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Application of Monodisperse PEGs in Pharmaceutics: Monodisperse Polidocanols

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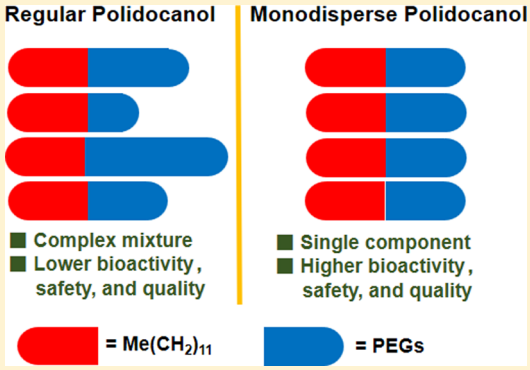
S *Supporting Information*

ABSTRACT: Polydisperse PEGs are ubiquitously used in pharmaceutical industry and biomedical research. However, the monodispersity in PEGs may play a role in the development of safe and effective PEGylated small molecular drugs. Here, to avoid the polydispersity in polidocanol, the active ingredient in a clinically used drug, a macrocyclic sulfate-based strategy for the efficient and scalable synthesis of monodisperse polidocanols, their sulfates, and their methylated derivatives, was developed. TLC and HPLC analysis indicated a complex mixture in regular polidocanol and high purities in monodisperse polidocanols and their derivatives. Assay on HUVEC, L929, and HePG2 cells showed that monodisperse polidocanols have much higher cytotoxicity and safety than that of regular polidocanol. It was found that the monodispersity of PEGs in polidocanols is crucial for achieving the optimal therapeutic results. Therefore, based on this case study, it would be beneficial to optimize PEGylated small molecular drugs with monodisperse PEGs in pharmaceutical research and development.



KEYWORDS: *monodisperse, polydisperse, polyethylene glycols (PEGs), polidocanol, PEGylated drugs*

INTRODUCTION

Polyethylene glycols (PEGs) are the most used polymer in pharmaceutical industry and biomedical research to improve solubility and stability, reduce dosing frequency and immunogenicity, and prolong blood circulation.[1−4] The so-called "stealth behaviors" of PEGs are the "golden standards" for biomedical polymers.[3] Until 2015, there are 17 PEGylated drugs approved by US FDA.

Although the importance of enantiomer purity in chiral drugs has been well recognized by pharmaceutical industry since the thalidomide tragedy, little attention has been paid to the monodispersity of PEGs used in either approved drugs or those in the pipeline. Unfortunately, regular PEGs even with an excellent polydispersity index (PDI) are still complex mixtures of oligomers.[5] There are two main reasons that led to the dilemma: (1) Replacing regular PEGs with monodisperse PEGs will dramatically increase the cost due to the high price and limited availability of monodisperse PEGs. (2) The polydispersity of PEGs is always supposed to play a minor role in the efficacy and safety of drugs. To address the first issue, many novel synthetic strategies have recently been developed for synthesizing monodisperse PEGs with high efficacy and low cost.[6−14] However, as far as we know, there is no research on whether the polydispersity of regular PEGs can compromise the efficacy and safety of PEGylated drugs or not.

Asclera is an FDA approved drug which has been widely used to sclerosis small spider veins and reticular veins in the lower extremities. Its active ingredient, polidocanol, is an ether mixture of hydrophobic dodecyl alcohol and hydrophilic polydisperse polyethylene glycols with an average molecular weight of 400 Da (Scheme 1). As a polymer prepared from dodecyl alcohol and ethylene oxide, regular polidocanol contains many analogs with n = 9 as the major component. The inherent heterogeneity in regular polidocanol may not only complicate its purification, characterization, quality control, and clinic application, but also compromise its therapeutic efficacy and safety. Therefore, it is necessary to identify the most effective and safe component in regular polidocanol from which monodisperse polidocanol, the next generation Asclera, may be developed. Herein, a macrocyclic sulfate-based strategy for efficient synthesis of monodisperse polidocanols, their sulfates, and methylated derivatives was developed for a comparative

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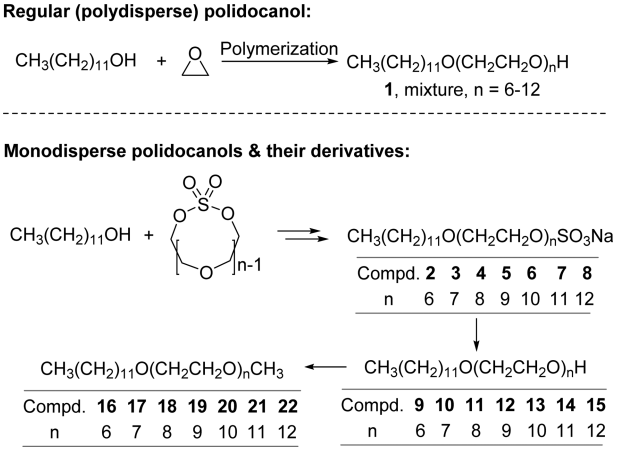
Scheme 1. Synthetic Strategies for Regular Polidocanol 1, Monodisperse Polidocanols 9-15, and Their Derivatives 2-8 and 16-22



study on the polydisperse and monodisperse polidocanols (Scheme 1). This study is intended to address the issue if it is necessary to replace regular polydisperse PEGs with monodisperse PEGs in clinically used small molecular drug polidocanol.

