Supporting Information

A toolbox of biodegradable dendritic (poly glycerol sulfate)-SS-poly(ester) micelles for cancer treatment: stability, drug release, and tumor targeting

Daniel Braatz,[a] Mathias Dimde,[a] Guoxin Ma,[a] Yinan Zhong,[b] Michael Tully,[a] Carsten Grötzinger,[c,d] Yuanyuan Zhang,[b] Alexandros Mavroskoufis,[a] Michael Schirner,[a] Zhiyuan Zhong,[e] Matthias Ballauff,[a*] Rainer Haag[a*]

[a] Institut für Chemie und Biochemie, Freie Universität Berlin, Takustr. 3, 14195 Berlin, Germany

[b] Department of Pharmaceutical Engineering, China Pharmaceutical University, Nanjing, 211198, P.R. China

[c] Department of Hepatology and Gastroenterology, Charité Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany

[d] German Cancer Consortium (DKTK), partner site Berlin, 13353 Berlin, Germany

[e] Biomedical Polymers Laboratory, College of Chemistry, Chemical Engineering and Materials Science, and State Key Laboratory of Radiation Medicine and Protection, Soochow University, Suzhou 215123, P.R. China

*Corresponding Authors

Email: mballauff@zedat.fu-berlin.de; haag@zedat.fu-berlin.de

Table of Contents

03.03.2021

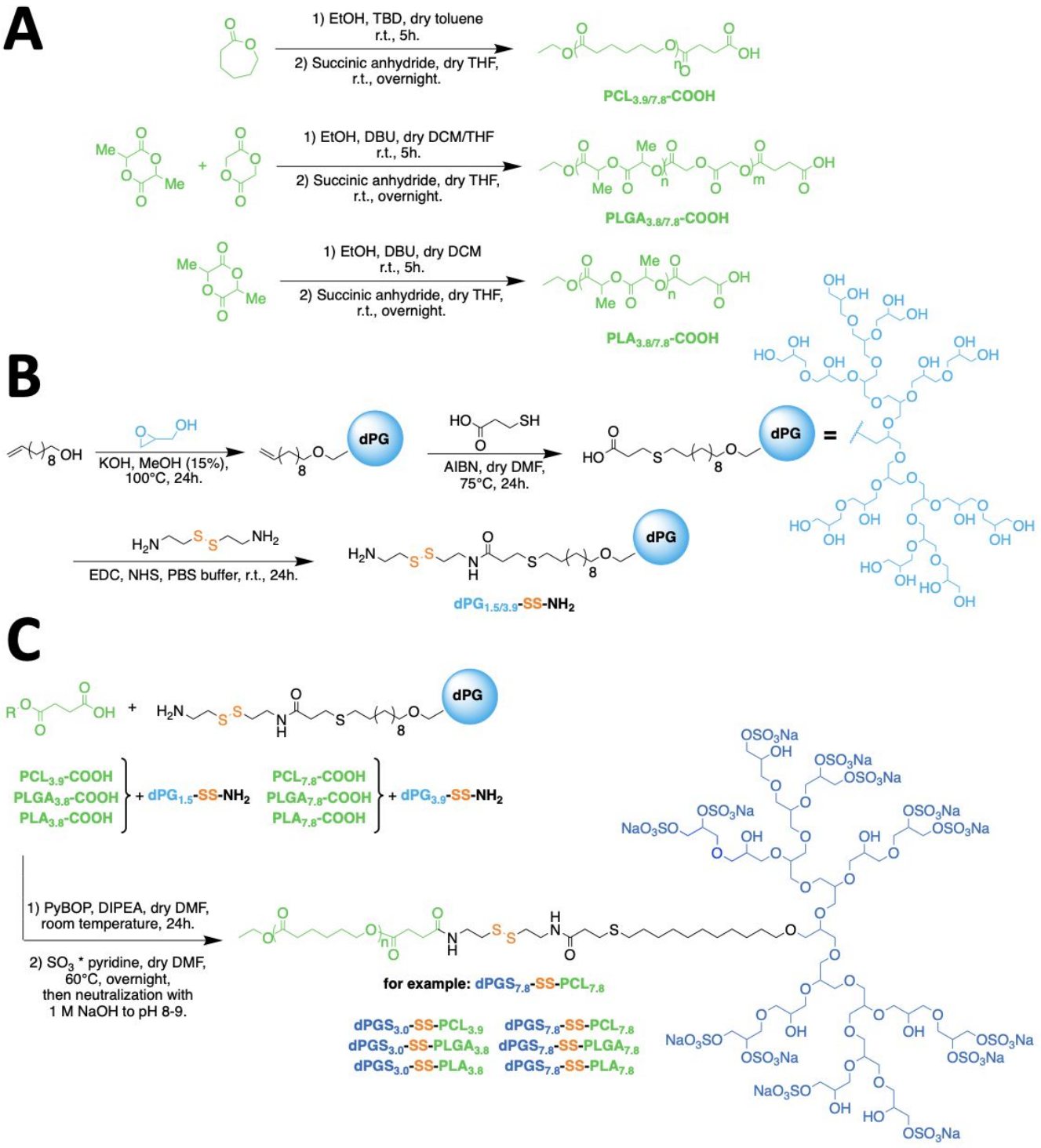
Synthesis of the hydrophobic block copolymer: PCL-COOH/PLGA-COOH/ PLA-COOH

For the hydrophobic precursors, all polymerizations were conducted using organocatalysts, either DBU or TBD, by ring-opening polymerization of caprolactone, lactide, and glycolide under strict avoidance of metal catalysts such as $Sn(oct)_2$. It has been pointed out that traces of metal-based catalysts lead to toxicity of polymer systems.[1, 2] The polymers were synthesized to obtain molecular weights of 4 and 8 kDa. The PCL-COOH was synthesized via TBD-mediated[3, 4] polymerizations of ε-caprolactone initiated by ethanol. An adapted procedure of Hedrick *et al.* was performed to synthesize PLA-COOH.[5] The synthesis of PLGA-COOH was obtained by conducting the following modified method of Hoye *et al.* under the use of DBU and sequential monomer addition of glycolide to yield a random-like distribution of lactide and glycolide.[6] The ratio of lactide to glycolide was fixed as L:G 75:25. The addition of succinic anhydride quenched the reactions and introduced the carboxylic acid functional group (**Scheme 1**). For purification, the polymers got precipitated from acetone into cold methanol three times and dried under vacuum. The obtained polymers were analyzed by ^{1}H NMR and GPC (THF) (**Table S 1**).

Synthesis of the hydrophilic block copolymer: dPG-SS-NH_2

The hydrophilic polymers dPG-SS-NH_2 were targeted to yield molecular weights of either 2 or 4 kDa. The hydrophilic segment presents as monofunctional dendritic polyglycerol, was synthesized through anionic ring-opening polymerization of glycidol initiated by 10-undecenol-1-ol. The conversion of the reaction was monitored by ^{1}H NMR. Subsequently, the allyl group was modified by thiol-ene click-reaction of mercaptopropionic acid underuse of AIBN. The *in-situ* thiol-ene reaction to monofunctional dPG-thioether-COOH occurred within 4h. After purification by precipitation in acetone and TFF dialysis against water, the polymer was analyzed by GPC (water), revealing a molecular weight of 3.9 kDa and dispersity of 1.6 and 1.5 kDa and M_w/M_n of 1.6. The degree of branching in both cases reached almost 56%.[7] Finally, to obtain amine end-capped copolymer capable of undergoing an amide coupling reaction, the dPG-thioether-COOH was reacted with Cystamine to either obtain amine end-capping as well as through implementation of Cystamine the redox-sensitive moiety present as disulfide bridge got introduced to the polymer structure. Therefore, monofunctional dPG-COOH was reacted with Cystamine underuse of EDC * HCl. After precipitation in acetone and running a TFF in water, the corresponding structure could be proven by ^{1}H NMR to obtain dPG-SS-NH_2 finally (**Table S 2**).

