

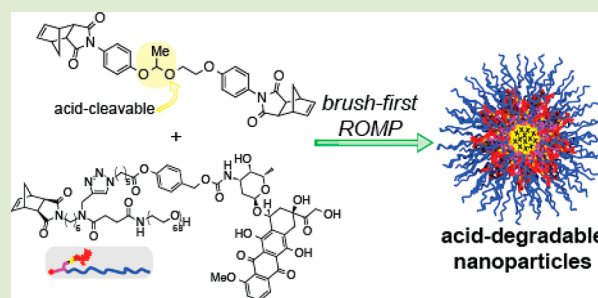
Synthesis of Acid-Labile PEG and PEG-Doxorubicin-Conjugate Nanoparticles via Brush-First ROMP

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Supporting Information

ABSTRACT: A panel of acid-labile bis-norbornene cross-linkers was synthesized and evaluated for the formation of acid-degradable brush-arm star polymers (BASPs) via the brush-first ring-opening metathesis polymerization (ROMP) method. An acetal-based cross-linker was identified that, when employed in conjunction with a poly(ethylene glycol) (PEG) macromonomer, provided highly controlled BASP formation reactions. A combination of this new cross-linker with a novel doxorubicin (DOX)-branch-PEG macromonomer provided BASPs that simultaneously degrade and release cytotoxic DOX in vitro.



Modular, multicomponent synthetic strategies have proven valuable for the discovery of novel polymeric materials.^{1–5} In this vein, we have focused on the development of highly convergent strategies for the construction of multifunctional polymer nanoparticles (NPs) directly from densely functionalized monomers with no extraneous formulation steps. For example, we recently reported the synthesis of brush-arm star polymer (BASP) NPs via a versatile “brush-first” method that involves graft-through ring-opening metathesis polymerization (ROMP) of a norbornene-terminated macromonomer (MM) followed by in situ cross-linking with a bis-norbornene derivative (Figure 1a).^{6,7} The remarkable efficiency and functional group tolerance of ROMP⁸ enables the benchtop synthesis⁷ of diversely functionalized BASPs within hours. We have used the brush-first ROMP method to prepare degradable BASPs with various sizes and corona compositions, including multidrug-loaded poly(ethylene glycol) (PEG)-based BASPs,⁹ two- and three-miktoarm BASPs,¹⁰ and chloride-functional BASPs¹¹ for subsequent azide exchange and copper-catalyzed azide–alkyne cycloaddition (CuAAC) “click”^{12–14} chemistry.

PEGylated BASPs are particularly versatile polymer architectures for drug delivery applications.⁹ They feature a unimolecular micelle-like structure with readily tunable core and shell functionality. Furthermore, they are easy to synthesize given appropriate MM and cross-linker precursors. In our continued effort to translate the brush-first method to cancer drug delivery, we sought to develop PEGylated BASPs that would degrade in the mildly acidic tumor microenvironment¹⁵ (pH ~ 6.0), in endosomes (pH ~ 5.5–6.5),¹⁶ or in lysosomes (pH ~ 4.5–5.0).^{17,18} The inclusion of acid-cleavable functional groups into nanostructures and polymers is a common strategy for tumor-targeted drug delivery.¹⁹ In the brush-first ROMP method, introduction of acid degradability can in principle be achieved through the use of an acid-cleavable bis-norbornene cross-linker. Herein, we report our efforts toward the

development of such a cross-linker and its use for the one-pot formation of novel acid-degradable doxorubicin (DOX)-conjugated BASPs.

Silyl ethers are widely studied acid-sensitive linkages.^{20–23} They can be readily prepared via addition of alcohols to dichlorosilanes; their rates of hydrolysis can be precisely tuned through choice of Si substituents.²⁰ Thus, we began our study with the synthesis of a panel of silyl ether-based bis-norbornene derivatives (1–4, Figure 1b). These compounds were prepared in 35–76% yield via exposure of 4-hydroxymethyl-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-*exo*-3,5-dione²⁴ to the corresponding dichlorosilane in the presence of *N,N*-diisopropylethylamine (see Supporting Information for synthetic details).