MATERIALS AND METHODS

General Information. Unless otherwise indicated, all reagents were obtained from commercial supplier and used without prior purification. THF and CH_2Cl_2 were dried and freshly distilled prior to use. Regular polidocanol was purchased from Sigma-Adrich. Flash chromatography was performed on silica gel (200−300 mesh) with either EtOAc/petroleum ether (PE, 60−90 °C) or MeOH/CH_2Cl_2 as eluents. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer. Chemical shifts are in ppm and coupling constants (J) are in Hertz (Hz). ^{1}H NMR spectra were referenced to tetramethylsilane (d, 0.00 ppm) using $CDCl_3$ as solvent. ^{13}C NMR spectra were referenced to solvent carbons (77.16 ppm for $CDCl_3$). The splitting patterns for ^{1}H NMR spectra are denoted as follows: s (singlet), d (doublet), q (quartet), m (multiplet). ESI Mass spectra were recorded on a Thermo Scientific Q Exactive Focus mass spectrometer.

Synthesis of Monodisperse Polidocanols and Their Derivatives. *Macrocyclic Sulfate **25**.* To a stirring solution of triethylene glycol **23** (27.1 g, 180.3 mmol), triethylamine (87.6 g, 865.4 mmol), and DMAP (1.1 g, 9.0 mmol) in CH_2Cl_2 (3.0 L) at 0 °C was slowly added a solution of $SOCl_2$ (53.7 g, 360.6 mmol, in 50 mL CH_2Cl_2). After the addition, the stirring mixture was warmed to 25 °C and monitored with TLC until the complete consumption of triethylene glycol. The reaction was quenched with 1.5 L water. The organic layer was collected, filtrated through a pad of silica gel, and concentrated under vacuum to provide the macrocyclic sulfite intermediate as brownish oil which was used directly in the next step. To the macrocyclic sulfite in a mixture of CH_2Cl_2 (150 mL), CH_3CN (150 mL), and water (225 mL) at 0 °C was added $NaIO_4$ (57.9 g, 270.5 mmol) and $RuCl_3{\cdot}3H_2O$ (0.23 g, 0.9 mmol). The stirring mixture was gradually warmed to 25 °C and monitored with TLC. Upon complete consumption of the macrocyclic sulfite, the reaction mixture was filtered through a pad of Celite. Organic layer was collected, washed with brine, concentrated under vacuum, and recrystallized in methanol at −15 °C to give the macrocyclic sulfate **25** as clear crystal (16.1 g, 42% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.69 (s, 4H), 3.84−3.95 (m, 4H), 4.43−4.52 (m, 4H).

*Macrocyclic Sulfate **26**.* **26** was prepared from tetraethylene glycol **24** by following the same procedure for macrocyclic sulfate **25** as white solid (26.6 g, 50% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.62−3.78 (m, 8H), 3.80−3.95 (m, 4H), 4.36−4.64 (m, 4H).

*Alcohol **27**.* Under an atmosphere of nitrogen, dodecyl alcohol (3.7 g, 20.0 mmol) was added to a suspension of NaH (0.96 g, 60% in mineral oil, 24.0 mmol) in anhydrous THF (50 mL) and the mixture was stirred for additional 30 min at 0 °C. Then, a solution of macrocyclic sulfate **25** (6.4 g, 30.0 mmol) in anhydrous THF (50 mL) was added and the reaction was monitored with TLC until no dodecyl alcohol could be detected. The reaction was quenched with water (2.0 mL), acidified with concentrated sulfuric acid to pH 2, and refluxed for 2 h. After cooled to room temperature, the reaction mixture was neutralized with saturated $NaHCO_3$ and extracted with CH_2Cl_2 (100 mL, 3 times). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum. The residue was purified by flash column chromatography on silica gel (petroleum ether/ethyl acetate = 1/1 as eluent) to give alcohol **27** as yellowish oil (5.3 g, 83% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.23−1.36 (m, 18H), 1.49−1.75 (m, 2H), 3.46 (t, J = 8.0 Hz, 2H), 3.56−3.78 (m, 12H); HRMS (ESI) calcd for $C_{18}H_{38}NaO_4^+$ [(M+Na)$^+$] 341.2662, found 341.2659.

*Alcohol **28**.* **28** was prepared from dodecyl alcohol and macrocyclic sulfate **26** by following the same procedure for alcohol **27** as clear oil (10.8 g, 86% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (t, J = 8.0 Hz, 3H), 1.14−1.42 (m, 18H), 1.46−1.70 (m, 2H), 3.34 (s, 1H), 3.43 (t, J = 8.0 Hz, 2H), 3.53−3.78 (m, 16H); HRMS (ESI) calcd for $C_{20}H_{42}NaO_5^+$ [(M+Na)$^+$] 385.2924, found 385.2917.

*Monodisperse Polidocanol **9**.* **9** was prepared from alcohol **27** and macrocyclic sulfate **25** by following the same procedure for polidocanol **27** as clear oil (5.4 g, 85% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.18−1.37 (m, 18H), 1.52−1.62 (m, 2H), 3.45 (t, J = 8.0 Hz, 2H), 3.53−3.78 (m, 24H); HRMS (ESI) calcd for $C_{24}H_{54}NO_7^+$ [(M+NH$_4$)$^+$] 468.3895, found 468.3935.