Synthetic scheme of PCL/PLGA/PLG-COOH, dPG-SS-NH_2, and dPGS-SS-PCL/PLGA/PLA



Scheme S 1*. synthetic pathways to* ***A)*** *hydrophobic segments of PCL/PLGA/PLA-COOH,* ***B)*** *hydrophilic segment of dPG-SS-*NH_2*, and* ***C)*** *amphiphilic block copolymers of dPGS-SS-PCL/PLGA/PLA.*

Analytical data of the hydrophobic block copolymers: PCL/PLA/PLGA-COOH

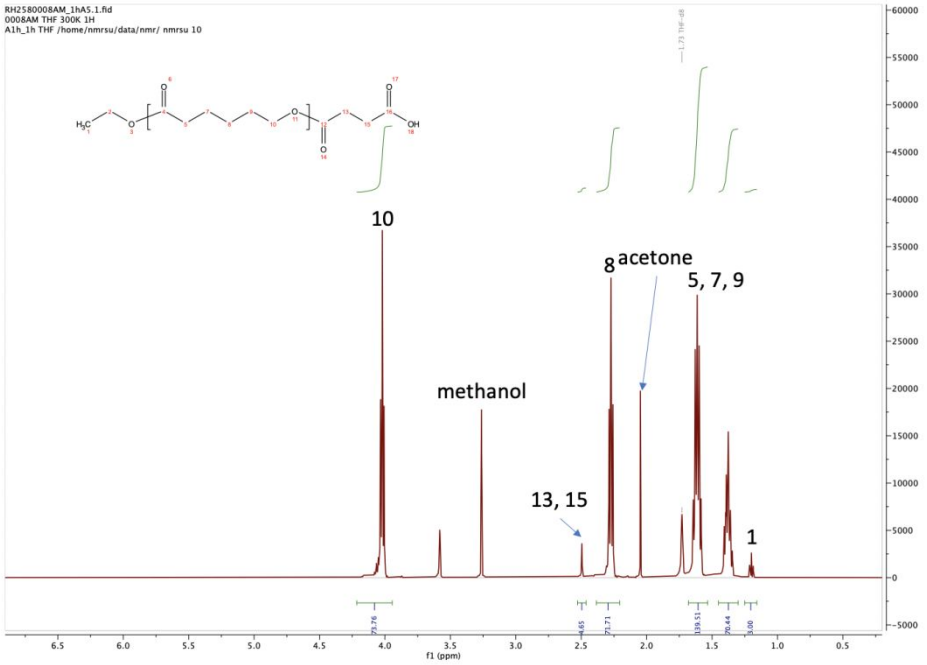


Figure S 1. *^{1}H NMR spectrum (500 MHz, THF-d_8) of $PCL_{3.9}$-COOH synthesized by ROP of caprolactone with ethanol catalyzed by TBD.*

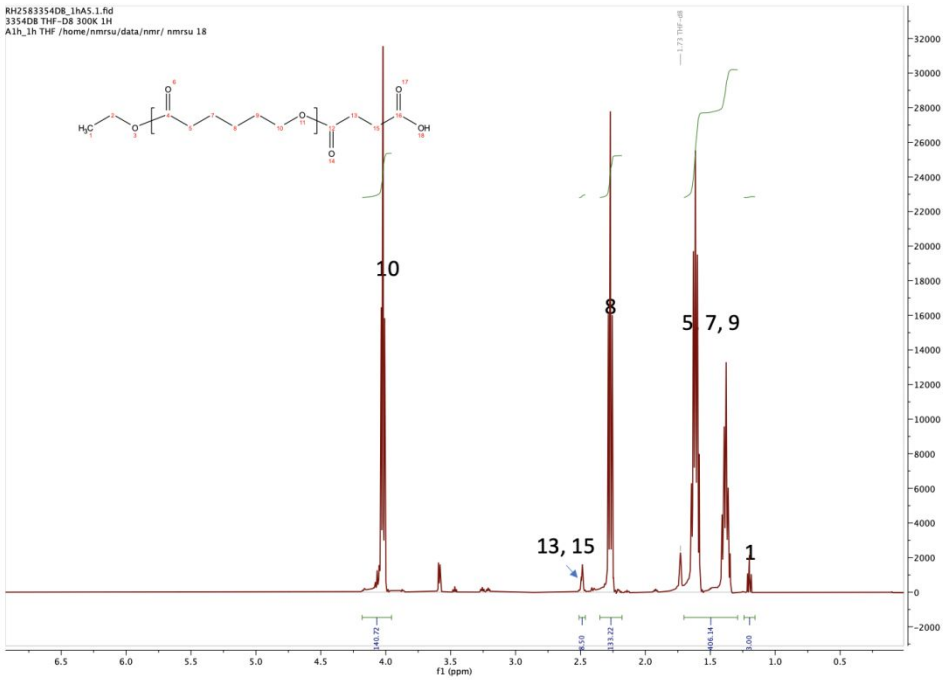


Figure S 2. *^{1}H NMR spectrum (500 MHz, THF-d_8) of $PCL_{7.8}$-COOH synthesized by ROP of caprolactone with ethanol catalyzed by TBD.*

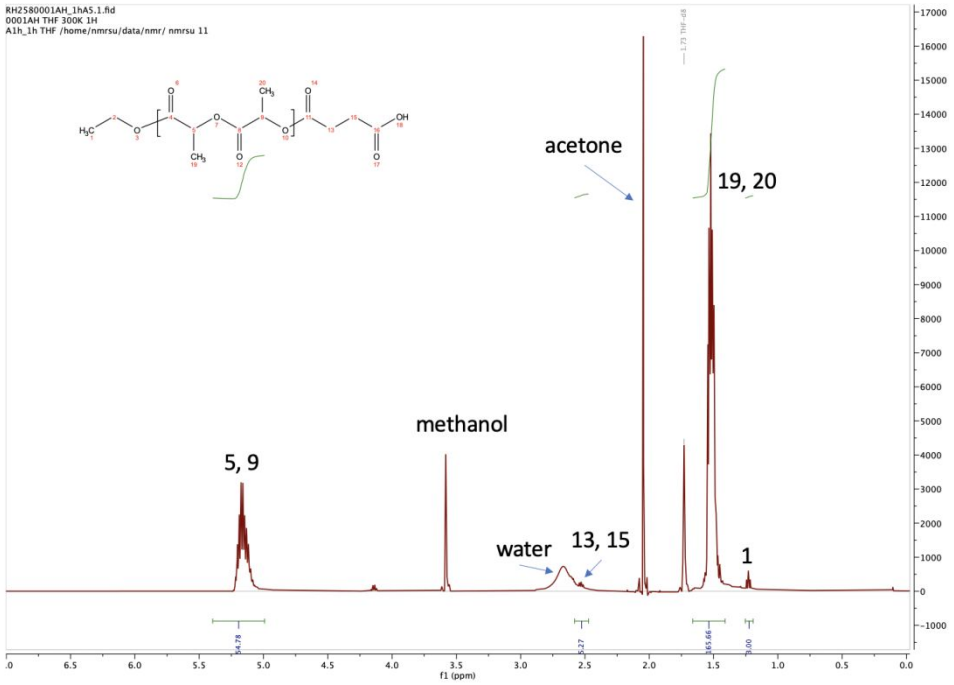


Figure S 3. *^{1}H NMR spectrum (500 MHz, THF-d_8) of $PLA_{3.8}$-COOH synthesized by ROP of lactide with ethanol catalyzed by DBU.*

