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Poly(2-ethyl-2-oxazoline) Featuring a Central Amino Moiety

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The incorporation of an amino group into a bifunctional initiator for the cationic ring-opening polymerization (CROP) is achieved in a two-step reaction. Detailed kinetic studies using 2-ethyl-2-oxazoline demonstrate the initiators' eligibility for the CROP yielding well-defined polymers featuring molar masses of about 2000 g mol^{-1}. Deprotection of the phthalimide moiety subsequent to polymerization enables the introduction of a cyclooctyne group in central position of the polymer which is further exploited in a strain-promoted alkyne-azide click reaction (SpAAC) with a Fmoc-protected azido lysine representing a commonly used binding motif for site specific polymer–protein/peptide conjugation. In-depth characterization via electrospray ionization mass spectrometry (ESI) confirms the success of all post polymerization modification steps.

1. Introduction

Enhancing the blood circulation times of pharmaceutically active proteins and peptides represents a key factor for an optimized therapeutic efficacy.[1] The covalent attachment of hydrophilic polymers featuring a "stealth" effect, such as the "gold standard" poly(ethylene glycol) (PEG), has been widely applied for that purpose. A wide variety of parameters including the polymer molar mass, applied coupling chemistry and polymer architectures have been reported.[2,3] For example, an early study reported that the use of PEG featuring a central binding motif enhances the stability and blood circulation time whereas the immunogenicity of the conjugate was reduced when compared to a linear PEG with a similar molar mass.[4] This observation can be attributed to the so-called "umbrella effect," resulting in an enhanced surface coverage of the protein.[5,6]

In general, polymer–protein conjugates can be accessed by exploitation of functional moieties, such as free amino or thiol moieties, present in the wild type of the proteins, respectively.[7] However, these reactions are mostly unspecific, since any functional amino acid could undergo the coupling reaction. In contrast, unique functional moieties can be introduced by the incorporation of non-natural amino acids. These protein mutants can be conjugated in a site specific manner,[8] allowing the attachment of a precisely pre-determined number of polymer chains at specific locations within the bio-macromolecule.

In particular, azides gained interest for this purpose as they can efficiently undergo 1,3-dipolar cycloadditions, either in a copper-catalyzed (CuAAC) process[9,10] or, more interestingly, for biomedical applications, in a copper-free biorthogonal approach with strained triple bonds as present in cyclooctynes (SpAAC).[11,12]

However, due to the excessive use of PEG in everyday products, such as cosmetics or food additives, an increasing amount of publications dealing with anti-PEG antibodies, that reduce the therapeutic efficiency of PEGylated substrates, have been reported.[13–16] This issue could be avoided by substitution of PEG by alternative polymers. Promising alternatives include hydrophilic poly(2-oxazoline)s (POx) featuring a similar "stealth" effect. This polymer class is widely applied in polymer-protein conjugations as recently summarized in an overview article by Hoogenboom and coworkers.[17] The conjugation strategy is mostly based on either α- or ω-end group functionalization. To exploit the "umbrella effect," a central binding motif for biorthogonal conjugation is required. From a retrosynthetic point of view, the utilization of a bifunctional CROP initiator featuring a protected functional moiety could serve this purpose. Although bifunctional initiators have been utilized in the cationic ring-opening polymerization (CROP) of 2-oxazolines,[18–22] additional functional moieties enabling peptide attachments are, to the best of our knowledge, missing up to now.

We present the development of a straightforward synthesis route yielding poly(2-ethyl-2-oxazoline)s (PEtOx) featuring an amino moiety in central position that can be utilized to introduce suitable linkers for a polymer-peptide/protein conjugation (**Scheme 1**). For this purpose, a phthalimide functionalized bifunctional benzyl halide-based CROP initiator was synthesized and investigated in-depth regarding the polymerization