*Monodisperse Polidocanol **10**.* **10** was prepared from alcohol **28** and macrocyclic sulfate **25** by following the same procedure for polidocanol **27** as yellowish oil (6.0 g, 88% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.22−1.36 (m, 18H), 1.54−1.63 (m, 2H), 3.45 (t, J = 8.0 Hz, 2H), 3.56−3.75 (m, 28H); HRMS (ESI) calcd for $C_{26}H_{58}NO_8^+$ [(M+NH$_4$)$^+$] 512.4157, found 512.4228.

*Monodisperse Polidocanol **11**.* **11** was prepared from alcohol **28** and macrocyclic sulfate **26** by following the same procedure for polidocanol **27** as yellowish oil (5.7 g, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.22−1.35 (m, 18H), 1.54−1.62 (m, 2H), 3.44 (t, J = 8.0 Hz, 2H), 3.56−3.74 (m, 32H); HRMS (ESI) calcd for $C_{28}H_{62}NO_9^+$ [(M+NH$_4$)$^+$] 556.4419, found 556.4503.

*Monodisperse Polidocanol **12**.* **12** was prepared from alcohol **9** and macrocyclic sulfate **25** by following the same procedure for polidocanol **27** as yellowish oil (5.4 g, 77% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.18−1.34 (m, 18H), 1.51−1.64 (m, 2H), 3.45 (t, J = 8.0 Hz, 2H), 3.55−3.76 (m, 36H); HRMS (ESI) calcd for $C_{30}H_{66}NO_{10}^+$ [(M+NH$_4$)$^+$] 600.4681, found 600.4776.

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Monodisperse Polidocanol ***13***. **13** was prepared from alcohol **10** and macrocyclic sulfate **25** by following the same procedure for polidocanol **27** as yellowish oil (1.7 g, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.21−1.37 (m, 18H), 1.51−1.64 (m, 2H), 3.44 (t, J = 8.0 Hz, 2H), 3.57−3.75 (m, 40H); HRMS (ESI) calcd for $C_{32}H_{70}NO_{11}^+$ [$(M+NH_4)^+$] 644.4943, found 644.4973.

Monodisperse Polidocanol ***14***. **14** was prepared from alcohol **10** and macrocyclic sulfate **26** by following the same procedure for polidocanol **27** as yellowish solid (4.0 g, 48% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.21−1.38 (m, 18H), 1.52−1.64 (m, 2H), 3.45 (t, J = 8.0 Hz, 2H), 3.56−3.75 (m, 44H); HRMS (ESI) calcd for $C_{34}H_{74}NO_{12}^+$ [$(M+NH_4)^+$] 688.5206, found 688.5254.

Monodisperse Polidocanol ***15***. **15** was prepared from alcohol **12** and macrocyclic sulfate **25** by following the same procedure for polidocanol **27** yellowish solid (1.8 g, 59% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.22−1.35 (m, 18H), 1.53−1.64 (m, 2H), 3.45 (t, J = 8.0 Hz, 2H), 3.55−3.76 (m, 48H); HRMS (ESI) calcd for $C_{36}H_{78}NO_{13}^+$ [$(M+NH_4)^+$] 732.5468, found 732.5497.

Monodisperse Polidocanol Sulfate ***2***. Under an atmosphere of nitrogen, to a suspension of NaH (0.8 g, 60% in mineral oil, 20.0 mmol) in anhydrous THF (50 mL) at 0 °C was slowly added a solution of alcohol **27** (5.3 g, 16.7 mmol) in anhydrous THF (50 mL). After stirring for 30 min at 0 °C, a solution of macrocyclic sulfate **25** (5.3 g, 25.1 mmol) in anhydrous THF (50 mL) was added and the reaction was monitored until no alcohol **27** could be detected by TLC. Then, the reaction was quenched with water (2.0 mL), concentrated under vacuum, purified with flash column chromatography on silica gel (dichloromethane/methanol = 10/1 as eluent) to give monodisperse polidocanol sulfate **2** as yellowish wax (6.0 g, 65% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.20−1.38 (m, 18H), 1.53−1.63 (m, 2H), 3.44 (t, J = 8.0 Hz, 2H), 3.52−3.80(m, 22H), 4.13−4.21 (m, 2H); HRMS (ESI) calcd for $C_{24}H_{49}Na_2O_{10}S^+$ [$(M+Na)^+$] 575.2836, found 575.2841.

Monodisperse Polidocanol Sulfate ***3***. **3** was prepared from alcohol **28** and macrocyclic sulfate **25** by following the same procedure for monodisperse polidocanol sulfate **2** as yellowish wax (8.9 g, 86% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.19−1.39 (m, 18H), 1.52−1.64 (m, 2H), 3.44 (t, J = 8.0 Hz, 2H), 3.55−3.82 (m, 26H), 4.16−4.24 (m, 2H); HRMS (ESI) calcd for $C_{26}H_{53}Na_2O_{11}S^+$ [$(M+Na)^+$] 619.3098, found 619.2982.