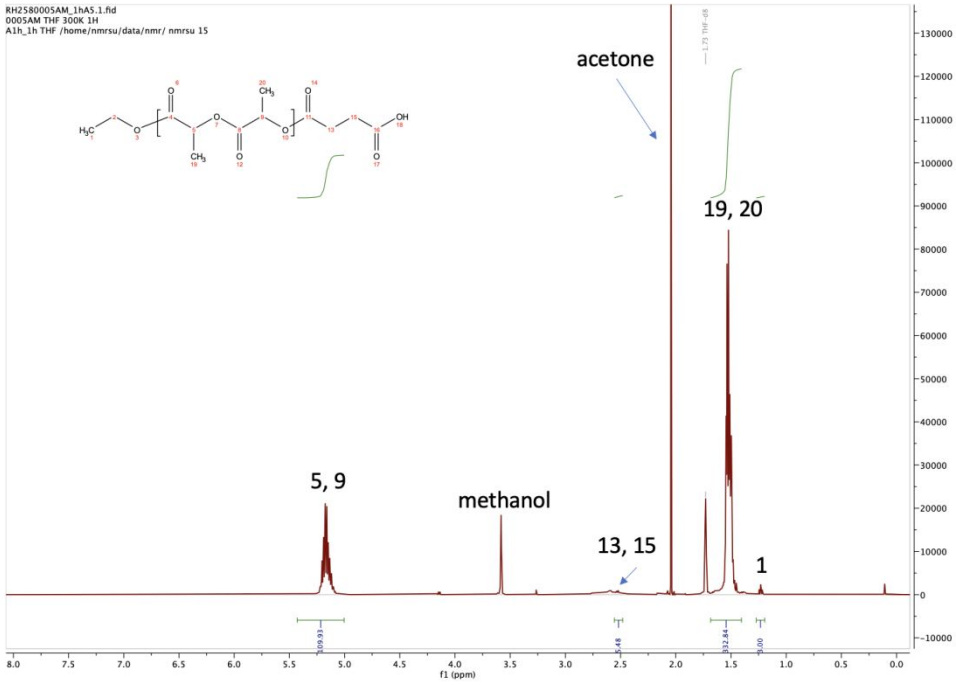


Figure S 4. *^{1}H NMR spectrum (500 MHz, THF-d_8) of $PLA_{7.8}$-COOH synthesized by ROP of lactide with ethanol catalyzed by DBU.*

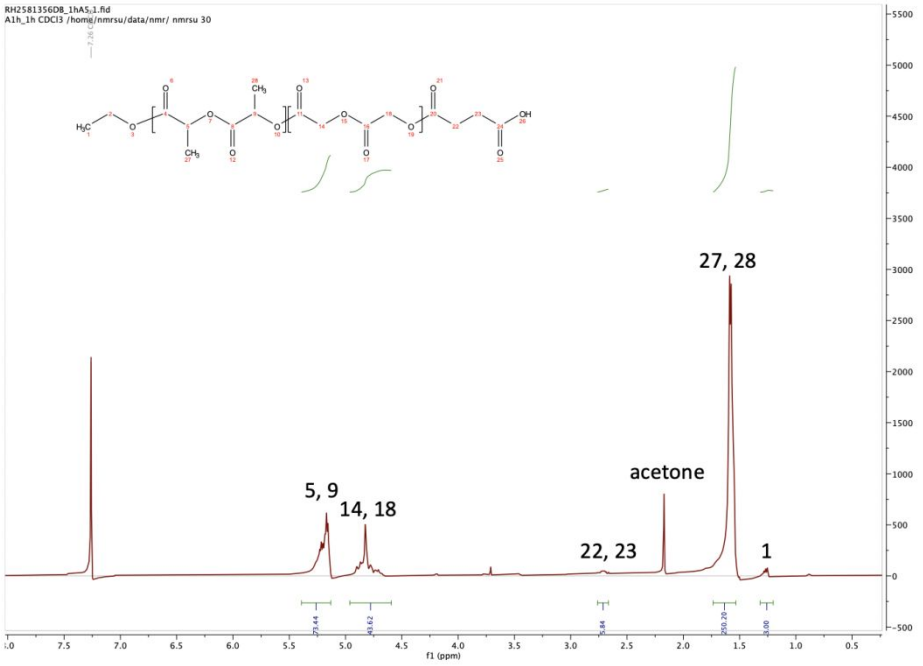


Figure S 5. *^{1}H NMR spectrum (500 MHz, $CDCl_3$) of $PLGA_{7.8}$-COOH synthesized by ROP of lactide/glycolide (75:25) with ethanol catalyzed by DBU.*

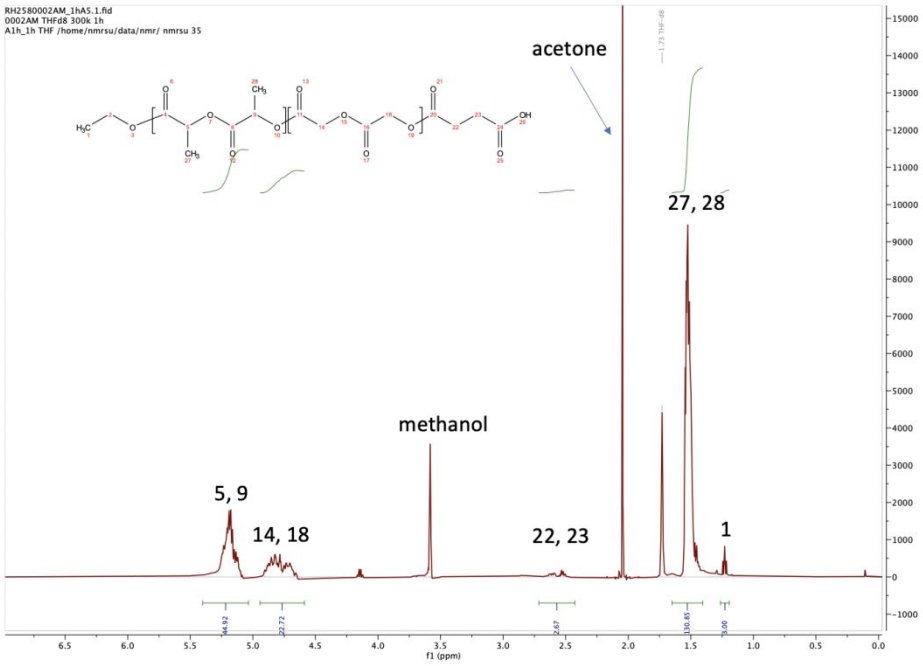


Figure S 6. *^{1}H NMR spectrum (500 MHz, THF-d_8) of $PLGA_{3.8}$-COOH synthesized by ROP of lactide/glycolide (75:25) with ethanol catalyzed by DBU.*

Table S 1. *characteristics of PCL-COOH, PLA-COOH, and PLGA-COOH copolymers (^{1}H NMR, GPC).*

	M_n (kDa)				
Compound	theoretical	^{1}H NMR	GPC (THF)	*Đ*	Conversion (%)
PCL-COOH	8.0	7.8	9.8	1.15	98.8
PCL-COOH	4.0	3.9	7.1	1.42	99.7
PLA-COOH	8.0	7.8	12.1	1.29	98.6
PLA-COOH	4.0	3.8	9	1.38	97.5
PLGA-COOH	8.0	7.8	9.6	1.43	98.6
PLGA-COOH	4.0	3.8	7.7	1.36	97.5

Analytical data of the hydrophilic block copolymer: dPG-SS-NH_2

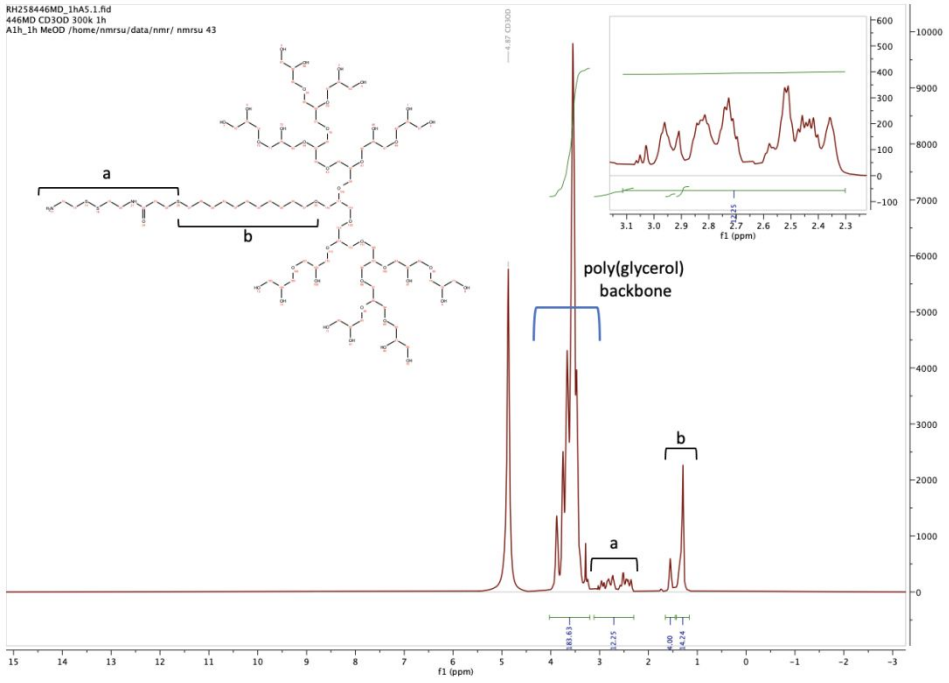


Figure S 7. *^{1}H NMR spectrum (500 MHz, CD_3OD) of $dPG_{1.5}$-SS-NH_2 synthesized by ROP of glycidol from 10-undecenol with subsequent click-reaction of mercaptopropionic acid and coupling of cystamine.*

