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Solvent-free mechanochemical synthesis of a bicyclononyne tosylate: A fast route towards bioorthogonal clickable poly(2-oxazoline)s

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The mechanochemical synthesis of a bicyclononyne tosylate (BCN-OTs) is presented. BCN-OTs is demonstrated to be a good initiator for the cationic ring-opening polymerization of 2-ethyl-2-oxazoline directly yielding BCN functional poly(2-ethyl-2-oxazoline) (PEtOx-BCN) with high chain end fidelity. Subsequent strain-promoted cycloadditions of the resulting PEtOx-BCN enable efficient additive-free conjugation reactions as demonstrated for the formation of a block copolymer and a PEtOx-protein conjugate.

Introduction

The development and application of bioorthogonal chemical reactions that enable the tracking of biomolecules in living systems has received increasing interest in recent years.[1] One of the most prominent reactions in this context is the strain-promoted azide-alkyne cycloaddition of cyclooctynes (SPAAC).[2] Among the various cyclooctynes that have been developed for SPAAC reactions, derivatives of bicyclo[6.1.0]non-4-yne (BCN) can be prepared with the least synthetic effort on the one hand while BCN is one of the least apolar cyclooctynes facilitating aqueous conjugation reactions.[3] BCN analogues have been show to undergo fast cycloadditions not only with azides but also with nitrones (SPANC)[3] and via inverse electron-demand Diels–Alder cycloaddition (SPIEDAC) with tetrazines.[4] In addition an activatable conjugation of BCN via the strain-promoted oxidation-controlled cyclooctyne–1,2-quinone cycloaddition (SPOCQ) has recently been reported.[5] In parallel, the rise of synthetic polymers for biomedical applications[6] created the demand for polymers with end-groups that enable efficient conjugation reactions, often referred to as *click* reactions.[7] Poly(2-oxazolines) (PAOx) which are accessible via living cationic ring-opening polymerization (CROP) of 2-oxazolines represent one of the most promising classes of polymers for biomedical applications.[8] One advantage of PAOx is that clickable end-groups such as alkynes[9], azides[10] or cyclopentadiene[11] can be easily incorporated employing functional initiators or terminators.[12] However, only one example of a cyclooctyne end-functional PAOx has been reported, in which a BCN group is attached via a multistep post-polymerization modification.[13]

Here, we report the mechanochemical synthesis of a bicyclononyne tosylate (BCN-OTs). BCN-OTs is subsequently employed as initiator for the CROP of 2-ethyl-2-oxazoline (EtOx) resulting in BCN functional poly(2-ethyl-2-oxazoline) (BCN-PEtOx), which is then used for additive-free (bio-)conjugations at ambient temperature.

Experimental

Materials and Instrumentation

Acetonitrile (Aldrich) was dried in a solvent purification system (J. C. Meyer) before use as a polymerization solvent. 2-Ethyl-2-oxazoline (EtOx; Aldrich) was distilled over barium oxide and stored under argon. Potassium hydroxide and potassium carbonate (Aldrich) were finely grounded in a mortar and dried in a vacuum oven at 50 °C prior to use. Tosyl chloride (Aldrich) was recrystallized from petroleum ether and dried under vacuum. (1*R*,8*S*,9*s*)bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN-OH) was purchased from SynAffix and stored at -20°C. Copper (I) bromide (Aldrich) was purified by sequential washing with glacial acetic acid, absolute ethanol and diethyl ether, dried under vacuum and stored under argon. Styrene was passed through a column of basic alumina (Merck) to remove inhibitor and stored at -20°C. Ethyl α-bromoisobutyrate (EBiB) (Sigma Aldrich), *N*,*N*,*N*',*N*'',*N*''-pentamethyldiethylenetriamine (PMDETA) (Sigma Aldrich), sodium azide (Fisher Chemical), bovine serum albumin (BSA, Sigma Aldrich) and NHS-PEG$_4$-N$_3$ (Life Technologies) were use as received. Benzyl azide[14] and 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine[15] were synthesized according to literature procedures.