Monodisperse Polidocanol Sulfate ***4***. **4** was prepared from alcohol **28** and macrocyclic sulfate **26** by following the same procedure for polidocanol sulfate **2** as yellowish wax (4.1 g, 52% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.19−1.38 (m, 18H), 1.51−1.63 (m, 2H), 3.45 (t, J = 8.0 Hz, 2H), 3.55−3.82 (m, 30H), 4.18−4.28 (m, 2H); HRMS (ESI) calcd for $C_{28}H_{57}Na_2O_{12}S^+$ [$(M+Na)^+$] 663.3361, found 663.3378.

Monodisperse Polidocanol Sulfate ***5***. **5** was prepared from alcohol **9** and macrocyclic sulfate **25** by following the same procedure for polidocanol sulfate **2** as yellowish wax (8.7 g, 78% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, J = 8.0 Hz, 3H), 1.13−1.42 (m, 18H), 1.48−1.70 (m, 2H), 3.45 (t, J = 8.0 Hz, 2H), 3.49−3.88 (m, 34H), 4.09−4.23 (m, 2H); HRMS (ESI) calcd for $C_{30}H_{61}Na_2O_{13}S^+$ [$(M+Na)^+$] 707.3623, found 707.3607.

Monodisperse Polidocanol Sulfate ***6***. **6** was prepared from alcohol **10** and macrocyclic sulfate **25** by following the same procedure for polidocanol sulfate **2** as yellowish wax (9.7 g, 82% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81 (t, J = 8.0 Hz, 3H), 1.11−1.32 (m, 18H), 1.43−1.59 (m, 2H), 3.37 (t, J = 8.0 Hz, 2H), 3.45−3.76 (m, 38H), 4.08−4.21 (m, 2H); HRMS (ESI) calcd for $C_{32}H_{65}Na_2O_{14}S^+$ [$(M+Na)^+$] 751.3885, found 751.4060.

Monodisperse Polidocanol Sulfate ***7***. **7** was prepared from alcohol **10** and macrocyclic sulfate **26** by following the same procedure for polidocanol sulfate **2** as yellowish wax (5.6 g, 59% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.20−1.38 (m, 18H), 1.50−1.65 (m, 2H), 3.44 (t, J = 8.0 Hz, 2H), 3.53−3.81 (m, 42H), 4.14−4.29 (m, 2H); HRMS (ESI) calcd for $C_{34}H_{69}Na_2O_{15}S^+$ [$(M+Na)^+$] 795.4147, found 795.4167.

Monodisperse Polidocanol Sulfate ***8***. **8** was prepared from alcohol **11** and macrocyclic sulfate **26** by following the same procedure for polidocanol sulfate **2** as yellowish wax (13.7 g, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.22−1.33 (m, 18H), 1.52−1.63 (m, 2H), 3.44 (t, J = 8.0 Hz, 2H), 3.55−3.79 (m, 46H), 4.18−4.27 (m, 2H); HRMS (ESI) calcd for $C_{36}H_{73}Na_2O_{16}S^+$ [$(M+Na)^+$] 839.4409, found 839.4357.

Methylated Monodisperse Polidocanol ***16***. Under an atmosphere of nitrogen, to a suspension of NaH (88 mg, 60% in mineral oil, 2.2 mmol) in anhydrous THF (50 mL) at 0 °C was slowly added a solution of alcohol **9** (501 mg, 1.1 mmol) in anhydrous THF (50 mL). After stirring for 30 min at 0 °C, a solution of CH_3I (310 mg, 2.2 mmol) in anhydrous THF (50 mL) was added and the reaction was monitored until no alcohol **9** could be detected by TLC. Then, the reaction was quenched with water (2.0 mL), concentrated under vacuum, purified with flash column chromatography on silica gel (dichloromethane/methanol = 10/1 as eluents) to give methylated monodisperse polidocanol **16** as yellowish wax (450 mg, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.22−1.43 (m, 18H), 1.51−1.64 (m, 2H), 3.39 (s, 3H), 3.45 (t, J = 8.0 Hz, 2H), 3.53−3.76 (m, 24H); HRMS (ESI) calcd for $C_{25}H_{52}NaO_7^+$ [$(M+Na)^+$] 487.3605, found 487.3594.

Methylated Monodisperse Polidocanol ***17***. **17** was prepared from alcohol **10** and CH_3I by following the same procedure for methylated monodisperse polidocanol **16** as yellowish wax (1.0 g, 94% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 8.0 Hz, 3H), 1.19−1.39 (m, 18H), 1.52−1.64 (m, 2H), 3.38 (s, 3H), 3.45 (t, J = 8.0 Hz, 2H), 3.52−3.75 (m, 28H); HRMS (ESI) calcd for $C_{27}H_{56}NaO_8^+$ [$(M+Na)^+$] 531.3867, found 531.3857.

Methylated Monodisperse Polidocanol ***18***. **18** was prepared from alcohol **11** and CH_3I by following the same procedure for methylated monodisperse polidocanol **16** as yellowish wax (371 mg, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, J = 8.0 Hz, 3H), 1.22−1.39 (m, 18H), 1.53−1.65 (m, 2H), 3.40 (s, 3H), 3.46 (t, J = 8.0 Hz, 2H), 3.49−3.82 (m, 32H); HRMS (ESI) calcd for $C_{29}H_{60}NaO_9^+$ [$(M+Na)^+$] 575.4130, found 575.4114.