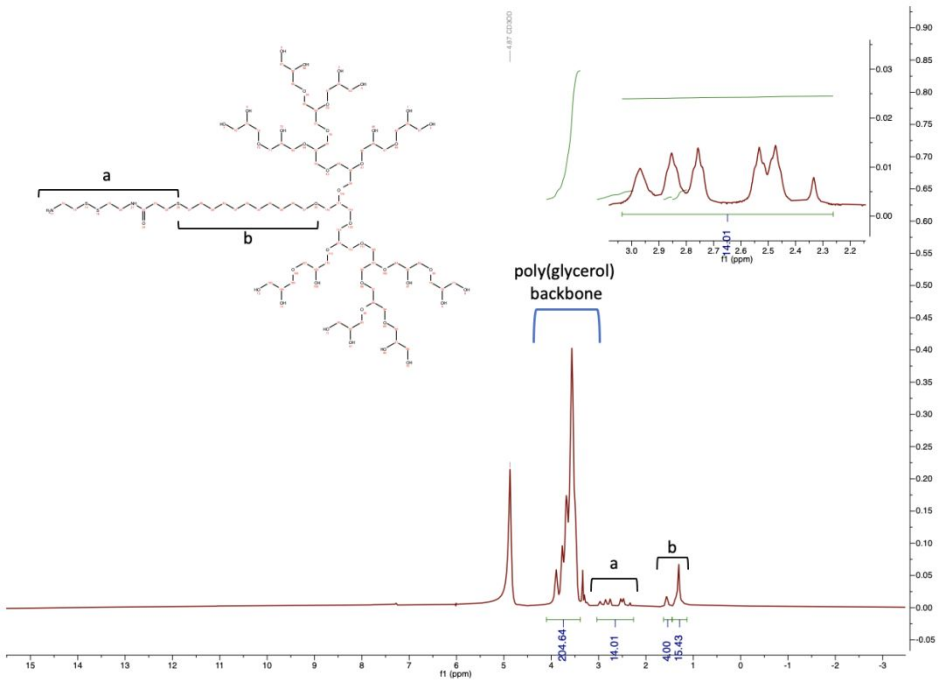


Figure S 8. *^{1}H NMR spectrum (500 MHz, CD_3OD) of $dPG_{3.9}$-SS-NH_2 synthesized by ROP of glycidol from 10-undecenol with subsequent click-reaction of mercaptopropionic acid and coupling of cystamine.*

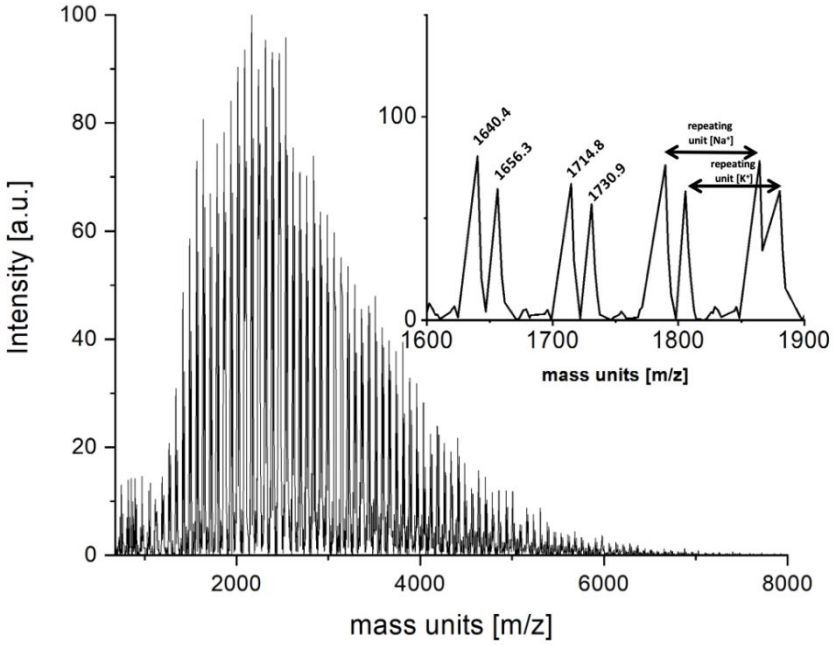


Figure S 9. *MALDI-TOF MS spectrum of the isolated polymer of $dPG_{3.9}$-COOH, the two peak panel corresponds to sodium and potassium ions, the peak difference determines the molecular weight of the respective repeating unit (glycidol).*

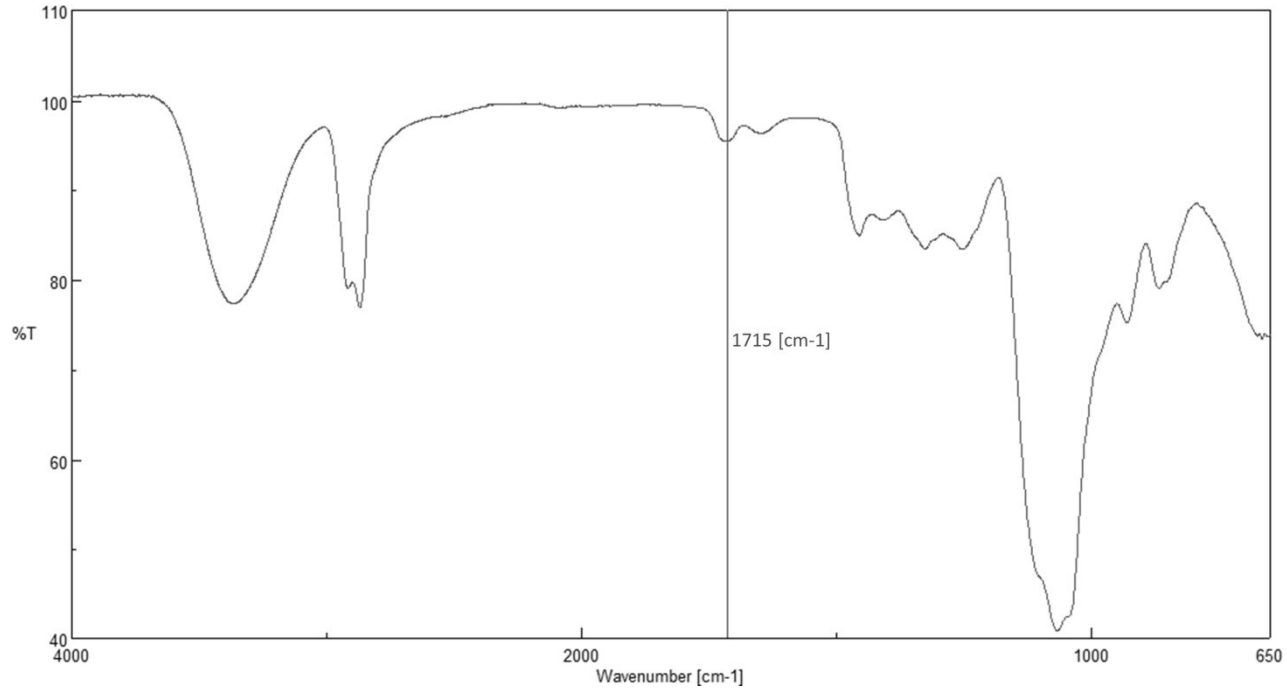


Figure S 10. *IR spectrum of $dPG_{3.9}$-COOH showing the characteristic ν_{as} (C=O) band at 1715 cm^{-1}.*

