as eq. 1 (Reid et al., 2015).

$$t_{rg} = t_{1/2,L2} \bullet \ln[(1-f)/0.39]/\ln(2) \quad [1]$$

where $t_{1/2,L2}$ is the half-life for cleavage of an individual crosslink and f is the fraction of cleaved crosslinks initially present in the hydrogel.

The β-eliminative cleavage is first-order in hydroxide, and changes 10-fold for each pH unit change according to eq. 2, where the k_{pH2} at pH_2 is calculated from the known rate k_{pH1} at pH_1 (Santi, Schneider, Reid, Robinson, & Ashley, 2012).

$$k_{pH2} = k_{pH1} \bullet 10^{(pH2 - pH1)} \quad [2]$$

The predominant mechanism of degradation of β-eliminative crosslinks of hydrogels during autoclaving is the accelerated linker cleavage at high temperatures. The cleavage rate of individual crosslinks at a particular temperature and pH can be estimated through the study of analogous PEG-linker-probes which represent an individual crosslink unit of a hydrogel. The temperature dependence of the reaction is described by the Arrhenius equation (eq. 3)

$$k = A \bullet e^{(-Ea/RT)} \quad [3]$$

where k is the rate constant, T is the temperature in ^oK, A is a preexponential factor, E_a is the activation energy, and R is the universal gas constant. A and E_a are determined through study of the change in reaction rate as a function of temperature, and may be used to predict reaction rates at different temperatures (see main text for parameters).

While the rate of cleavage increases exponentially with temperature, there is a corresponding exponential decrease in cleavage rate as the pH is lowered. By combining eqs. 2 and 3, the decrease in pH required to compensate for the increased rate of cleavage due to temperature change from T_1 to T_2 (in degrees Kelvin) can be calculated by eq. 4.

$$\Delta pH = \frac{E_a}{R \cdot \ln(10)} \cdot \left[\frac{1}{T_1} - \frac{1}{T_2}\right] \quad [4]$$

If the stability of a hydrogel with β-eliminative linkers is known at given T_1 and pH_1, using the activation energy E_a for the cleavage reaction a pH value, pH_2, can be estimated in which the

hydrogel is equally stable at temperature T_2. Thus, eq. 4 can be used to estimate suitable conditions for the autoclave sterilization of hydrogels comprising beta-eliminative linkers.

By designating an acceptable level of crosslinker cleavage – for example, by setting limits on the variability in the t_{RG} – during the sterilization process and knowing E_a, suitable sterilization pH values can be estimated. From eq. 1, the effect on t_{rg} of changing the extent of crosslinking from f_1 to f_2 in a hydrogel with crosslinks having cleavage half-life of $t_{1/2,L2}$ is given by eq. 5

$$\Delta t_{RG} = \frac{t_{1/2,L2}}{\ln(2)} \cdot \ln\left[\frac{(1-f_2)}{(1-f_1)}\right] \qquad [5]$$

which after dividing by eq. 1 can by expressed as a fractional change in Δt_{rg} by eq. 6.

$$\Delta t_{rg}/t_{rg} = [\ln(1-f_2) - \ln(1-f_1)]/[\ln(1-f_1) - \ln(0.39)] \qquad [6]$$

Thus, for a perfect hydrogel comprised of 100% initial crosslinks (i.e. $f_1 = 0$), cleavage of 10% of the crosslinks during sterilization (i.e., $f_2 = 0.1$) should result in an 11% decrease in t_{rg}. For an initially imperfect hydrogel, the effect of a loss in crosslinking on t_{rg} is greater. As examples, when $f_1 = 0.2$ a 10% loss in crosslinking to $f_2 = 0.28$ results in a 15% change in t_{rg}, and when $f_1 = 0.3$ a 10% loss in crosslinking to $f_2 = 0.37$ results in a 18% change in t_{rg}.

Conversely, if it is desired to maintain the t_{rg} within a factor of $\Delta t_{rg}/t_{rg} = X$, the value of f_2 must be maintained as in eq. 7.

$$f_2 \leq 1 - \exp[(1+X)\bullet\ln(1-f_1) - X\bullet\ln(0.39)] \qquad [7]$$

From eq. 7, **Table S1** shows the maximum allowable loss in crosslinking $\Delta f = f_2 - f_1$ that will maintain $\Delta t_{rg}/t_{rg}$ within a given tolerance. From the table, it can be seen that to maintain a tolerance of 5% for t_{rg} will require a loss of less than 4.6% of the crosslinks from a perfect hydrogel ($f_1 = 0$), and less than 2.8% of the crosslinks from a hydrogel initially having 80% of the theoretical number of crosslinks ($f_1 = 0.2$).

Table S1. Calculated Δf values for a resulting fractional change in degelation time $\Delta t_{rg}/t_{rg}$ for a hydrogel having an initial fraction of broken crosslinks f_1.

	$\Delta f = f_2 - f_1$			
$\Delta t_{rg}/t_{rg}$	*-0.05*	*-0.1*	*-0.15*	*-0.2*
f_1				
0	0.0460	0.0899	0.1317	0.1717
0.05	0.0414	0.0809	0.1188	0.1550
0.1	0.0369	0.0722	0.1061	0.1386
0.15	0.0325	0.0637	0.0937	0.1226
0.2	0.0282	0.0555	0.0817	0.1071
0.25	0.0241	0.0475	0.0701	0.0919
0.3	0.0202	0.0398	0.0588	0.0773
0.35	0.0164	0.0324	0.0479	0.0631
0.4	0.0128	0.0253	0.0375	0.0495

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