Methylated Monodisperse Polidocanol ***19***. **19** was prepared from alcohol **12** and CH_3I by following the same procedure for methylated monodisperse polidocanol **16** as yellow wax (891 mg, 94% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (t, J = 8.0 Hz, 3H), 1.15−1.34 (m, 18H), 1.47−1.65 (m, 2H), 3.34 (s, 3H), 3.41 (t, J = 8.0 Hz, 2H), 3.47−3.73 (m,

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36H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 22.6, 26.1, 29.3, 29.5, 29.58, 29.63, 31.9, 59.0, 70.0, 70.4, 70.5, 70.6, 71.5, 71.9; HRMS (ESI) calcd for $C_{31}H_{64}NaO_{10}^+$ [(M+Na)$^+$] 619.4392, found 619.4371.

*Methylated Monodisperse Polidocanol **20***. **20** was prepared from alcohol **13** and CH_3I by following the same procedure for methylated monodisperse polidocanol **16** as yellow wax (900 mg, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, J = 8.0 Hz, 3H), 1.09−1.47 (m, 18H), 1.52−1.69 (m, 2H), 3.40 (s, 3H), 3.46 (t, J = 8.0 Hz, 2H), 3.54−3.71 (m, 40H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 22.7, 26.1, 29.4, 29.5, 29.6, 29.7, 31.9, 59.0, 70.0, 70.5, 70.6, 71.6, 71.9; HRMS (ESI) calcd for $C_{33}H_{68}NaO_{11}^+$ [(M+Na)$^+$] 663.4654, found 663.4636.

*Methylated Monodisperse Polidocanol **21***. **21** was prepared from alcohol **14** and CH_3I by following the same procedure for methylated monodisperse polidocanol **16** as yellow solid (1.3 g, 82% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, J = 8.0 Hz, 3H), 1.22−1.35 (m, 18H), 1.53−1.64 (m, 2H), 3.40 (s, 3H), 3.46 (t, J = 8.0 Hz, 2H), 3.54−3.76 (m, 44H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 22.6, 26.0, 29.3, 29.6, 29.55, 29.58, 29.6, 31.9, 59.0, 70.0, 70.46, 70.52, 71.5, 71.9; HRMS (ESI) calcd for $C_{35}H_{72}NaO_{12}^+$ [(M+Na)$^+$] 707.4916, found 707.4895.

*Methylated Monodisperse Polidocanol **22***. **22** was prepared from alcohol **15** and CH_3I by following the same procedure for methylated monodisperse polidocanol **16** as yellow solid (1.5 g, 63% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, J = 8.0 Hz, 3H), 1.19−1.41 (m, 18H), 1.51−1.69 (m, 2H), 3.39 (s, 3H),3.45 (t, J = 8.0 Hz, 2H), 3.53−3.75 (m, 48H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 22.6, 26.1, 29.3, 29.4, 29.55, 29.58, 29.6, 31.9, 59.0, 70.0, 70.47, 70.53, 71.5, 71.9; HRMS (ESI) calcd for $C_{37}H_{76}NaO_{13}^+$ [(M+Na)$^+$] 751.5178, found 751.5154.

Purity Analysis by HPLC-ELSD. Polydisperse polidocanol **1**, monodisperse polidocanols **9**−**15**, and their derivatives **16**−**22** were dissolved in water at a concentration of about 5 mg/mL. Three mixtures of monodisperse polidocanols and their derivatives solutions were also prepared, respectively. After filtration, the solutions were analyzed by HPLC-ELSD using 0.1% (V/V) trifluoroacetic acid in water (mobile phase A) and 0.1% (V/V) trifluoroacetic acid in acetonitrile (mobile phase B) under the following gradient elution: from 0 to 20 min a linear increase from 0 to 60% of B, and from 21 to 80 min a linear increase from 60% to 100% of B (for polydisperse polidocanol **1** and monodisperse polidocanol **9**−**15**); from 0 to 10 min a linear increase from 0 to 80% of B, from 11 to 35 min a linear increase from 80% to 100% of B, and from 36 to 40 min an isocratic step at 100% of B (for methylated monodisperse polidocanol **16**−**22**). The flow rate was set at 0.5 mL/min and the gas flow rate was set at 2.9 L/min. The temperature of the evaporation chamber was 109 °C.

CMC Measurement.[15,16] To a 10 mL brown bottle was added a solution of pyrene (0.10 mL, 6.08 mg/L in acetone) and acetone was allowed to volatize in dark. Then, an aqueous solution of the polidocanol or its derivative (10 mL with accurate concentration) was added to the bottle which was covered with aluminum foil to avoid light. The bottle was first put in an ultrasound bath for 30 min, then in a water bath at 60 °C for 40 min and 40 °C for 12 h. After it was cooled to room temperature, pyrene fluorescent intensities at 373 and 384 nm were measured at 25 °C and the intensity ratio of I_{373}/I_{384} was calculated. Then, the intensity ratios of I_{373}/I_{384} at a series of polidocanols or their derivatives concentrations were measured with the above method. Finally, the pyrene intensity ratios of I_{373}/I_{384} were plot against concentrations to calculate the CMC.