We screened each of these new cross-linkers in the context of brush-first ROMP as follows. First, norbornene-terminated poly(ethylene glycol) (PEG) macromonomer PEG-MM (10 equiv, Figure 1a) was exposed to Grubbs third generation bispyridyl initiator^{25,26} (1 equiv) for 15 min to generate living PEG bottlebrush polymers with a target average degree of polymerization (DP) of 10. Aliquots of this solution of living polymer were transferred to a series of empty vials. A THF stock solution of *N* = 10, 15, or 20 equiv of cross-linker was slowly added to each of the vials to initiate cross-linking reactions. After 1 h, the reactions were quenched with ethyl vinyl ether and directly analyzed by gel permeation chromatography (GPC, 0.02 M LiBr in *N,N*-dimethylformamide). The molar mass distributions for these BASPs (e.g., Figure S1 with 4) invariably possessed large fractions of uncoupled bottlebrush polymers. We attempted a wide range of alternative bottlebrush polymer lengths and *N* values, both of

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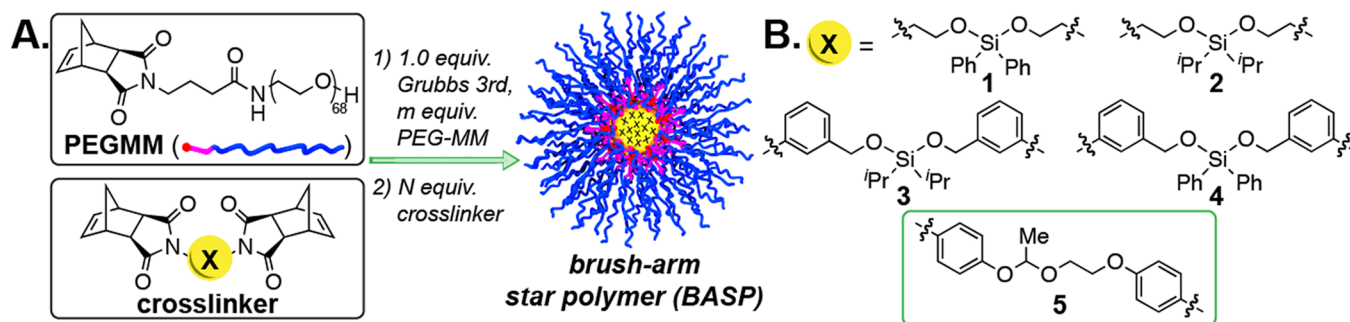


Figure 1. (A) Schematic for the brush-first ROMP process. (B) Acid-labile cross-linkers studied in this report.

which we have previously shown⁶ can dramatically effect the efficiency of the BASP formation process; no improvements were realized. The steric bulk of the Si-substituents of cross-linkers 1–4 may enhance competitive cyclopolymerization²⁷ reactions via a Thorpe-Ingold-like effect.^{28,29} Such reactions reduce cross-linking efficiency through nonproductive consumption of norbornene groups.⁶

In an effort to identify a suitable cross-linker that would provide uniform BASPs, we turned to acetal-based cross-linker 5. Acetals are another class of acid-cleavable functional groups that are widely used to impart pH-sensitivity to polymers.^{30–38} Cross-linker 5 was prepared in three steps starting from *cis*-5-norbornene-*exo*-2,3-dicarboxylic anhydride (see Supporting Information for details). Acetal-core BASPs with $N = 10, 15$, and 20 equiv of 5 were prepared following the same procedure described above for cross-linkers 1–4. GPC analysis (Figure 2)

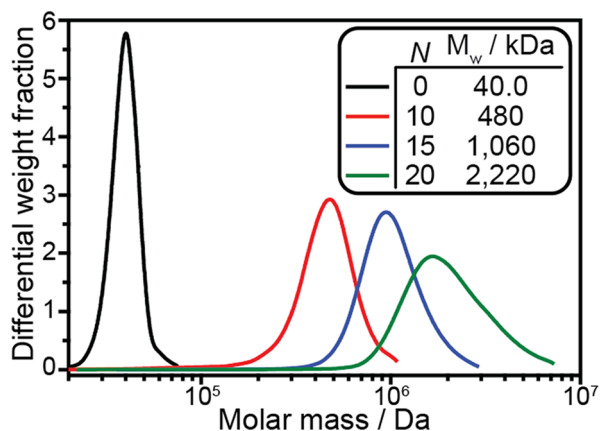


Figure 2. Gel permeation chromatography (GPC) traces for $N = 10, 15$, and 20 BASPs prepared from PEGMM and 5.