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The CROP was performed in a capped vial in a microwave reactor (Biotage Initiator Sixty) equipped with an IR temperature sensor.

High-speed vibration milling was performed using a mini-mill pulverisette 23 (Fritsch) equipped with a 10 mL stainless steel grinding bowl and a stainless steel grinding ball (∅ 15 mm) at a frequency of 50 Hz at ambient temperature.

^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 300 MHz spectrometer.

Size exclusion chromatography (SEC) measurements were performed on an Agilent 1260-series equipped with a 1260 ISO-pump, a 1260 Diode Array Detector (DAD), a 1260 Refractive Index Detector (RID), and a PSS Gram30 column in series with a PSS Gram1000 column inside a 1260 Thermostated Column Compartment (TCC) at 50°C using *N*,*N*-dimethylacetamide containing 50 mM of LiCl (flow rate of 0.59 mL min^{-1}) as solvent. Molar masses were calculated against poly(methyl methacrylate) standards.

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) was performed on an Applied Biosystems Voyager De STR MALDI-TOF mass spectrometer equipped with 2 m linear and 3 m reflector flight tubes. All mass spectra were obtained with an accelerating potential of 20 kV in positive ion mode and in reflectron mode (matrix: trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB); salt: sodium trifluoroacetate).

Gas chromatography (GC) was performed on a 7890A from Agilent Technologies with an Agilent J&W Advanced Capillary GC column (30 m, 0.320 mm, and 0.25 mm). Injections were performed with an Agilent Technologies 7693 auto sampler. Detection was done with a FID detector. Injector and detector temperatures were kept constant at 250 and 280 °C, respectively. The column was initially set at 50 °C, followed by two heating stages: from 50 °C to 120 °C with a rate of 20 °C /min and from 100 °C to 300 °C with a rate of 50 °C /min, and then held at this temperature for 0.5 minutes. For the ATRP, conversion of the monomer was determined based on the integration of the monomer peak using the polymerization solvent, anisole, as an internal standard.

((1R,8S)-bicyclo[6.1.0]non-4-yn-9-yl)methyl 4-toluenesulfonate (BCN-OTs)

BCN-OH (150 mg, 1 mmol) and KOH (280 mg, 5 mmol) were added to a ball mill vessel along with a stainless steel ball. The ball mill was set to vibrate at a frequency of 50 Hz for 3 min at ambient temperature. Tosyl chloride (384 mg, 2 mmol) and K_2CO_3 (500 mg, 3.6 mmol) were then added to the vessel and ball-milling was continued for 3 min. Finally, additional KOH (280 mg, 5 mmol) was added to the vessel and ball-milling was continued for 2 min to destroy the remaining excess of tosyl chloride. The solid material was extracted with ethyl acetate and the insoluble salts were removed by centrifugation. After removal of the solvent under reduced pressure at ambient temperature the crude material was purified by a filtration column over silica (*n*-hexane/ethyl acetate 5:1). BCN-OTs (81 mg, 27%) was obtained as yellowish oil and used immediately for the next step.

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.80 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.35 (d, *J* = 8.2 Hz, 2H, Ar-H), 4.14 (d, *J* = 8.3 Hz, 2H, CH_2-OTs), 2.45 (s, 1H, Ar-CH_3), 2.35–2.20 (m, 6H, BCN), 1.50–1.29 (m, 3H, BCN), 1.04–0.90 (m, 2H, BCN).