Procedures for Biological Assay. HUVEC cells were selected as the representative cells. The cells were cultured in a cell incubator under 5% CO_2 at 37 °C with DMEM containing 10% fetal bovine serum and 1% streptomycin double antibody as the medium. Test compounds were diluted with PBS buffer, pH 7.4 (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was employed to evaluate the cell viability. HUVEC cells were harvested in logarithmic growth phase and digested with trypsin to prepare cell suspension with a concentration of about 3×10^4 cells/mL. Cell suspension (100 μL) was added to each well of 96-well plates and incubated in a 5% CO_2 cell incubator at 37 °C. After 24 h, polidocanol solution of different concentrations was added and diluted with DMEM medium to the final concentration. Each concentration is set to six identical wells and blank control wells were also set. The cells were incubated for 24 h. Then 20 μL MTT was added to each well and the cells were incubated for another 4 h. Afterward, the culture medium was discarded and the cells were dissolved by 200 μL DMSO and absorbance was read at 570 and 490 nm by a microplate reader (Thermo, USA). Cell viability (%) was calculated by the formula:

$$\text{Cell Viability}(\%) = [(A_{\text{Test}} - A_{\text{Blank}})/(A_{\text{Control}} - A_{\text{Blank}})] \times 100\%$$

A_{Test}, A_{Control}, and A_{Blank} represented the absorbance of cells with different treatments, untreated cells, and blank culture media, respectively. The IC_{50} was calculated by Origin software. The cytotoxicity assay on L929 cells and HePG2 cells employed the same procedure as the HUVEC cells. DMEM and MEM were used as the mediums for HePG2 and L929 cells, respectively.

RESULTS

The synthesis of each monodisperse component in regular polidocanol is based on a macrocyclic sulfate strategy developed in this group (Scheme 2).[4] Besides monodisperse polidocanols, the corresponding sulfates are also highly valuable because sodium tetradecyl sulfate (STS) has been clinically used as a substitute for Asclera since 1940s. Interestingly, this macrocyclic sulfate strategy can conveniently provide these sulfates by nucleophilic ring opening of the macrocyclic sulfate.

Macrocyclization of triethylene glycol **23** and tetraethylene glycol **24** with thionyl chloride followed by oxidation with in situ generated RuO_4 gave macrocyclic sulfates **25** and **26**, respectively. Nucleophilic ring opening of macrocyclic sulfates **25** and **26** with dodecyl alcohol followed by hydrolysis in the presence of sulfuric acid provided alcohols **27** and **28**, respectively. From alcohols **27** and **28**, iterative nucleophilic ring opening reaction on macrocyclic sulfates **25** and **26** followed by acidic hydrolysis of sulfate intermediates provided all the designed monodisperse polidocanols **9**−**15** on multigram scale, respectively. The corresponding monodisperse polidocanol sulfates **2**−**8** were directly prepared by the nucleophilic ring opening reaction of macrocyclic sulfates **25** and **26** without hydrolysis. Then their methylated derivatives **16**−**22** were synthesized by methylation of monodisperse polidocanols **9**−**15** with iodomethane (Scheme 2).

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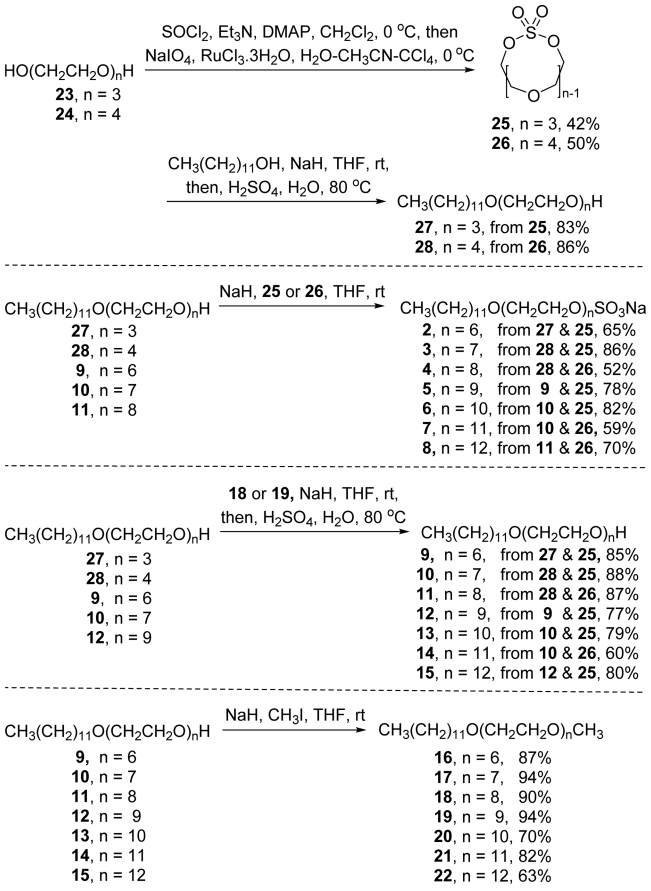
Scheme 2. Synthesis of Monodisperse Polidocanols 9−15, Their Sulfates 2−8, and Methylated Derivatives 16−22



It is noteworthy that the synthesis is efficient and convenient. On one hand, no protecting or activating group was used in this synthesis which dramatically shorten the synthetic route. On the other hand, by simple combination of these building blocks, all the designed monodisperse polidocanol analog can be conveniently synthesized. Finally, it is also interesting to point out that these monodisperse polidocanol sulfates are very difficult to prepare through other means.

