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Revealing the Cytotoxicity of Residues of Phosphazene Catalysts Used for Synthesis of Poly(ethylene oxide)

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ABSTRACT. We herein report a case study on the toxicity of residual catalyst in metal-free polymer. Eight-arm star-like poly(ethylene oxide)s were successfully synthesized via phosphazene-catalyzed ring-opening polymerization of ethylene oxide using sucrose as an octahydroxy initiator. The products were subjected to MTT assay using human cancer cell lines (MDA-MB-231 and A2780). Comparison between the crude and purified products clearly revealed that the residual phosphazanium salts were considerably cytotoxic regardless of the anionic species, and that the cytotoxicity of more bulky *t*-BuP₄ salt was higher than that of *t*-

BuP₂ salt. Such results have therefore put forward the necessity for removal of the catalyst residues from PEO-based polymers synthesized through phosphazene catalysis for bio-related applications, and for the development of less or non-toxic organocatalysts for such polymers.

Introduction

In the unprecedentedly fast development of organocatalytic polymerization during the past 15 years,^{1, 2} phosphazene bases (PBs) have shown great utilities and strengths for (pseudo)anionic polymerization of a number of monomers, in particular heterocycles and methacrylates.^{3, 4} For instance, the highly basic, non-nucleophilic, and bulky natures have enabled PBs (especially *t*-BuP₄, Figure 1) to deprotonate weakly acidic groups and generate highly nucleophilic species on the initiator allowing rapid and controlled growth of polyether chains therefrom.^{5, 6} So far, PB has been the most frequently employed type of organocatalyst for the ring-opening polymerization (ROP) of epoxides, including ethylene oxide (EO) whose polymer, poly(ethylene oxide) (PEO), is one of the most appealing polymers for biomedical applications because of its desirable properties such as hydrophilicity, biocompatibility, non-toxicity, etc.^{7, 8}

One major motive and expectation for using metal-free catalysts is that they are non-toxic thus the resulting polymers are more environmentally friendly and suitable for bio-applications as compared with the polymers synthesized by heavy-metal catalysts. However, it has been shown recently that some organocatalysts are in fact remarkably cytotoxic.⁹ Therefore, regarding the most widely used organocatalysts for the synthesis of the most important biomedical polymer, the answer is urgently needed to the question whether the residues of PBs would render the PEO products toxic. To this end, we have utilized the synthesis of eight-arm star-like PEOs as a specific case to study the toxicity of residual phosphazanium salts, which are generated upon

1
2
3 quenching of the anionic ROP of EO with protic acids, by performing *in vitro* cytotoxicity tests
4 of the crude and purified star-PEO products through the well-established MTT [3-(4,5-
5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay.
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10 Star-like PEO has been extremely valued for its structural compactness, low viscosity,
11 multiple functional end groups as well as the ease of its synthesis, which have provided
12 additional merits for a variety of biomedical and biotechnological applications.^{10, 11} Although
13 four-arm star-PEOs derived from pentaerythritol have been most frequently used, those with
14 more arms (especially eight) have shown to be more competent in many cases because of their
15 unique properties associated with higher local densities of PEO chains and end functionalities.¹²⁻
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¹⁵ On this basis, we are also interested to use sucrose as an octahydroxy initiator for the synthesis
of such PEOs. Even though there is a large diversity of commercially available eight-arm star-
PEOs derived from hexaglycerol and tripentaerythritol, the non-toxicity, bio-renewability,
inexpensiveness, readily achieved high purity are believed to make sucrose an ideal alternative.