BCN-OTs initiated CROP of EtOx

A mixture of freshly prepared BCN-OTs (81 mg, 0.266 mmol) and EtOx (791 mg, 7.98 mmol) in acetonitrile was prepared in a glove-box (Vigor Gas Purification Technologies) with a monomer to initiator ratio of 30:1 and an initial monomer concentration of 4M and crimped air-tight inside the glove-box before transfer to the microwave reactor. The polymerization was carried out under microwave irradiation at 100°C for 14 minutes aiming for full conversion. After cooling to ambient temperature, the polymerization was terminated by addition of tetramethylammonium hydroxide solution (25 wt% in methanol, 1.2 equivalents relative to initiator) and stirred for 2 h at ambient temperature. After removal of the solvents under reduced pressure the residue was redissolved in dichloromethane, passed through a short column of neutral aluminium oxide, precipitated in diethyl ether and dried under vacuum. Yield: 710 mg (90%), M_n (theo) = 3000 g mol^{-1}; M_n (SEC_{DMA}) = 5000 g mol^{-1}; $Đ$ = 1.10, M_n (MALDI) = 3000 g mol^{-1}

Strain-promoted cycloadditions of PEtOx-BCN with small molecules

A solution of PEtOx-BCN (10 mg, 3.3 mmol) and benzyl azide or 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (30 mmol) in 1 mL dichloromethane was stirred at ambient temperature over night. The solvent was removed under reduced pressure and the residue was dissolved in milliQ water. In case of the tetrazine, the insoluble part was separated by centrifugation. The solution was passed through a PD-10 desalting column and the PEtOx containing fraction was freeze-dried.

Synthesis of bromine-terminated polystyrene (PS-Br) by ATRP

A 5 mL glass vial was charged with styrene 3 mL (25.5 mmol), CuBr 14.66 mg (0.102 mmol), PMDETA 17.7 mg (0.102 mmol), EBiB 19.9 mg (0.102 mmol) and anisole 0.5 mL. The vial was crimped air-tight and bubbled with Argon for 30 minutes. Then the vial was placed in a drysyn heating block preheated to 110 °C. The monomer conversion was determined by GC. After a definite time, the vial was removed from the drysyn block and the reaction was quenched by cooling it in ice water and diluted with THF. The solution was filtered through a neutral alumina column to remove the catalyst. The polymer was precipitated in methanol and dried under vacuum. Conversion (GC) = 29%, M_n^{theo} = 9000 g mol^{-1}, M_n (SEC_{DMA}) = 3700 g mol^{-1}, $Đ$ = 1.11, M_n (NMR) = 9300 g mol^{-1}

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.30–6.30 (m, Ph), 4.53–4.40 (C*H*(Ph)Br), 3.63–3.44 (m, CH_2O), 2.15–1.17 (m, backbone), 1.06–0.71 (m, C(CH_3)$_2$, CH_3CH$_2$O).

Synthesis of azide end-functional Polystyrene (PS-N_3)

0.72 g (0.08 mmol) PS-Br and 20.0 mg (0.3 mmol) NaN_3 were dissolved in 6 mL DMF. The solution was stirred at 25°C for 48 h under argon atmosphere. The polymer was precipitated in methanol and dried under vacuum. M_n (SEC_{DMA}) = 3700 g mol^{-1}, $Đ$ = 1.11, M_n (NMR) = 9300 g mol^{-1}
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.30–6.30 (m, Ph), 4.03–3.85 (C*H*(Ph)N_3), 3.63–3.44 (m, CH_2O), 2.15–1.17 (m, backbone), 1.06–0.71 (m, C(CH_3)$_2$, CH_3CH_2O).

Synthesis of PS-*b*-PEtOx

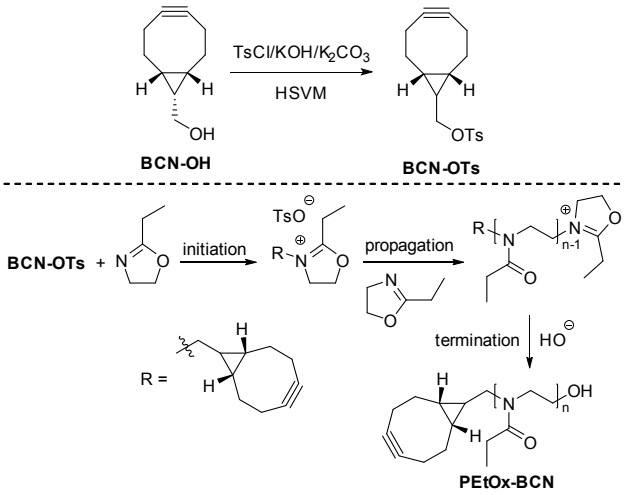
A solution of PS-N_3 (50 mg, 5.38 mmol based on M_n(NMR)) and PEtOx-BCN (19 mg, 6.45 mmol based on M_n(MALDI)) in 1 mL dichloromethane was stirred at ambient temperature overnight. The resulting block copolymer was precipitated in cold methanol and dried under vacuum.