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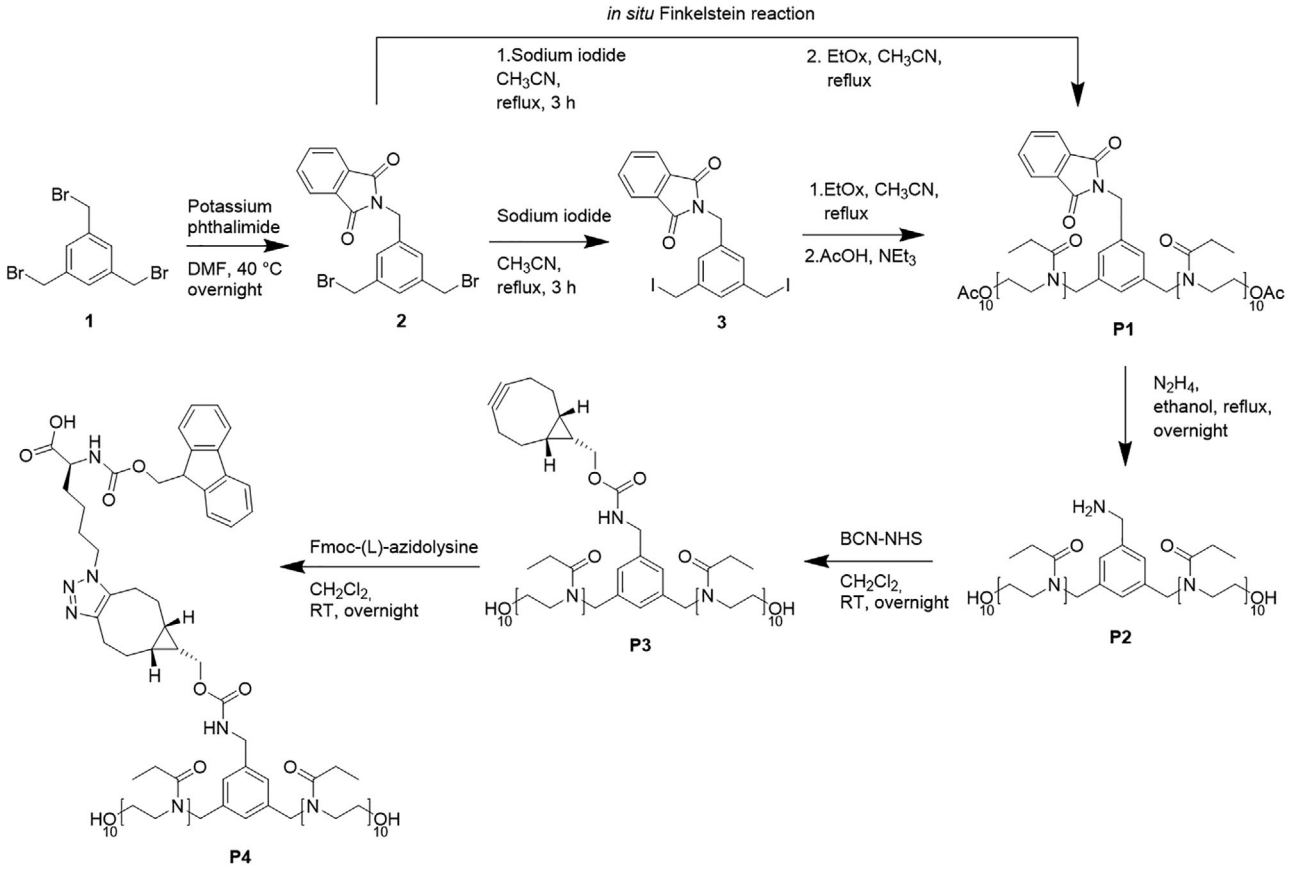

Scheme 1. Schematic representation of the synthesis route toward functional poly-2-ethyl-2-oxazolines with a central amino moiety including postpolymerization modifications.

of 2-ethyl-2-oxazoline (EtOx). In a proof of principle approach, post polymerization modifications including deprotection of the amine, functionalization with a cyclooctyne moiety and SpAAC with Fmoc-(L)-azidolysine as a model system were performed to demonstrate the applicability of the synthesis route toward protein conjugates benefitting from the "umbrella" effect.