Table S 2. *characteristics of dPG-SS-NH_2 copolymers (^{1}H NMR, Degree of branching by inverse gated ^{13}C NMR, GPC).*

	M_n (kDa)				
Compound	theoretical	^{1}H NMR	GPC (H_2O)	*Đ*	Degree of Branching (%)[8]
dPG-SS-NH_2	4.0	3.4	3.9	1.63	58
dPG-SS-NH_2	2.0	1.6	1.5	1.59	56

Analytical data of the amphiphilic copolymers: dPGS-SS-PCL/PLA/PLGA

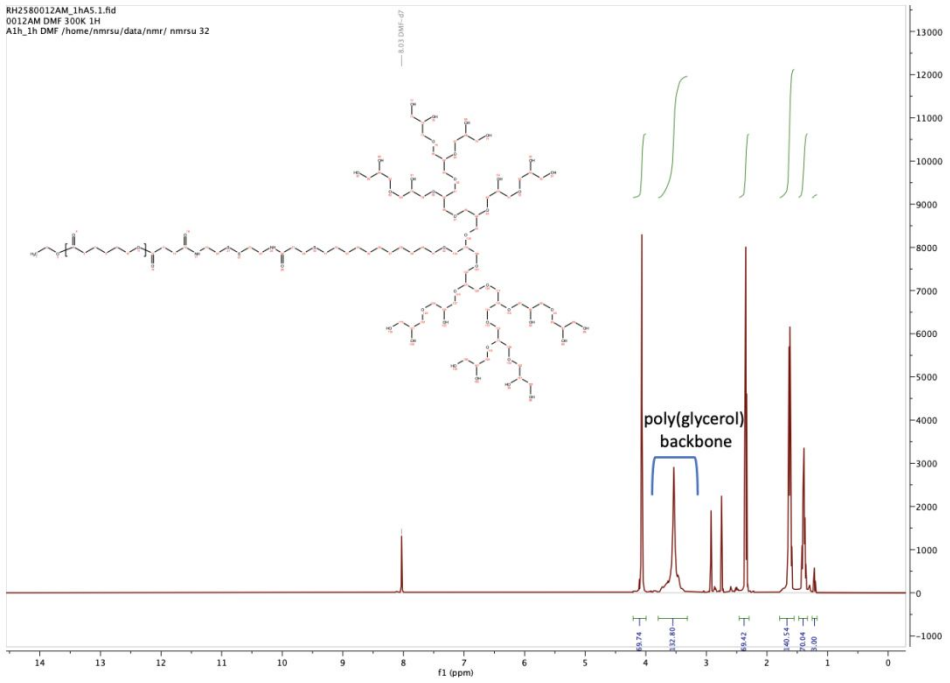


Figure S 11. *^{1}H NMR (500 MHz, DMF-d_7) of $dPG_{1.5}$-SS-$PCL_{3.9}$ synthesized by amide coupling between $dPG_{1.5}$-SS-NH_2 and $PCL_{3.9}$-COOH.*

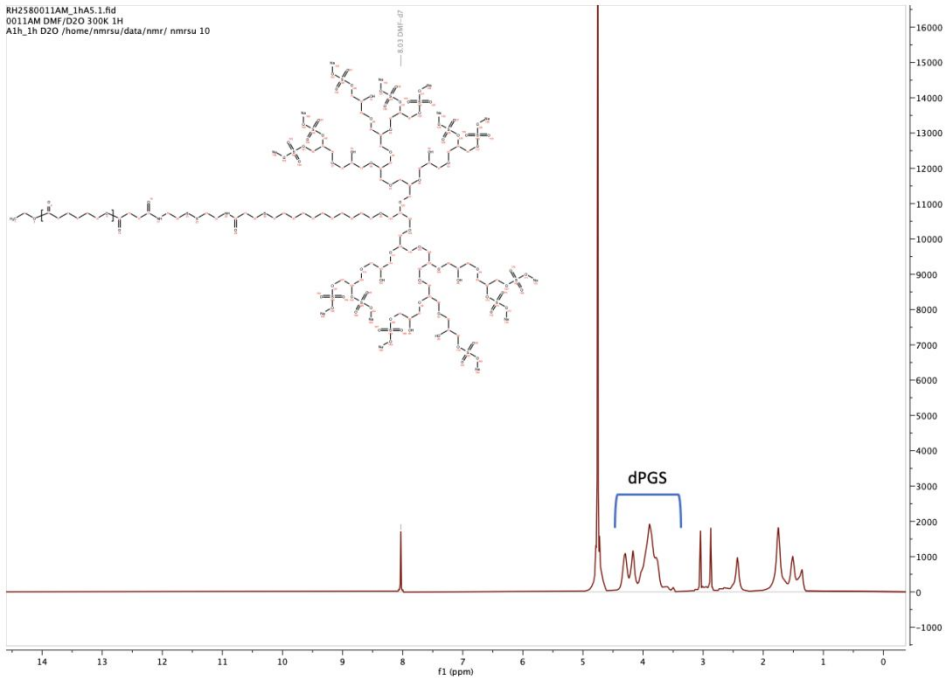


Figure S 12. *^{1}H NMR (500 MHz, DMF-d_7:D_2O 1:1) of $dPGS_{3.0}$-SS-$PCL_{3.9}$.*

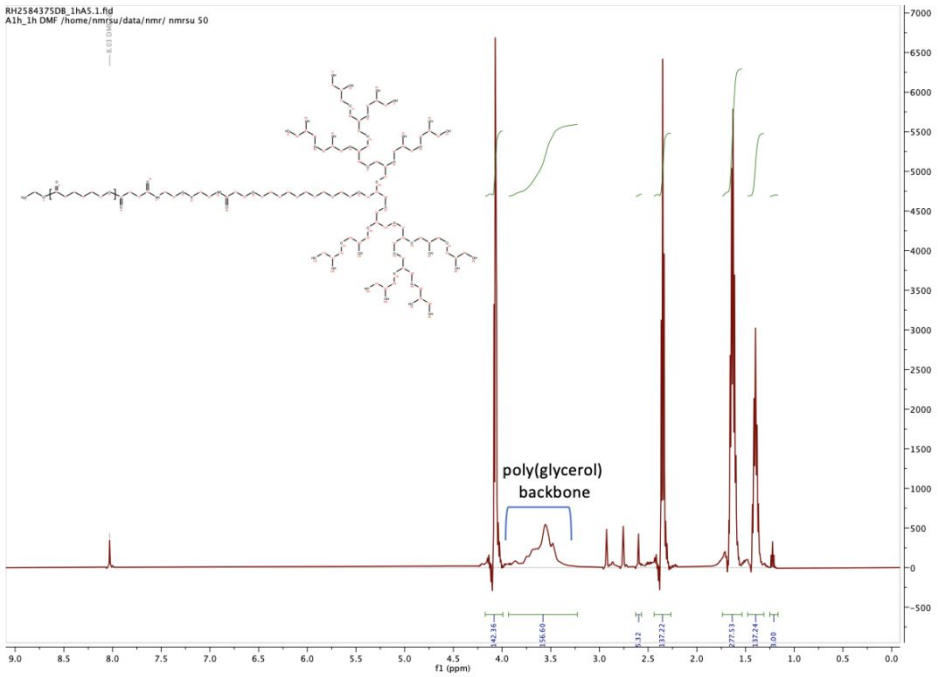


Figure S 13. *^{1}H NMR (500 MHz, DMF-d_7) of $dPG_{3.9}$-SS-$PCL_{7.8}$ synthesized by amide coupling between $dPG_{3.9}$-SS-NH_2 and $PCL_{7.8}$-COOH.*