Conjugation of PEtOx-BCN with NHS-PEG_4-N_3 modified BSA.

Stock solutions of BSA (1.16 x 10^{-4} M, 7.7 mg/mL in PBS pH 7.4) and NHS-PEG_4-N_3 (2.57 x 10^{-2} M, 10 mg/mL in DMSO) were prepared. BSA (1.16 x 10^{-4} mmol, 1 mL) was modified with a 5-, 10- or 20-fold molar excess of NHS-PEG_4-N_3 (22.5, 45 or 90 µL respectively). After overnight reaction, the protein solutions were dialyzed against DI water (6 x 5L) for 2 days using a MWCO of 8000 Da and freeze-dried. Stock solutions of BSA-PEG_4-N_3 (1.16 x 10^{-4} M, 7.7 mg/mL in PBS pH 7.4) and PEtOx-BCN (3.33 x 10^{-3} M, 10 mg/mL in PBS pH 7.4) were combined to obtain a molar ratio of protein:polymer 1:10 or 1:20. The reaction mixtures were diluted with PBS to obtain a final protein concentration of 1.16 x 10^{-5} M and incubated overnight at ambient temperature with continuous stirring. Conjugation efficiency was evaluated by SDS-PAGE. Quantification of protein conjugation was done by automated integration of optical density by ImageJ software.

Results and discussion

Tosylation of the commercially available BCN-OH (Scheme 1) should directly result in a BCN tosylate (BCN-OTs) that can be employed as initiator for the CROP of 2-oxazolines. However, the limited stability of BCN-OTs due to ring-strain in combination with the high reactivity of tosylates makes its synthesis by conventional protocols in solution challenging, if not impossible as several attempts using standard conditions were unsuccessful due to partial degradation of the product. However, Kazemi *et al*. reported an extremely fast synthesis of alkyl tosylates by solvent-free manual grinding of alcohols with tosyl chloride and a solid base.[16] This procedure has also been successfully employed for the synthesis of the rather unstable benzyl tosylate, which was subsequently used as initiator for the CROP of 2-oxazolines.[17] Here, we adapted this procedure for the synthesis of BCN-OTs using high-speed vibration milling (HSVM) instead of manual grinding. HSVM is a valuable tool for organic synthesis as it facilitates efficient and reproducible mixing on laboratory scale.[18]



Scheme 1 Synthesis of BCN-OTs via high-speed vibration milling (top) and mechanism of the CROP of EtOx initiated by BCN-OTs (bottom).

Analysis by ^{1}H NMR spectroscopy confirmed that HSVM of BCN-OH with KOH, followed by milling with tosyl chloride and K_2CO_3 results in complete conversion and formation of BCN-OTs in less than 10 minutes (Figure 1). After rapid purification by a filtration column, BNC-OTs was immediately used for the CROP of EtOx as shown in Scheme 1. The microwave assisted polymerization was conducted in acetonitrile at 100°C. This temperature was identified as an optimal compromise between a fast polymerization and the stability of the BCN group. Size-exclusion chromatography (SEC) of the resulting PEtOx shows the formation of a well-defined polymer with a narrow molar mass distribution (M_n = 5000 g mol^{-1}, $Đ$ = 1.10, Figure S1) indicating a controlled/living polymerization. To investigate the end-group fidelity, PEtOx-BCN was further analyzed by MALDI-TOF mass spectrometry. Inspection of Figure 2 shows one major distribution which can be assigned to the sodium adduct of the expected BCN functionalized PEtOx (see Table S1 for theoretical and measured *m*/*z* ratios).