revealed unimodal molar mass distributions and very efficient conversion of bottlebrush polymer ($N = 0$, black trace) to BASP for $N = 10$ and 15; the molar mass distribution broadened for the $N = 20$ case. As we observed previously for photocleavable BASPs,^{6,7} the weight-average molar mass (M_w) for these acetal-based particles increased geometrically with each addition of five more equivalents of cross-linker 5, which is indicative of a kinetically limited step-growth coupling mechanism.⁶

Encouraged by these results, we pursued further studies with BASPs constructed from cross-linker 5. To demonstrate BASP degradation under acidic conditions, the $N = 15$ acetal-core BASP was dissolved in pH 4.0 PBS buffer (1 mg of BASP per mL PBS). Samples of this solution were taken at various times

and subjected to LC/MS analysis (Figure 3). The continuous shift of the initial BASP peak (red trace) to shorter retention

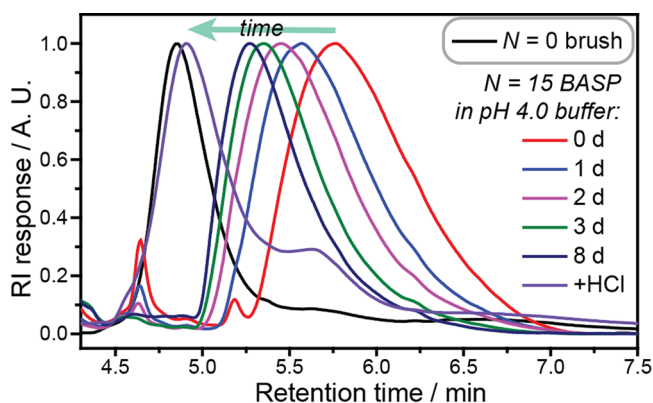


Figure 3. LC-MS traces of the $N = 0$ bottlebrush polymer and $N = 15$ acetal core BASP after exposure to pH 4.0 buffer for up to 8 d. After this time, 1 drop of concentrated HCl was added to produce the +HCl trace.

times over the course of 8 days is consistent with particle degradation.¹¹ After 1 week, a drop of HCl (12.1 M) was added. Complete acetal cleavage should regenerate ~ 40 kDa bottlebrush polymers. In line with this expectation, the LC/MS trace of the HCl degradation product (+HCl trace, Figure 3) nearly overlapped with that of the parent bottlebrush polymer (black trace), though a significant fraction of the BASP remained partially in tact (right shoulder in +HCl chromatogram). In agreement with this result, the GPC trace of BASP exposed to excess trifluoroacetic acid in organic solvent (THF) overlapped with that of the parent bottlebrush with a small shoulder for high molecular weight species (Figure S2). Collectively, these results confirm that BASPs constructed from cross-linker 5 degrade slowly in acidic media.

Next, we turned our attention to the synthesis of multiresponsive drug-conjugated BASPs that could degrade under acidic conditions and release a drug molecule in response to an enzymatic trigger. Building off of our established “branched macromonomer” platform,^{39,40} we designed a novel doxorubicin (DOX)-conjugated MM (DOX-MM) that can release free DOX via enzymatic hydrolysis followed by rapid 1,6-elimination (Figure 4A).⁴¹ ¹H NMR and matrix-assisted laser desorption/ionization (MALDI) analyses confirmed the structure of DOX-MM (see Supporting Information).

We prepared a DOX-loaded BASP (DOX-BASP, Figure 4a) with $N = 15$ equiv of 5 following identical procedures as

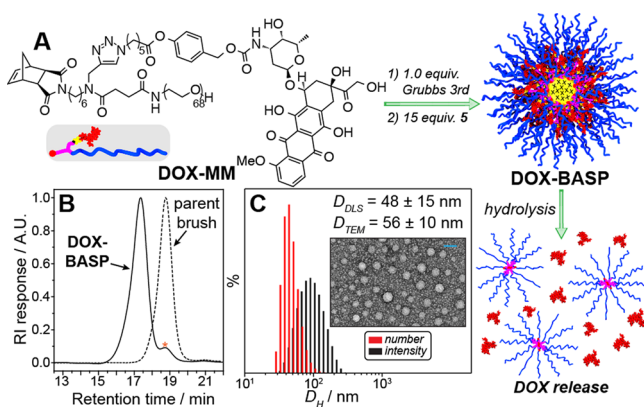


Figure 4. (A) Scheme for synthesis of DOX-BASP from DOX-MM and 5. (B) GPC trace of $N = 15$ DOX-BASP. (C) DLS histograms and negatively stained TEM image (inset) of $N = 15$ DOX-BASP. Scale bar in TEM image is 100 nm.