2. Results and Discussion

The initiator was designed as a symmetric molecule composed of one protected amino moiety and two chemically identical initiation sites for the CROP. Phthalimide was selected as a protective group since it remains unaffected throughout the course of the CROP. It can be removed by hydrazinolysis in a Gabriel-like synthesis.[23] For this purpose, 1,3,5-tris(bromomethyl)benzene (**1**) was reacted with potassium phthalimide in order to yield the 2-(3,5-bis(bromomethyl)benzyl)isoindoline-1,3-dione (**2**). Due to the similar reactivities of the bromomethyl groups, a mixture of mono-, di-, and tri-substituted molecules were formed during the reaction. The mono-substituted species was isolated by silica gel column chromatography applying a procedure known from literature reports.[24] The purified compound was further subjected to initial test polymerizations. Although benzyl bromides are applicable in the CROP of 2-oxazolines, these attempts resulted in broad molar mass distributions, most likely due to a slow initiation, as has previously been observed for similar initiators.[25,26]

In order to accelerate the polymerization, an in situ Finkelstein reaction,[27] aiming at the respective iodide-based initiator, was performed prior to the monomer addition. Indeed, high monomer conversions were obtained in a shorter polymerization time, demonstrating the successful bromine to iodine exchange. In addition, the resulting PEtOx featured a narrow molar mass distribution as evident from size-exclusion chromatography (SEC). Aside the suitability of an in situ approach, the bis-iodo compound (**3**) was isolated. ^{1}H-NMR spectroscopy indicated a successful substitution of bromine by iodine after a reaction time of 3 h (Figure S1, Supporting Information). The phthalimide signals were not affected by the reaction whereas signals of the benzene core and the methylene protons adjacent to the halide shifted to lower ppm values. In addition, isotopic patterns that would result from bromine were absent in the ESI mass spectrum of **3** (Figure S1, Supporting Information). The most abundant signal was assigned to the desired product ionized with a sodium cation ($[M + Na]^+$). Less abundant signals corresponded to a potassium adduct ($[M + K]^+$) and adducts consisting of two molecules with either sodium ($[2M + Na]^+$) or potassium ($[2M + K]^+$). This initiator was applied in preliminary polymerization attempts yielding comparable results as for the in situ polymerizations.

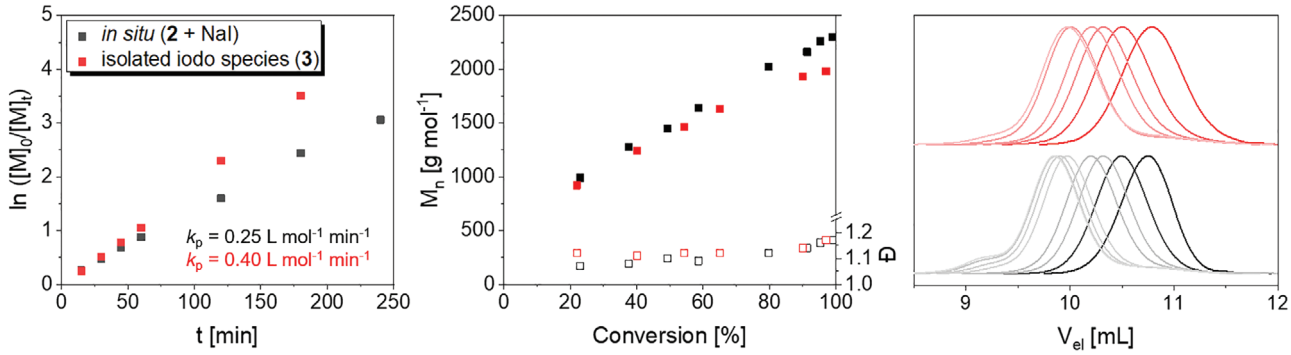

Figure 1. Kinetic studies of the CROP of EtOx ($[M]_0 = 1$ mol L^{-1}, $[M]_0/[I]_0 = 20$ in acetonitrile at reflux temperature) using the isolated iodo-based initiator **3** (red) and the in situ Finkelstein approach utilizing the bromo-based initiator **2** with sodium iodide (black). Left: Semi-logarithmic plot. Middle: Evolution of the molar mass and dispersities with increasing monomer conversion. Right: Overlay of the SEC elugrams ($CHCl_3$, NEt_3, *i*PrOH, RI-detection).