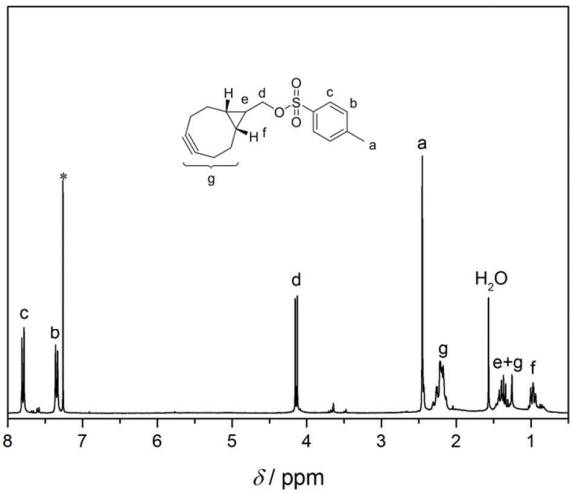


Figure 1 ^{1}H NMR spectrum BCN-OTs in $CDCl_3$.* = $CHCl_3$

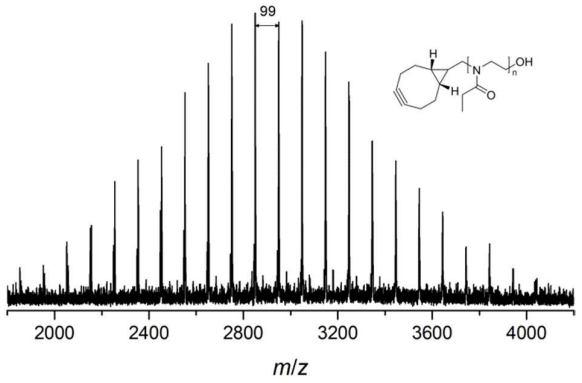
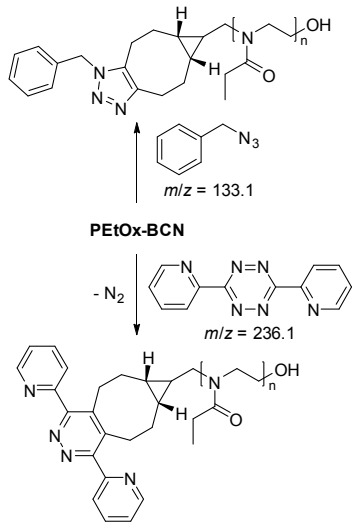


Figure 2 MALDI-TOF mass spectrum of PEtOx-BCN.

To explore the ability of PEtOx-BCN to undergo strain-promoted cycloadditions, it was reacted in dichloromethane with an excess of benzyl azide for SPAAC and 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine for SPIEDAC, respectively (Scheme 2). Monitoring of the reactions by MALDI-TOF MS (Figure 3) confirms the quantitative transformation of the BCN end-group into the respective cycloadduct. The success of the SPAAC and SPIEDAC reactions is further confirmed by ^{1}H NMR analysis of the resulting polymers. Inspection of the spectra depicted in Figure 4 reveals the presence of signals that can be unambiguously assigned to the respective cycloadduct. The peak at 5.5 ppm arising from the benzylic protons adjacent to the triazole ring for the SPAAC product (Figure 4 top) and the signals assigned to the pyridinyl protons of the SPIEDAC product (Figure 4 bottom) are in agreement with the spectra that have been reported for the cycloadducts of corresponding small molecules.[3, 4b]



Scheme 2 Strain-promoted cycloadditions of PEtOx-BCN with benzyl azide (SPAAC) and 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (SPIEDAC).