Experimental Section

Materials

ϵ -Caprolactone (CL; 99%), and tetrahydrofuran (THF; AR) were purchased from Aladdin. CL was dried over calcium hydride and distilled under vacuum; THF was dried successively by molecular sieve (4 Å), calcium hydride and *n*-butyllithium. D-(+)-Sucrose (Sucrose; >99%) was purchased from TCI and was used as received. Ethylene oxide (EO; 99.5%), *t*-BuP₄ (0.8 M in *n*-hexane) and *t*-BuP₂ (2.0 M in THF) were purchased from Aldrich. EO was dried successively by sodium hydride and *n*-butyllithium, *t*-BuP₄ and *t*-BuP₂ were used as received. Cation-exchange resin (Dowex50WX4, 200-400 mesh) was purchased from J&K. The human cancer cell lines

MDA-MB-231 (breast cancer cells) and A2780 (ovarian cancer cells) were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Eggenstein, Germany) containing 10% fetal bovine serum.

Instrumentation

Size exclusion chromatography (SEC) coupled with RI detector was conducted in THF at 35 °C using two identical PLgel columns (5 μ m, MIXED-C) at a flow rate of 1.0 mL min⁻¹. Calibration was done with a series of poly(ethylene oxide) (PEO) standards (Fluka) to obtain number-average molar mass ($M_{n,SEC}$) and molar mass distribution (D_M) of the polymers. Nuclear magnetic resonance (NMR) spectra were recorded at room temperature (RT) on a Bruker AV400 NMR spectrometer operating at 400 MHz using deuterated dimethyl sulfoxide (DMSO- d_6) as a solvent, and were used to calculate the monomer conversion using integrals of the characteristic signals from the monomer and the polymer, as well as the number-average molar mass ($M_{n,NMR}$) using integrals of the characteristic signals from the end group and/or polymer main body. Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) measurement was performed on a Bruker Autoflex III Smartbeam MALDI-TOF mass spectrometer (Bruker, Germany). Sample was dissolved in THF (10 mg mL⁻¹) and mixed with a solution of sodium trifluoroacetate (NaTFA) in THF (10 mg mL⁻¹) in a volume ratio of 5:1. This solution was then mixed with a THF solution of matrix (2,5-dihydroxybenzoic acid, 20 mg mL⁻¹) in a volume ratio of 1:10. Then, 0.4 μ L of the final solution was spotted on the target plate (dried-droplet method). The reflective positive ion mode was used to acquire the mass spectra of the sample. Calibration was done externally with poly(methyl methacrylate) standard using the nearest neighbor positions.

Polymer Synthesis

Eight-Arm Star-Poly(ethylene oxide) Initiated by Sucrose

Typical procedure of SCEO2 (Table 1): Sucrose (106.0 mg, 0.31 mmol) was charged in a reaction flask which was docked on the vacuum line and evacuated. THF (50 mL) was cryo-condensed into a flask followed by the addition of *t*-BuP₄ solution (0.15 mL, 0.12 mmol). Then THF was cryo-distilled off, and the remaining solid was dried substantially under vacuum with constant pumping for 16 hours. After that, THF (50 mL) and EO (7.0 mL, 140.0 mmol) was cryo-condensed into the flask cooled at −20 °C ([EO]₀ = 2.5 M). The flask was then sealed by a stopcock and the temperature was slowly elevated to 60 °C. After heating and stirring for 72 h, the reaction was finally quenched by the addition of acetic acid (0.5 mL). Then the solution was poured into *n*-hexane to precipitate the crude product. The white powder was then collected, dried in vacuum and used for SEC and ¹H NMR analysis. Theoretical number-average molar mass ($M_{n,theor}$, assuming complete EO conversion) = 20.0 kg mol^{−1}. $M_{n,SEC}$ (THF, PEO standards) = 10.4 kg mol^{−1}, D_M = 1.08. ¹H NMR(400 MHz, DMSO-*d*₆): δ /ppm = 4.57-4.54 (−PEO−OH), 3.65-3.43 (−OCH₂CH₂O−), 3.41-3.38 (−PEO−OCH₂CH₂OH), 2.63-2.59 ((CH₃)₂N− in phosphazanium cation); $M_{n,NMR}$ = 2.5 kg mol^{−1} × 8.