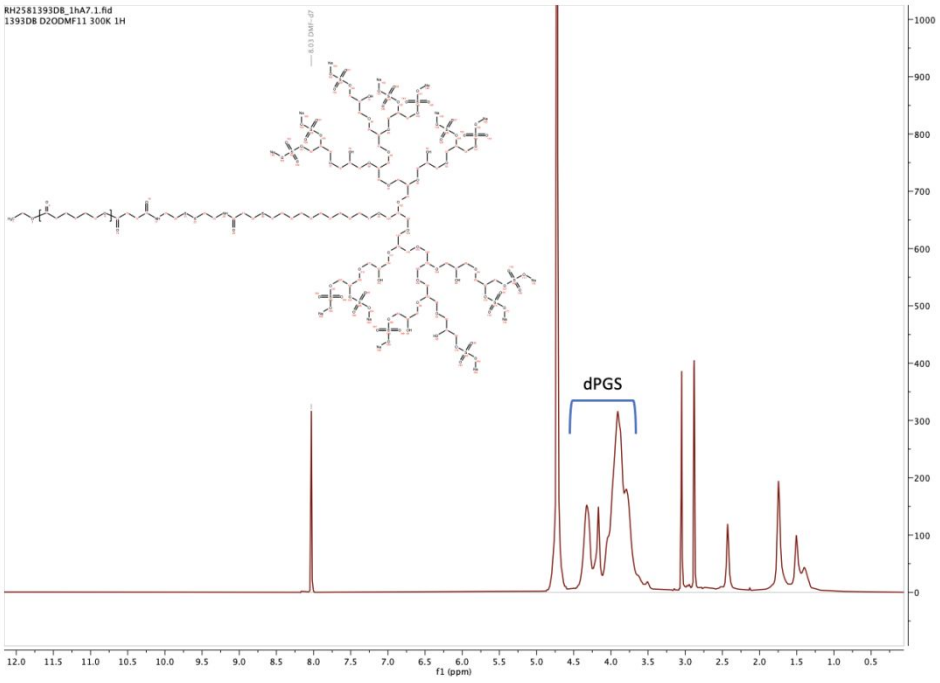
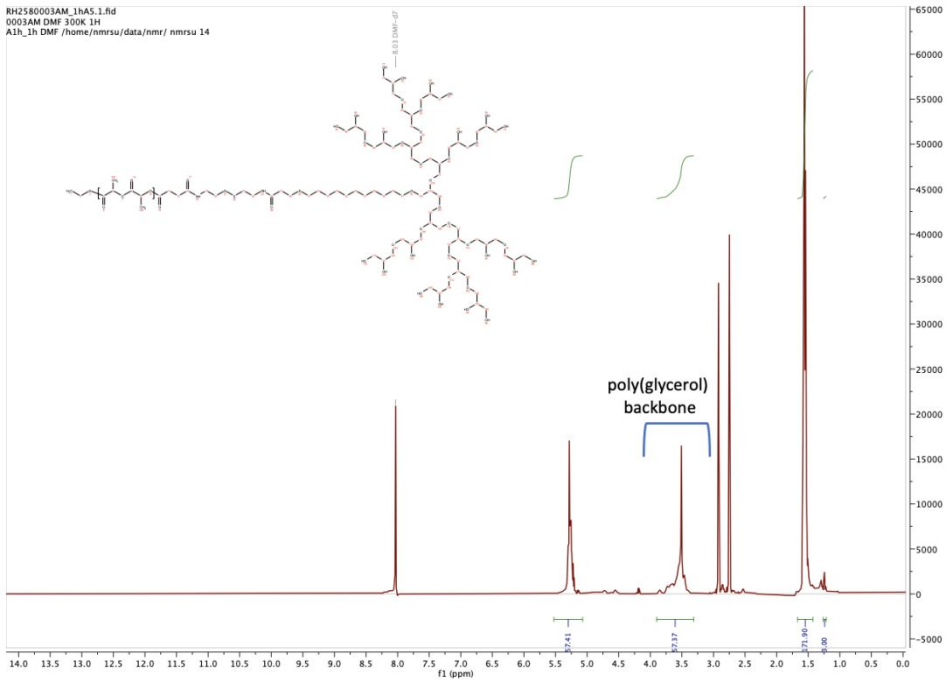
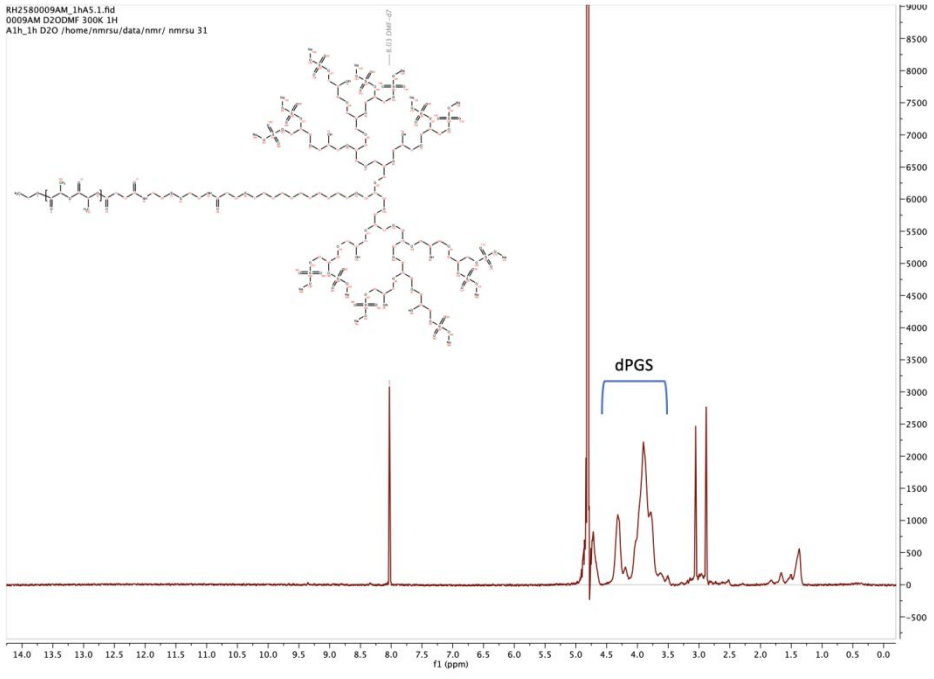


Figure S 14. *^{1}H NMR (500 MHz, DMF-d_7:D_2O 1:1) of $dPGS_{7.8}$-SS-$PCL_{7.8}$.*



134

135 ***Figure S 15.*** *^{1}H NMR (500 MHz, DMF-d_7) of $dPG_{1.5}$-SS-$PLA_{3.8}$ synthesized by amide*
136 *coupling between $dPG_{1.5}$-SS-NH_2 and $PLA_{3.8}$-COOH.*



137

138 ***Figure S 16***. *^{1}H NMR (500 MHz, DMF-d_7:D_2O 1:1) of $dPGS_{3.0}$-SS-$PLA_{3.8}$.*

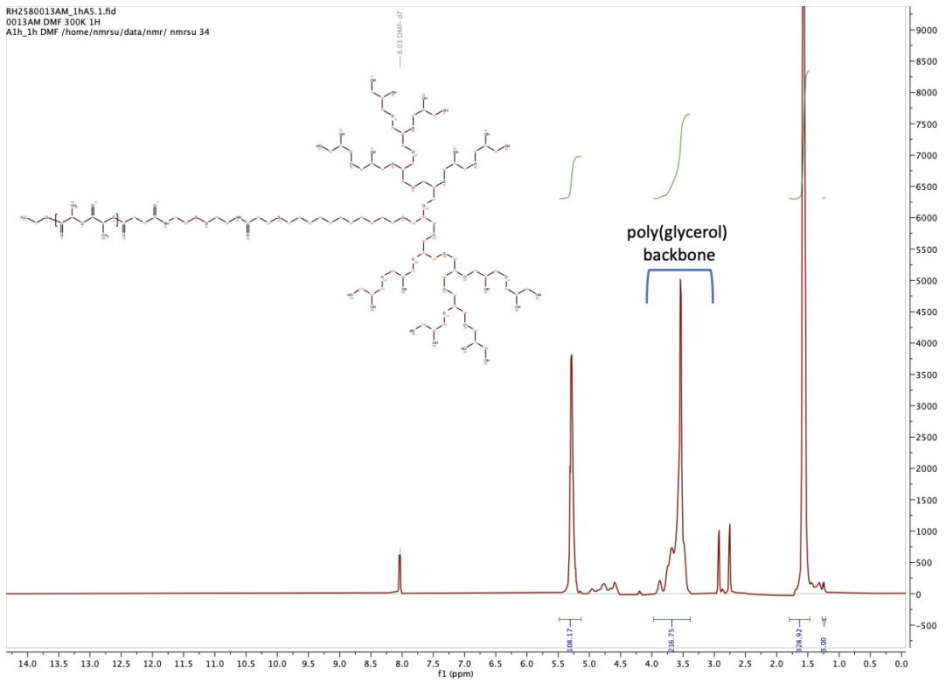


Figure S 17. *^{1}H NMR (500 MHz, DMF-d_7) of $dPG_{3.9}$-SS-$PLA_{7.8}$ synthesized by amide coupling between $dPG_{3.9}$-SS-NH_2 and $PLA_{7.8}$-COOH.*