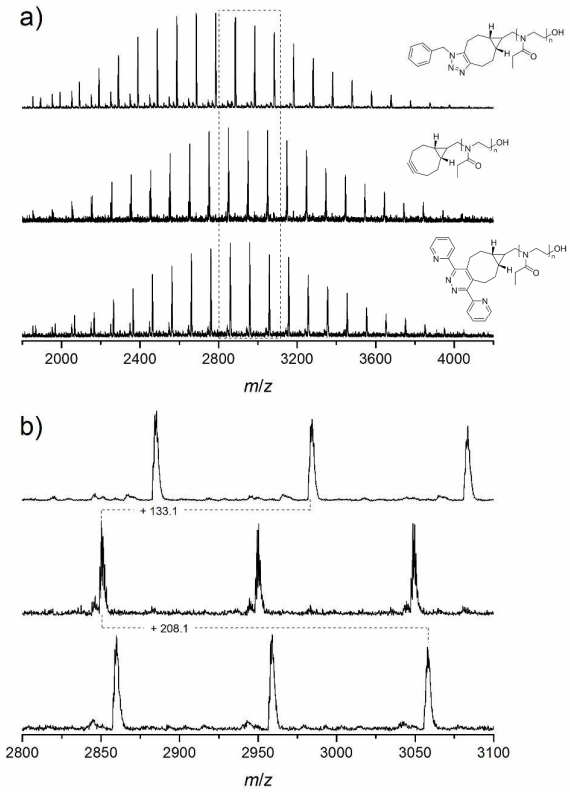


Figure 3 a) MALDI-TOF mass spectra of PEtOx-BCN (middle spectrum) and the products of the SPAAC with benzyl azide (top spectrum) and the SPIEDAC with 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (bottom spectrum). b) Zoom of the *m/z*-region highlighted in a). The main distributions can be assigned to the sodium adducts of the structures shown in a) (see Table S1 ESI).

To demonstrate the applicability of PEtOx-BCN as building block for the modular construction of larger macromolecular architectures, a block copolymer was prepared via the SPAAC reaction with an azido functional polystyrene synthesized by ATRP as shown in Scheme 3.

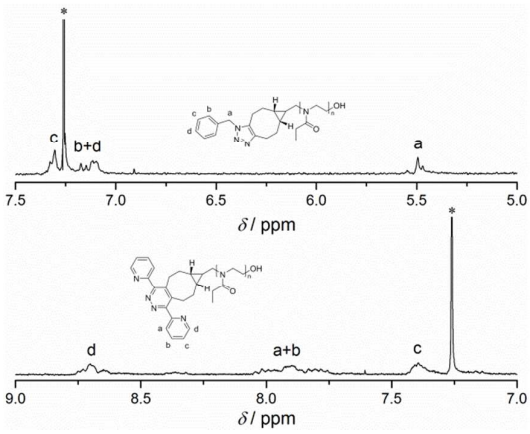
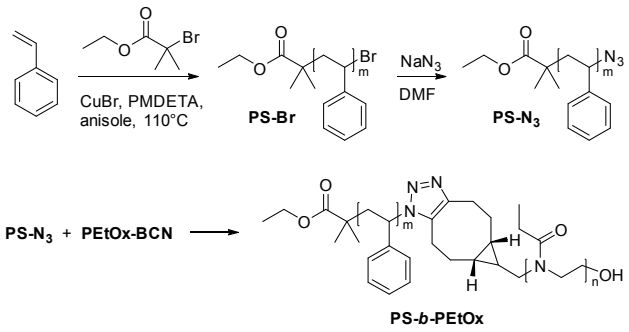


Figure 4 Expanded regions of the ^{1}H NMR spectra of the cycloadducts of PEtOx-BCN with benzyl azide (top) and 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (bottom) in $CDCl_3$. (See Figure S2 ESI for full spectra). * = $CHCl_3$.



Scheme 3 Synthesis of PS-N_3 and modular construction of PS-*b*-PEtOx via the SPAAC reaction with PEtOx-BCN.

The coupling reaction between PEtOx-BCN (1.2 eq) and PS-N_3 (1 eq) was performed in dichloromethane and the resulting block copolymer was precipitated in methanol to remove the excess of PEtOx-BCN. Figure 5 shows the SEC traces of the two homopolymers and the coupling product. A clear shift to lower retention times indicates the successful formation of PS-*b*-PEtOx. Importantly no remaining PS or PEtOx can be detected by SEC analysis.