For removal of the phosphazanium acetate salt, the crude product was dissolved in Millipore-Q water and stirred with cation-exchange resin (Dowex50WX4) for 10 min followed by freeze-drying (SCEO2-E), or dialyzed in regenerated cellulose membrane (tubing, flat width = 45 mm, molecular weight cut-off = 1 kDa) against Millipore-Q water for 3 days and freeze-dried (SCEO2-D).

Eight-Arm Poly(ethylene oxide)-b-Poly(ε-caprolactone) Star-Diblock Copolymer

Synthetic procedure for SCEO5CL (Table 1): SCEO5 was synthesized similarly as SCEO2 using sucrose (106.0 mg, 0.31 mmol), THF (50 mL), *t*-BuP₂ solution (0.105 mL, 0.21 mmol), and EO

(7.0 mL, 140.0 mmol). After heating at 60 °C and stirring for 72 h, the flask was cooled down to RT. Then a small aliquot (0.1 mL) was withdrawn in an argon flow and injected into a mixture of THF (2 mL) and a few drops of acetic acid for SEC measurement. $M_{n,theor} = 20.0 \text{ kg mol}^{-1}$. $M_{n,SEC} = 12.5 \text{ kg mol}^{-1}$, $D_M = 1.06$.

The solution (10.0 mL) containing star-PEO (1.23 g, 0.062 mmol) and *t*-BuP₂ (0.042 mmol) was withdrawn with a syringe in an argon flow and injected into another flask followed by addition of CL (3.0 mL, 27.1 mmol). The reaction mixture was stirred at 60 °C ($[CL]_0 = 2.1 \text{ M}$) for 5 days before it was quenched by addition of acetic acid (0.5 mL). An aliquot (0.05 mL) was withdrawn and injected into DMSO-*d*₆ (0.6 mL) containing one drop of acetic acid for ¹H NMR measurement to determine the conversion of CL. Then the rest of the solution was poured into diethyl ether to precipitate the PEO-*b*-PCL star-diblock copolymer. The white powder was then collected, dried in vacuum and used for SEC and ¹H NMR analysis. Conv.(CL) = 54%; $M_{n,theor}(\text{PCL in each arm}) = 3.4 \text{ kg mol}^{-1}$, $M_{n,theor}(\text{total}) = 47.2 \text{ kg mol}^{-1}$. $M_{n,SEC} = 41.3 \text{ kg mol}^{-1}$, $D_M = 1.11$. ¹H NMR(400 MHz, DMSO-*d*₆): $\delta/\text{ppm} = 4.34\text{--}4.31$ (–PCL–OH), 4.13–4.09 (–PEO–CH₂CH₂OCO–PCL), 4.01–3.93 (–CH₂CH₂CH₂CH₂CH₂OCO–), 3.64–3.43 (–OCH₂CH₂O–), 2.32–2.23 (–OCOCH₂CH₂CH₂CH₂CH₂–), 1.60–1.48 (–OCOCH₂CH₂CH₂CH₂CH₂–), 1.33–1.25 (–OCOCH₂CH₂CH₂CH₂CH₂–); $M_{n,NMR}(\text{PCL in each arm}) = 3.6 \text{ kg mol}^{-1}$, $M_{n,NMR}(\text{total}) = 48.8 \text{ kg mol}^{-1}$.

Cytotoxicity Assay in vitro

MDA-MB-231 and A2780 cells were seeded into 96-well plates (5000 cells per well) in 100 μL DMEM complete medium (DMEM supplemented with 10% fetal bovine serum, 100 U mL^{−1} penicillin and 100 mg mL^{−1} streptomycin), and then cultured overnight at 37 °C in a 5% CO₂ humidified atmosphere. Polymers were dissolved in DMEM complete medium and added into

wells at equally diluted concentrations (final polymer concentration = 0.01 to 10 mg mL⁻¹). Cells incubated in medium without polymers were used as controls. After 48 h incubation, cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (n = 5). Briefly, MTT (final concentration = 2 mg mL⁻¹) was added into wells and incubated for about 4 h at 37 °C. Then the medium was carefully replaced with DMSO (150 μL) to dissolve formazan crystals. After incubation at 37 °C for 30 min, absorbance at 490 nm was read by a microplate reader (Bio-Rad 680, Winooski, VT). The final relative cell viability (%) was calculated by the formula ([Abs]_{sample} - [Abs]_{blank}) / ([Abs]_{control} - [Abs]_{blank}) × 100%. All tests were repeated in three independent trials.