Then a purity analysis of monodisperse polidocanols **9−15** (Figure S1) and their methylated derivatives **16−22** (Figure S2) together with regular polidocanol **1** was carried out on HPLC. As no UV absorption of these compounds on HPLC, evaporative light-scattering detector (ELSD) was employed. A complex mixture on HPLC can be found from regular polidocanol **1** which clearly show its polydispersity. Over 17 components were observed in regular polidocanol and no complete separation was obtained on HPLC after many tries. In contrast, only one component can be find from each monodisperse polidocanol **9−15** and its methylated derivative **16−22**, respectively. In addition, a side-by-side comparison of regular and monodisperse polidocanols on TLC conveniently showed the obvious difference in purity (Figure S3).

With these polidocanols **9-15**, their sulfates **2-8**, and methylated derivatives **16−22** in hand, a physicochemical investigation was then carried out to reveal the relationship between their chemical structure and micelle formation property which may play a role during polidocanol's clinic application. Through a pyrene-based fluorescent method,[5] it was found that polidocanol sulfates **2−8** have about 4 times higher critical micelle concentration (CMC) than regular polidocanol **1** as a result of the sulfates' higher hydrophilicity (Figure 1 and Table S1). Monodisperse polidocanols **9−15** and

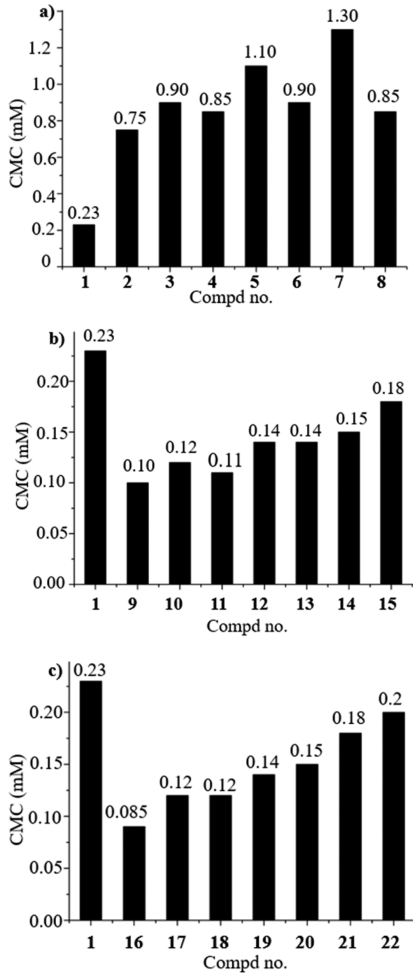


Figure 1. CMC of monodisperse polidocanols **9−15** (b), their sulfates **2−8** (a), and their methylated derivatives **16−22** (c).

their methylated derivatives **16−22** have lower CMC than polydisperse polidocanol **1**. It was also found that, with the increasing of ethylene glycol units in monodisperse polidocanols **9−15** and their methylated derivatives **16−22**, their CMCs also gradually increased. The noticeable CMC difference between monodisperse polidocanols and their methylated derivatives may result in different biological behaviors.

Then, cytotoxicity of monodisperse polidocanols **9−15**, their sulfates, and methylated derivatives **16−22** together with regular polidocanol **1** was studied with MTT cytotoxicity assay on a panel of selected cells, including human umbilical vein cells (HUVEC cells), fibroblast-like cells (L929 cells), and liver hepatocellular carcinoma cells (HePG2 cells) (Figures 2, S4, S5, and S6 and Table S2). HUVEC cells are the target cells in clinic treatment of sclerose small spider veins and reticular veins with Asclera. L929 cells and HePG2 cells were chosen as normal cells for safety assessment and cancer cells for anticancer efficacy assessment of these compounds, respectively. First, the sulfates of polidocanols has pretty low cytotoxicity toward the selected cell lines. Second, the cytotoxicity assay on HUVEC cells indicated that monodisperse polidocanols **9−15** exhibit higher cytotoxicity than regular