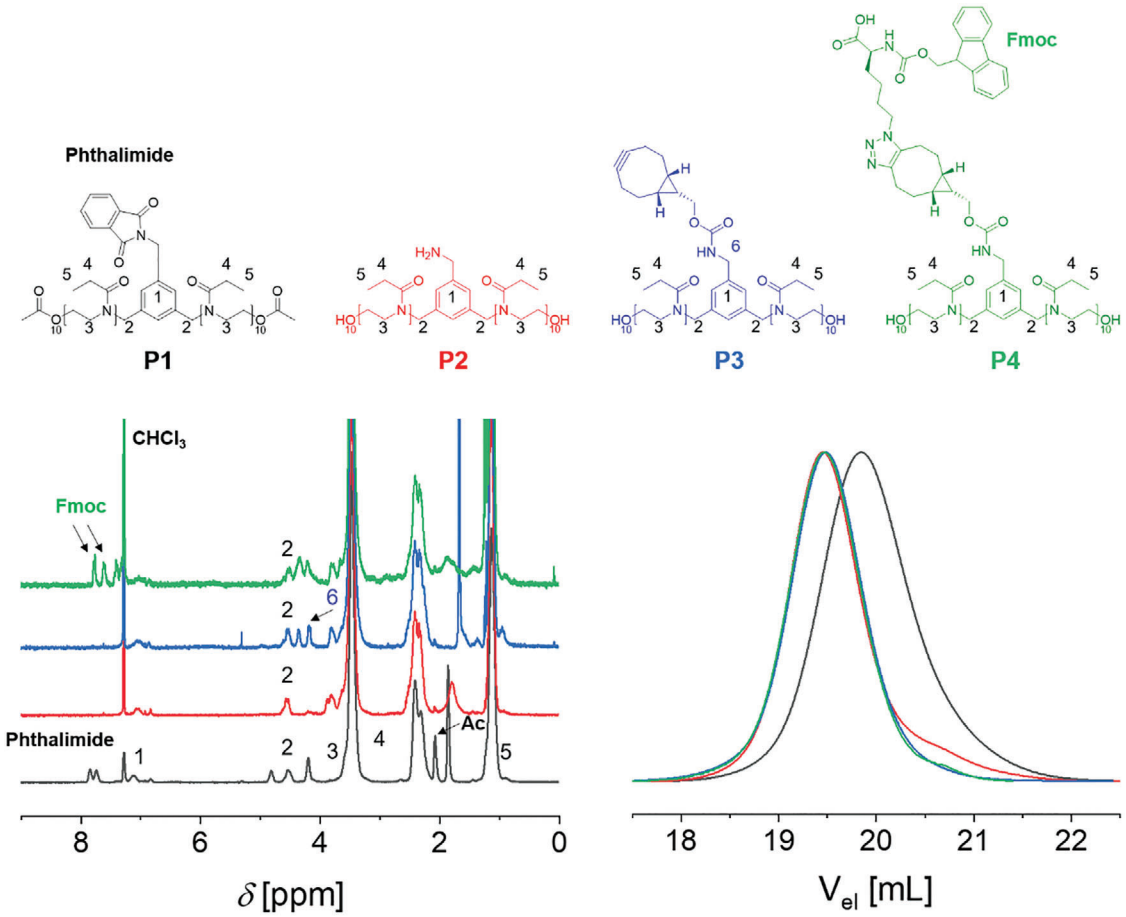

Figure 2. Characterization of the polymers **P1** to **P4**. Top: Schematic representations of the polymer structures. Left: Overlay of the ^{1}H-NMR spectra (300 MHz, $CDCl_3$). Signals marked with arrows were used to determine the DF (compare Table S1, Supporting Information). Right: Overlay of the SEC elugrams (DMAc, 0.21wt% LiCl).