Results and Discussion

Table 1. Experimental Conditions and Characteristics of the Eight-Arm Star-Polymers from Sucrose.

entry	PB	[M] ₀ /[OH] ₀ /[PB] ₀ ^a	<i>M</i> _{n,theor} ^b (kg mol ⁻¹)	<i>M</i> _{n,NMR} ^c (kg mol ⁻¹)	<i>M</i> _{n,SEC} ^d (kg mol ⁻¹)	<i>D</i> _M ^d	IC ₅₀ ^e (mg mL ⁻¹)
SCEO1	<i>t</i> -BuP ₄	11.4/1/0.01	0.5×8	0.5×8	2.4	1.08	0.005
SCEO2	<i>t</i> -BuP ₄	56/1/0.05	2.5×8	2.5×8	10.4	1.03	0.005
SCEO3	<i>t</i> -BuP ₄	114/1/0.08	5.0×8	5.0×8	32.3	1.14	0.006
SCEO4	<i>t</i> -BuP ₂	56/1/0.08	2.5×8	2.0×8	9.7	1.04	>0.056
SCEO5	<i>t</i> -BuP ₂	56/1/0.08	2.5×8	2.2×8	12.5	1.06	n.d. ^f
SCEO5CL ^g	<i>t</i> -BuP ₂	55/1/0.08	5.9×8	6.1×8	41.3	1.11	n.d.
SCEO6 ^h	<i>t</i> -BuP ₄	56/1/0.05	2.5×8	2.8×8	15.2	1.07	0.003

^a Feed ratio of monomer, hydroxyl and phosphazene base. ^b Theoretical number-average molar mass calculated from feed and monomer conversion. ^c Number-average molar mass calculated from ¹H NMR spectra of the isolated product using integrals of the characteristic signals. ^d Number-average molar mass and molar mass distribution obtained from SEC analysis (THF, 35 °C, PEO standards). ^e Concentration of phosphazenium salt that inhibits cell growth by 50% obtained from the MTT assay using MDA-MB-231 cells. ^f Not determined. ^g Conversion of CL reached 54% after 5 days at 60 °C. ^h

The reaction of SCEO6 was quenched by addition of hydrochloric acid, while others were quenched by acetic acid.

Although sucrose dissolves well in dimethyl sulfoxide (DMSO), the highly probable chain transfer reaction^{5, 6} and the inconvenient handling have restrained us from using this solvent. Instead, tetrahydrofuran (THF) has been used in this study which is easier to reach dryness via classic purification procedures for anionic polymerization.¹⁶ Sucrose is insoluble in the reaction media (a mixture of THF and EO) initially. However, complete dissolution is observed soon after the reaction mixture is heated at 60 °C, indicating the growth of PEO from the hydroxyls which helps gaining the solubility.

Figure 1. Schematic illustration (upper) for the synthesis of eight-arm star-PEOs via PB-catalyzed ROP using sucrose as initiator, SEC traces (lower and left; RI signal), and ¹H NMR spectra (lower and right; in DMSO-*d*₆) of a representative star-PEO synthesized via the catalysis of *t*-BuP₄ (SCEO2 in Table 1; 56 EO units per hydroxyl) before and after purification performed

by stirring with cation-exchange resin or dialysis against water (SCEO2-E and SCEO2-D, respectively).