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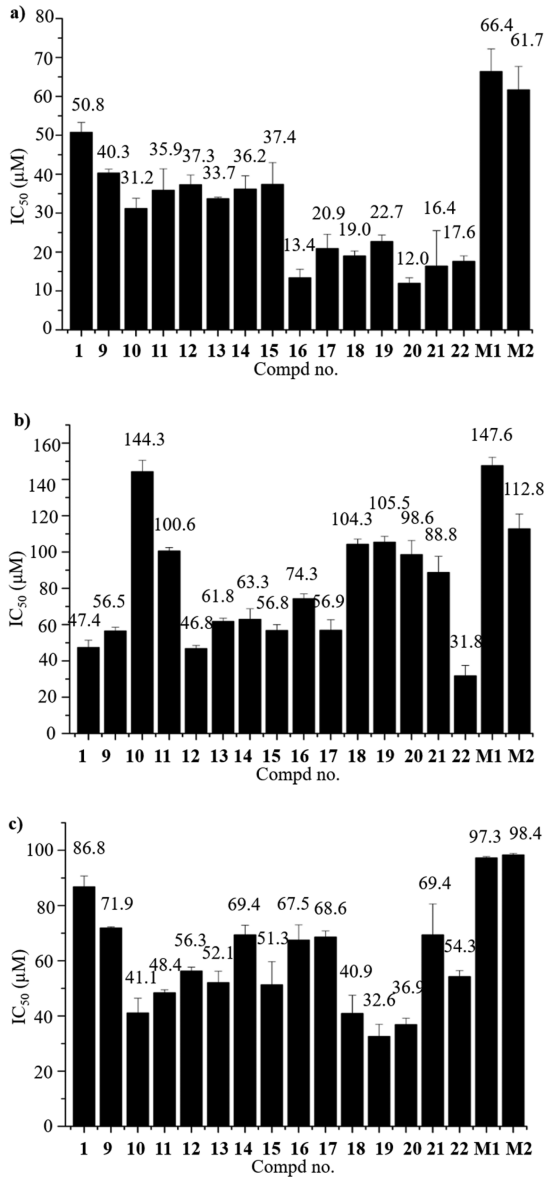

Figure 2. IC_{50} of regular polidocanol **1**, monodisperse polidocanols **9**–**15**, their methylated derivatives **16**–**22**, and mixtures with equal amount of **9**–**15** (M1) and **16**–**22** (M2) on HUVEC cells (a), L929 cells (b), and HePG2 cells (c).

polidocanol **1**. Although compound **12** with a nonaethylene glycol moiety is claimed as the major component in Asclera, its cytotoxicity toward HUVEC cells is actually not the most potent among monodisperse polidocanols **9**–**15**. It is noteworthy that methylation of the hydroxyl group in monodisperse polidocanols **9**–**15** significantly enhance the cytotoxicity toward HUVEC cells. Among them, methylated monodisperse polidocanol **20** with an IC_{50} of 12.0 μM is 4.2 and 2.8 times more potent than regular polidocanol **1** and the corresponding monodisperse polidocanol **13**, respectively. Third, the cytotoxicity assay on L929 cells showed that monodisperse polidocanols **9**–**15** have much lower cytotoxicity than regular polidocanol **1**, which is an indication of higher safety for monodisperse polidocanols. The cytotoxicity of monodisperse polidocanol **10** with an IC_{50} of 144.3 μM is 3 times lower than that of regular polidocanol **1**. The same trend was also found for methylated monodisperse polidocanols **16**–**22**. Forthyl, the cytotoxicity assessment of these compounds on HePG2 cells indicated that monodisperse polidocanols and their methylated derivatives have much higher anticancer efficacy than regular polidocanol **1**. Finally, cytotoxicity of two mixtures with equal amount of monodisperse polidocanols **9**-**15** (M1) and their methylated derivatives **16**–**22** (M2) was investigated. Comparing to the monodisperse polidocanols **9**–**15** and their methylated derivatives **16**–**22**, mixture M1 and M2 showed lower cytotoxicity toward the selected cell lines. Therefore, the polydispersity in polidocanol indeed plays a role in the cytotoxicity. It is interesting to point out that mixture M1 and M2 showed even lower cytotoxicity toward the selected cell lines than regular polidocanol **1** which indicated that some unknown components in regular polidocanol **1**, such as impurities derived from the polymerization process, interfered with the cytotoxicity. In all the cases of monodisperse polidocanols and derivatives, it was also found that the size of polyethylene glycol moiety has a considerable influence on their bioactivity, safety, and anticancer efficacy. Based on these observations, synthesis of monodisperse polidocanols and their derivatives would be an effective way to improve the drug efficacy and safety of Asclera.

CONCLUSIONS

In this study, we have developed a macrocyclic sulfate-based strategy for the convenient and scalable synthesis of monodisperse polidocanols, their sulfates, and methylated derivatives for a comparative study of monodisperse and polydisperse polidocanols. Through physicochemical study, HPLC analysis, and biological assay, it was found that polydispersity in PEGs can downgrade the purity, bioactivity, and safety of regular polidocanol. In contrast, monodisperse polidocanols and their derivatives exhibit a single component, predictable physicochemical properties, much higher bioactivity and safety than regular polidocanol. Furthermore, the size of ethylene glycol in monodisperse polidocanols and their derivatives also influenced their cytotoxicity and safety. Although the molecular mechanism behind these phenomena is still unknown, this study shows, for the first time, that polydispersity of PEGs indeed can compromise the therapeutic efficacy and safety of polidocanol. Therefore, in the era of accurate medicine, it is necessary to pay more attention to polydispersity of PEGs in small molecular drugs to avoid the issues in drug purity, efficacy, safety, quality control, regulatory approval, and beyond.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.7b00496.

HPLC chromatograms, TLC comparison of purification, CMC of compounds, MTT cytotoxicity assays, and copies of $^{1}H/^{13}C$ NMR, MS/HRMS spectra of compounds (PDF)

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Author Contributions

[⊥]Z.Y. and S.B. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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