With a focus on several postpolymerization modification steps to be established, an initial [M]:[I] ratio of 20 was selected for CROP experiments in order to enable a straightforward characterization by means of mass spectrometry. Kinetic studies (**Figure 1**) were hence performed using the in situ approach (**2** and NaI) as well as the isolated initiator **3**. A living polymerization character was observed for both systems, as evident from the linearity of the semi-logarithmic plot and the linear course of the evolution of the molar mass with increasing monomer consumption. In addition, SEC elugrams revealed monomodal molar mass distributions featuring low dispersity values (Đ < 1.17) that shifted to lower elution volumes with increasing

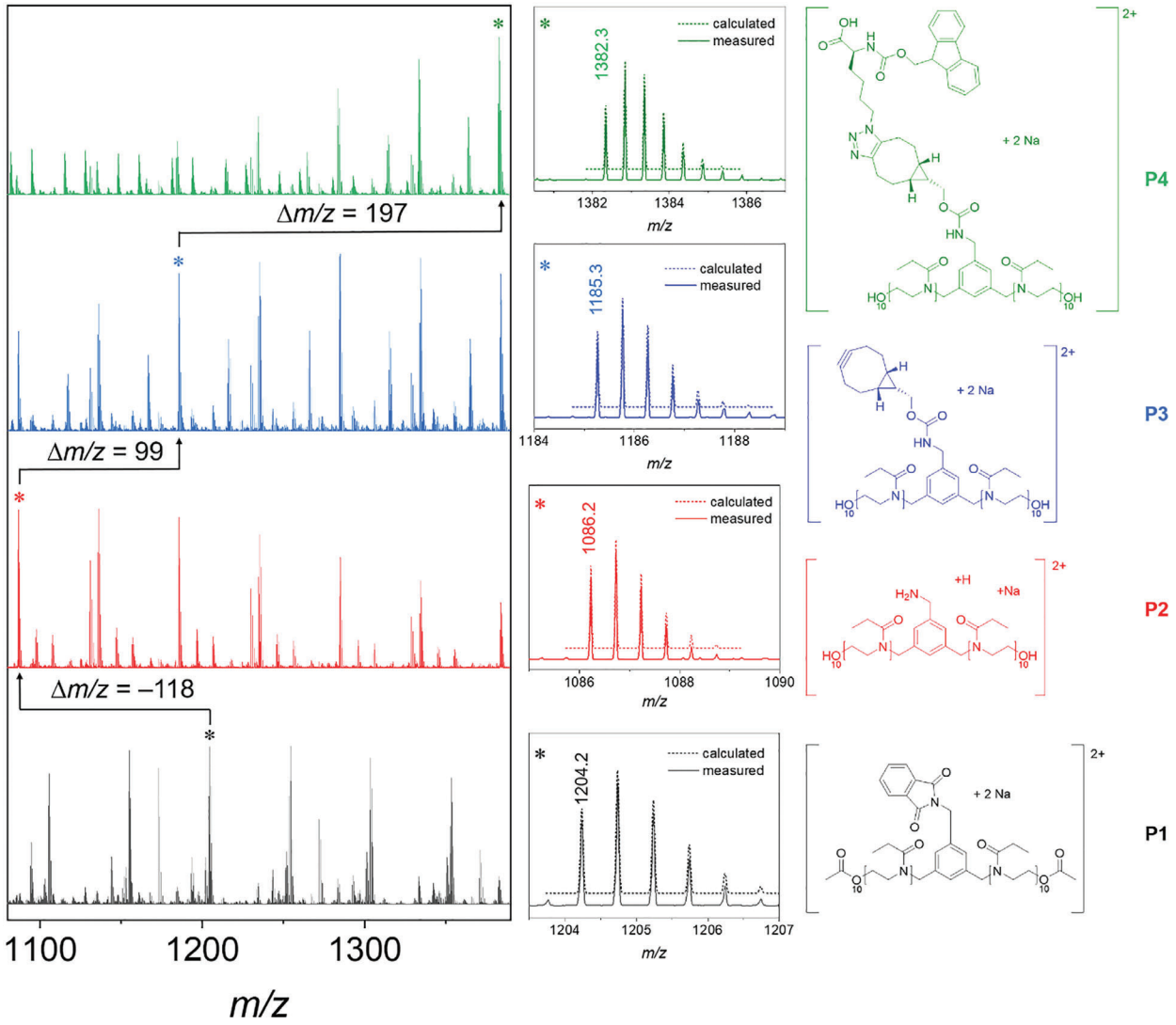

Figure 3. Zoom into the ESI mass spectra of **P1** to **P4** and overlay of the measured and calculated isotopic patterns for the indicated *m/z* species. Complete assignments are provided in the Supporting Information.