The strongest commercially available PB, *t*-BuP₄, is first used as the catalyst in the amount of <0.1 mol% of EO and <10 mol% of hydroxyls (Table 1). The feed ratio of EO and sucrose is varied so that eight-arm star-PEOs with different arm lengths can be achieved (SCEO1, 2, 3 in Table 1 with 11, 56, or 114 EO units per hydroxyl, respectively). After heating for 3 days, ¹H NMR analysis of the reaction solutions indicates complete consumption of EO, and size exclusion chromatography (SEC) presents unimodal and narrow molar mass distributions ($D_M < 1.15$). ¹H NMR spectra (in DMSO-*d*₆) of the isolated products exhibit all the characteristic signals of hydroxyl-terminated PEOs with the absence of signals from sucrose –OH (Figure 1 and S1) even for the star-PEO with the lowest molar mass. The number-average molar masses calculated by comparison of the signal integrals of hydroxy protons (–CH₂OH) and methylene protons (–OCH₂CH₂O–) agree well with the theoretical values (Table 1). Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of the low-molar-mass product further confirms the achievement of targeted macromolecular characteristics (Figure 2). It can therefore be inferred that all of the hydroxyls have participated in the reaction with EO despite the insolubility of sucrose in the initial stage and the low catalyst loading.

Figure 2. MALDI-TOF mass spectrum of a low-molar-mass eight-arm star-PEO (SCEO1 in Table 1) synthesized via ROP of EO initiated by sucrose and catalyzed by *t*-BuP₄. The sample was purified by stirring with cation-exchange resin in water (SCEO1-E).

It was previously demonstrated that *t*-BuP₂ (Figure 1), with a lower basicity, could also trigger ROP of EO.¹⁷ In this study, *t*-BuP₂-catalyzed ROP of EO from sucrose has succeeded under similar reaction conditions (Figure 1; SCEO4 and 5 in Table 1). Growth of a second block from each PEO arm upon addition of ϵ -caprolactone to the solution containing star-PEO and *t*-BuP₂ can also be accomplished allowing one-pot facile synthesis of well-defined eight-arm polyether-polyester star-diblock copolymer using a single organocatalyst (Figure 3; SCEO5 and SCEO5CL in Table 1).^{17, 18} Note that the stability of sucrose cores towards further modification and application of the star-like (co)polymers may need to be examined in each specific case.

Figure 3. Left: SEC traces of the eight-arm star-PEO precursor and the PEO-*b*-PCL star-diblock copolymer synthesized by one-pot sequential ROP of EO and CL from sucrose using *t*-BuP₂ as a single catalyst (SCEO5 and SCEO5CL in Table 1). Right: ^1H NMR spectrum (in $\text{DMSO}-d_6$) of the isolated PEO-*b*-PCL star-diblock copolymer.

In the ^1H NMR spectra of the star-PEO crude product, the existence of catalyst residue is clearly shown (Figure 1) because phosphazanium acetate salt is insoluble in non-polar *n*-hexane and precipitates together with the polymer. Comparison of the signal integrals of $-\text{N}(\text{CH}_3)_2$ and $-\text{OCH}_2\text{CH}_2\text{O}-$ indicates that the weight fraction of phosphazanium salt agrees well with the feed. Purification of the crude product by stirring with cation-exchange resin or by dialysis against water for 3 days removes the phosphazanium salt completely without affecting the molar mass or D_M (Figure 1 and S1). MTT assay *in vitro* is then performed using two human cancer cell lines MDA-MB-231 and A2780 to assess the cytotoxicity. As shown in Figure 4 and S2, none of the purified star-PEOs, regardless of molar mass, has shown noticeable toxicity towards MDA-MB-231 cells through the fact that the cell viability remains steady around 100% as the polymer concentration raises to 2 mg mL^{-1} (even up to 10 mg mL^{-1}). On the other hand, the crude

products are clearly cytotoxic as indicated by the gradual decrease of cell viability with increased polymer concentration. It is therefore evident that the cytotoxicity originates from the phosphazanium salts.