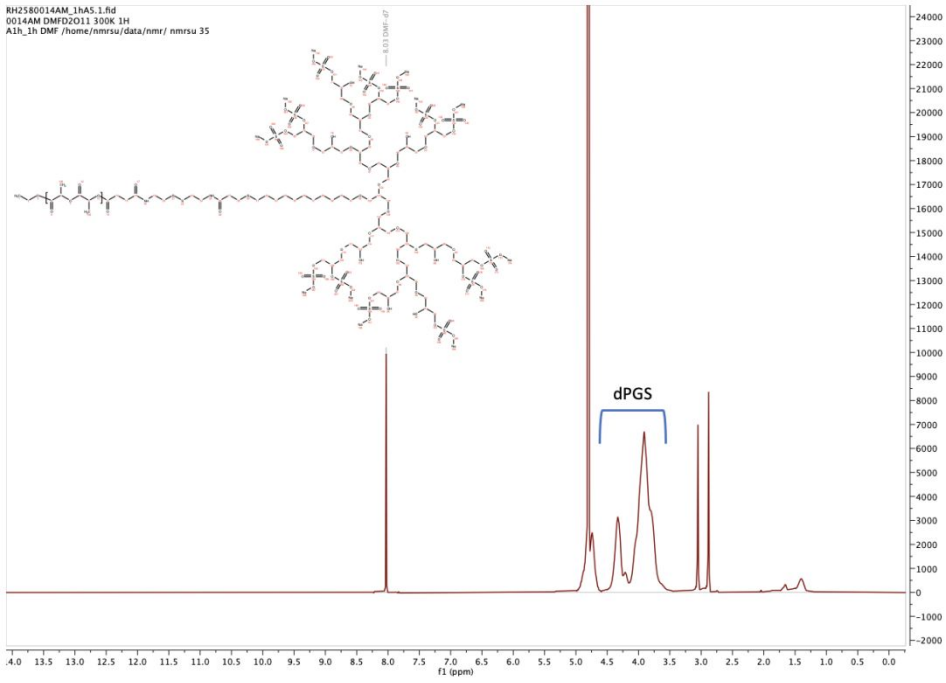
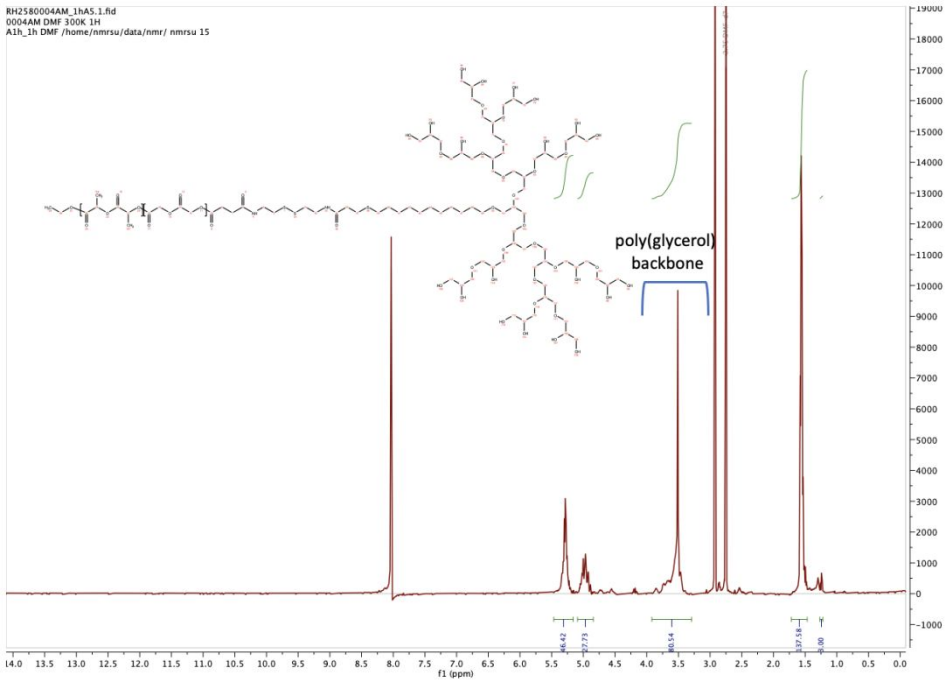
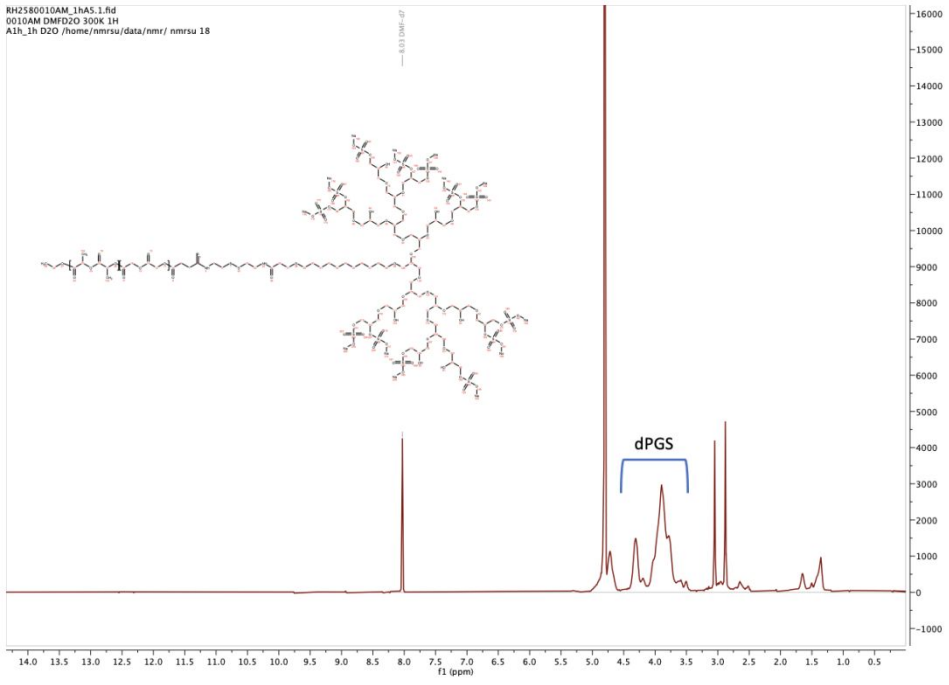


Figure S 18. *^{1}H NMR (500 MHz, DMF-d_7:D_2O 1:1) of $dPGS_{7.8}$-SS-$PLA_{7.8}$.*



144

145 ***Figure S 19.*** *^{1}H NMR (500 MHz, DMF-d_7) of $dPG_{1.5}$-SS-$PLGA_{3.8}$ synthesized by amide*
146 *coupling between $dPG_{1.5}$-SS-NH_2 and $PLGA_{3.8}$-COOH.*



147

148 ***Figure S 20.*** *^{1}H NMR (500 MHz, DMF-d_7:D_2O 1:1) of $dPGS_{3.0}$-SS-$PLGA_{3.8}$.*