polymerization times. The polymerization using the bis-iodo compound **3** as initiator proceeded somewhat faster compared to the CROP based on in situ halide exchange reaction. The respective apparent polymerization rate constants (k_p) were determined as 0.25 L mol^{-1} min^{-1} (0.125 L mol^{-1} min^{-1} per initiation site) and 0.40 L mol^{-1} min^{-1} (0.2 L mol^{-1} min^{-1} per initiation site), respectively. This might be due to the presence of solvated bromide ions in the polymerization mixture, resulting in lower propagation rates during the in situ approach. For comparison, 1,3-bis(bromomethyl)benzene, representing another bifunctional CROP initiator featuring the same initiation sites as **2**, was recently reported to successfully polymerize EtOx under the same reaction conditions.[28] The k_p was determined as 0.02 L mol^{-1} min^{-1} (0.01 L mol^{-1} min^{-1} per initiation site), demonstrating that the bromine to iodine exchange resulted in an enhancement of the k_p by one order of magnitude.

In order to verify the covalent attachment of the introduced moieties by the postpolymerization modifications, as well as the quantitative degree of functionalization evident from ^{1}H-NMR spectroscopy (**Figure 2**), mass spectrometry measurements were performed. Electrospray ionization mass spectrometry (ESI) was selected due to the mild ionization conditions, in consequence avoiding fragmentation throughout the measurement, which was of utmost importance for the unambiguous assignment of the post polymerization modification products. The first hint on successful modifications was evident from the *m/z* shifts of the molar mass distributions as mass discrimination effects were absent (compare Figure S4, Supporting Information). **Figure 3** depicts an overlay of a zoom into the ESI mass spectra of all synthesized polymers. For the assignment of the individual polymer species derived from the bifunctional initiator, regions displaying doubly charged distributions were selected since the intensity of singl y charged distributions was rather low. The highlighted peaks correspond to a macromolecule featuring a DP of 20. The signals of the most abundant polymer species were assigned to the macromolecules aimed at. The assignment

of other polymeric species can be found in the Supporting Information. In none of the spectra, species matching the individual starting materials were observed, supporting the quantitative degree of functionalization observed from the ^{1}H-NMR spectra. In detail, the molar mass loss of the phthalimide removal as well as the gain in molar mass by functionalization with the cyclooctyne moiety and azido-amino acid were observed. Any detected minor *m/z* species did not result from the post polymerization modification reactions at the new central unit but were rather caused by commonly observed CROP side reactions such as proton initiation through chain-transfer as evident already in **P1**.[26] Additional tandem MS investigations clearly confirmed that (see the Supporting Information).

3. Conclusions

We presented a straightforward synthesis route yielding PEtOx featuring a central amino moiety applicable in post polymerization modifications. 2-(3,5-Bis(iodomethyl)benzyl)isoindoline-1,3-dione proved to be a suitable CROP initiator for this purpose as the phthalimide moiety was unaffected by the polymerization conditions. In situ bromine to iodine exchange as well as isolation of the iodo-based compound represented suitable pathways for the polymerization. The resulting material featured narrow molar mass distribution and was functionalized in a quantitative manner. A cyclooctyne unit was introduced subsequent to deprotection of the amino moiety. In a proof of principle approach aiming at biorthogonal click reactions, the model amino acid Fmoc-(L)-azidolysine was coupled.

Having established a general synthetic route toward PEtOx that can be functionalized in its center, upcoming studies will exploit the new material to obtain polymer-peptide conjugates to benefit from the "stealth" behavior of PEtOx. Besides the polymerization of other 2-oxazolines as monomers with the new initiator, a multitude of other azide functional molecules or building blocks could be introduced to result in more complex polymer architectures such as, e.g., miktoarm star-shaped polymers. Such syntheses could be performed either via SpAAC or by exploiting the central amino moiety directly.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

2-ethyl-2-oxazoline, bifunctional initiator, cationic ring-opening polymerization, electrospray ionization mass spectrometry, strain promoted azide-alkyne click reaction

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