Figure 4. Cell viability in the presence of star-PEO products before or after removal of the phosphazanium acetate salts as a function of polymer concentration from MTT assay using MDA-MB-231 cells.

In the cases of star-PEOs with [t-BuP₄H]⁺Ac⁻, cell viability touches zero when polymer concentration reaches 2 mg mL⁻¹. While with [t-BuP₂H]⁺Ac⁻, cell viability remains >60% at the same concentration, indicating that *t*-BuP₄ salt is more cytotoxic than *t*-BuP₂ salt. To gain a more quantitative insight, concentrations of polymers inhibiting cell growth by 50% have been calculated by the simulation of log[crude product] vs. normalized response-Variable slope, which are further converted to concentrations of phosphazanium salts using the weight fractions calculated from ¹H NMR spectra (IC₅₀ in Table 1). Note that the weight fraction of [t-BuP₂H]⁺Ac⁻ is designed to be approximately the same as that of [t-BuP₄H]⁺Ac⁻ in star-PEO products (Figure 1 and Table 1).

Figure 5. Cell viability in the presence of sucrose-initiated eight-arm star-PEOs before or after removal of the phosphazanium acetate salts as a function of polymer concentration from MTT assay using A2780 cells.

The results of MTT assay using A2780 cells have shown similar trends (Figure 5), except that IC_{50} for both $[t\text{-BuP}_4\text{H}]^+\text{Ac}^-$ and $[t\text{-BuP}_2\text{H}]^+\text{Ac}^-$ salts are lower by almost one order of magnitude. Considering that phosphazanium acetates may be substantially basic and nucleophilic on the anionic moiety,¹⁹ we quenched one of the reactions with more acidic hydrochloric acid (Figure 1) so that catalyst residue could become less basic phosphazanium chloride (SCEO6 in Table 1). Nevertheless, the cytotoxicity of the crude star-PEO product is not noticeably alleviated as shown by MTT assay using MDA-MB-231 cells (Figure 6). The calculated IC_{50} of $[t\text{-BuP}_4\text{H}]^+\text{Cl}^-$ is close to $[t\text{-BuP}_4\text{H}]^+\text{Ac}^-$ (Table 1) indicating the cytotoxicity originates from the phosphazanium cation and is practically independent of the anionic species.

Figure 6. Cell viability in the presence of sucrose-initiated eight-arm star-PEO (SCEO6 in Table 1), quenched by hydrochloric acid, before removal of the phosphazanium chloride salt as a function of polymer concentration from MTT assay using MDA-MB-231 cells.

Conclusions

In conclusion, eight-arm star-PEOs can be synthesized in a facile and controlled manner via metal-free anionic ROP of EO in THF using PBs as catalysts and sucrose as an octahydroxy initiator. MTT assay shows that, while the purified star-PEOs are harmless, the crude products are considerably cytotoxic because of the existence of catalyst residues regardless of quenching agent used, and the cytotoxicity is considerably higher with more bulky phosphazanium cation. Therefore, removal of residual phosphazanium salts, or employing non-toxic organocatalysts to substitutes PBs, is crucial for the metal-free synthesis of PEOs and other polymers to be used in bio-related applications.

ASSOCIATED CONTENT

Supporting Information. Additional NMR spectra, SEC traces, and cell viability vs. polymer concentration plots. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Revealing the Cytotoxicity of Residues of Phosphazene Catalysts Used for Synthesis of Poly(ethylene oxide)

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and Guangzhao Zhang

TOC graphics

85x30mm (300 x 300 DPI)

Figure 1

80x95mm (300 x 300 DPI)

Figure 2

80x62mm (300 x 300 DPI)

Figure 3

160x76mm (300 x 300 DPI)

Figure 4

160x60mm (300 x 300 DPI)

Figure 5

80x58mm (300 x 300 DPI)

Figure 6
80x60mm (300 x 300 DPI)