The rapidly emerging area of biomedical applications of protein-polymer conjugates[19] motivated us to also investigate the conjugation of PEtOx-BCN to an azido functional protein. Bovine serum albumin (BSA) was modified with respectively a 5-, 10- or 20-fold molar excess NHS-OEG_4-N_3, followed by extensive dialysis to remove unreacted azides. In this step, azides linked to a tetraethyleneglycol spacer are coupled to lysine residues by amide bond formation. In a second step, PEtOx-BCN is conjugated via SPAAC to the protein using a protein to polymer molar ratio of 1:10 or 1:20. After overnight reaction, the conjugates were analyzed by SDS-PAGE (Figure 6A). Integration of the optical density of the gels (Figure 6B),[20] clearly reveals a shift in molecular weight of the BSA-OEG_4-N_3 towards higher molecular weight species, demonstrating successful conjugation. This shift depends both on the extent of azide modification of the BSA in the first step and on the excess of PEtOx-BCN added in the second step.

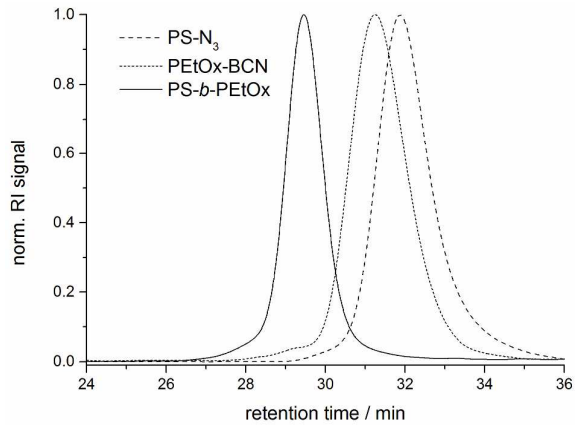


Figure 5 Overlay of SEC traces showing the formation of PS-*b*-PEtOx by the SPAAC reaction of PEtOx-BCN and PS-N_3.

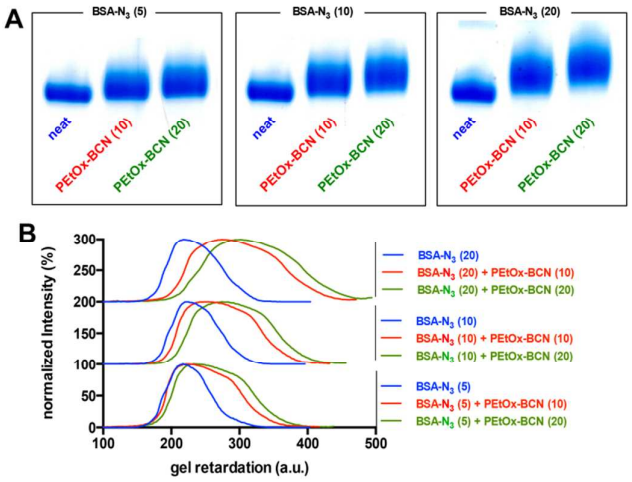


Figure 6 (A) SDS-PAGE analysis of the conjugation of PEtOx-BCN to azide-modified BSA. (B) Quantification of PEtOx-BCN to BSA conjugation by integration of the optical density.

Conclusions

In summary, we have presented the first synthesis of a BCN tosylate via solvent-free reaction conditions using high-speed vibration milling. Subsequent utilization of BCN-OTs as initiator for the CROP of EtOx represents a rapid route to prepare defined BCN functionalized PEtOx that can be used for bioorthogonal strain-promoted conjugation reactions. This strategy was successfully employed for the synthesis of a PS-*b*-PEtOx copolymer and a PEtOx-protein conjugate. We envisage that BCN-OTs will find future applications as intermediate in the synthesis of functional BCN derivatives. Furthermore the ball-milling methodology presented herein is a promising tool for the synthesis of other unstable/highly reactive tosylates.

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