described above using DOX-MM in place of PEG-MM. GPC analysis (Figure 4b) of DOX-BASP revealed a lower conversion ($\sim 95\%$) of brush to BASP compared to the studies described above and our previous studies with similar branched MMs.⁹ This difference is likely due to a combination of the increased steric hindrance of DOX-MM compared to PEG-MM and the fact that here we use only the DOX-MM to construct DOX-BASP, whereas we previously⁹ used a mixture of drug-conjugated MM and PEG-MM. Nevertheless, this combination of DOX-MM and cross-linker 5 yielded BASPs with diameters measured by DLS and TEM of 48 ± 15 and 56 ± 10 nm, respectively (Figure 4c) and an 11.4% DOX loading without need for extraneous particle formulation steps. If desired, residual bottlebrush polymer and MM can be removed via dialysis, centrifugation, or preparatory HPLC.³⁹

We next sought to determine if these DOX-conjugated acetal-core BASPs could release free DOX in vitro. First, the particles were incubated in pH 6.0 and 7.4 PBS buffers for 1 day. A small amount of DOX was observed in both cases, with a greater release at neutral pH (Figure S3).⁴¹ To assess the therapeutic efficacy of DOX-BASP in vitro, HeLa cells were exposed to DOX-BASP, non-drug-loaded acetal-core BASP (ABASP, $N = 15$), and free DOX for 72 h. Cell viability (Figure 5) was assessed via MTT assay; half-maximal inhibitory concentration (IC_{50}) values for each sample were obtained via standard fitting procedures. Non-drug-loaded acetal-core ABASP showed no toxicity over the range of concentrations studied (blue data, Figure 5). This result is encouraging; it suggests that BASPs constructed from cross-linker 5 are not inherently cytotoxic. In contrast, free DOX and DOX-BASP displayed IC_{50} values of 1.3 ± 0.3 and 8.4 ± 0.5 μM , respectively. As is common for polymer-drug conjugates, the IC_{50} of DOX-BASP was higher than free DOX.⁴² Nevertheless, the observation of significant in vitro toxicity strongly suggests that DOX-BASP releases therapeutically active DOX in cell culture.

This work describes the synthesis of novel hydrolytically labile BASP nanoparticles via the design of acid-cleavable cross-linkers. We identify an acetal-based bis-norbornene derivative (compound 5) that enables the brush-first synthesis of uniform BASPs that degrade in response to acidic pH. We interface this new cross-linker with a novel DOX-based branched macromonomer (DOX-MM) for the synthesis of a DOX-conjugated BASP (DOX-BASP) that degrades and releases therapeutic

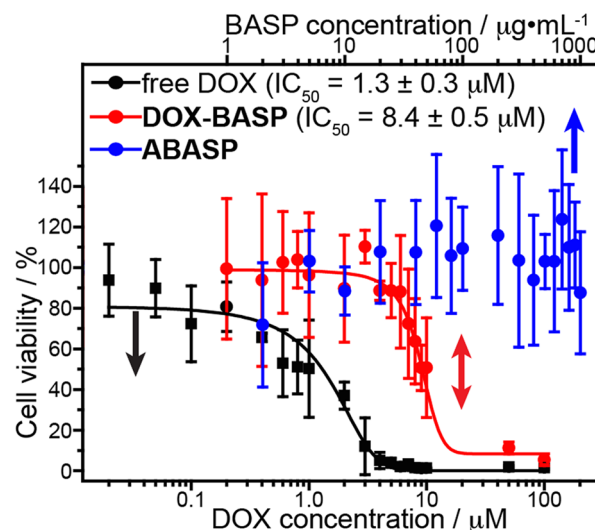


Figure 5. HeLa cell viability studies. "ABASP" refers to the acetal-core $N = 15$ BASP without DOX.

DOX in cell culture. These new compounds are important additions to our brush-first ROMP platform for BASP drug delivery.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic methods, NMR spectra, and other detailed experimental information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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