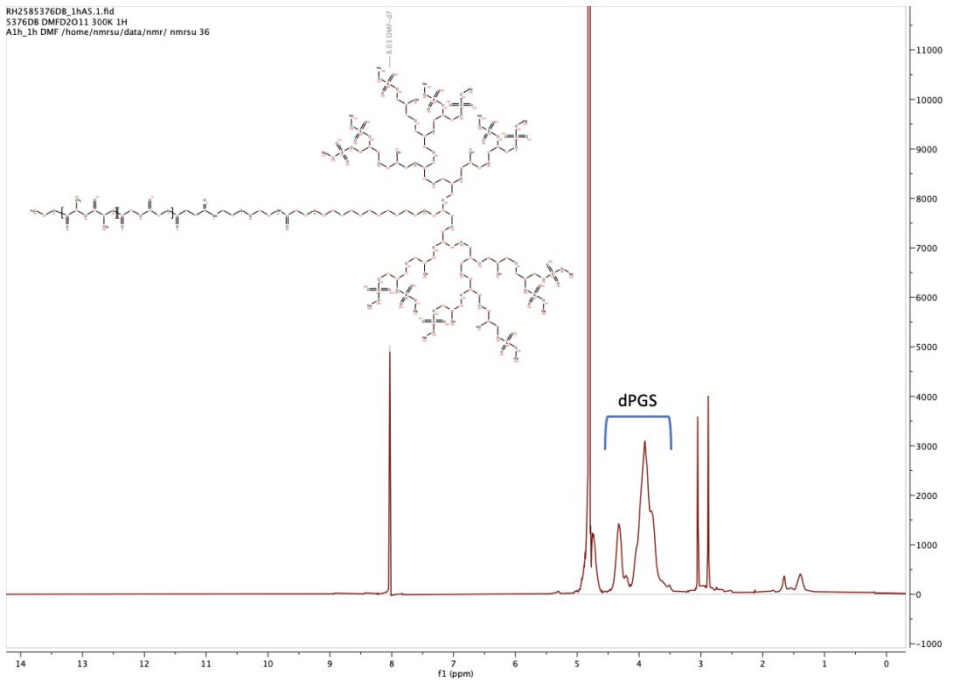


Figure S 21. *^{1}H NMR (500 MHz, DMF-d_7:D_2O 1:1) of $dPGS_{7.8}$-SS-$PLGA_{7.8}$.*

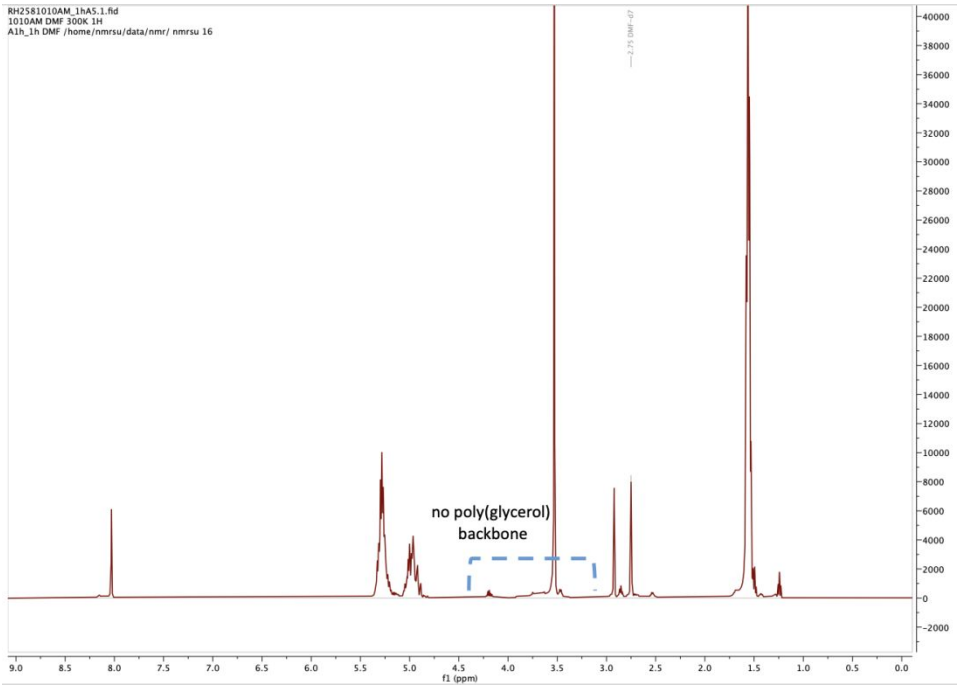


Figure S 22. *precipitation of $PLGA_{7.8}$-COOH after sulfation of $dPGS_{7.8}$-SS-$PLGA_{7.8}$ showing that separation was successful (no peaks of poly(glycerol); no $dPGS_{7.8}$-SS-$PLGA_{7.8}$ in precipitation).*

Table S 3. *characteristics of dPG-SS-PCL/PLA/PLGA. (*sulfation was run directly conducted after coupling)*

Compound	M_n (kDa) theoretical	^{1}H NMR	GPC (DMF)	$Đ$
$dPG_{3.9}$-SS-$PCL_{7.9}$	12	10.3	11.3	1.62
$dPG_{1.5}$-SS-$PCL_{4.0}$	6	5.9	11.7	1.43
$dPG_{3.9}$-SS-$PLA_{7.9}$	12	10.1	13.8	2.05
$dPG_{1.5}$-SS-$PLA_{3.9}$	6	4.9	13.6	1.48
$dPG_{3.9}$-SS-$PLGA_{7.9}$	12	-*	-*	-*
$dPG_{1.5}$-SS-$PLGA_{3.9}$	6	5.3	10.7	1.48

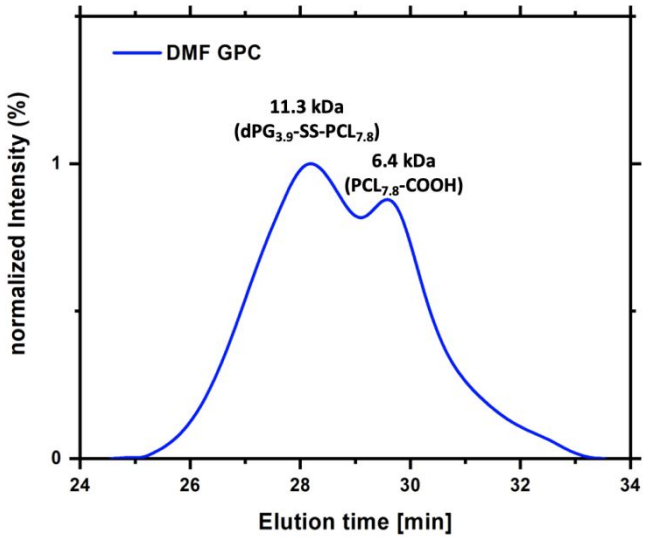


Figure S 23. *GPC chromatogram of $dPG_{3.9}$-SS-$PCL_{7.8}$ after amide coupling (main peak: $dPG_{3.9}$-SS-$PCL_{7.8}$, shoulder: non-coupled $PCL_{7.8}$-COOH; depicted molecular weights are according to the GPC results).*

Table S 4. *degree of sulfation of dPGS-SS-PCL/PLGA/PLA copolymers according to elemental analysis.*

Compound	theoretical S content $^{100\% \text{ functionalization}}$ (%)	S content ^{EA} (%)	Degree of sulfation (%)
$dPGS_{7.8}$-SS-$PCL_{7.8}$	14.7	11.4	78%
$dPGS_{3.0}$-SS-$PCL_{3.9}$	12.9	10.4	81%
$dPGS_{7.8}$-SS-$PLA_{7.8}$	14.7	11.6	79%
$dPGS_{3.0}$-SS-$PLA_{3.8}$	12.6	12.3	97%
$dPGS_{7.8}$-SS-$PLGA_{7.8}$	14.2	12.6	89%
$dPGS_{3.0}$-SS-$PLGA_{3.8}$	15.0	13.0	87%

Serum stability of $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles determined by FPLC

Table S 5. *serum stability of $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles detected by FPLC; micelles were incubated with 45 mg/mL HSA in PBS at 37 °C for 0h or 24 h and loaded on a Superdex 200 10/30 column; the calculated peak areas were normalized to 0h giving the serum stability in percentages.*

	Peak integral			n = 3				
Time (h)	**micelle**	**HSA 1**	**HSA 2**	**HSA 3**	**Micelle/ HSA**	**Micelle/HSA Average**	**normalized to 0h**	**micellar stability (%)**
0	46.16	104.15	349.53	104.42	0.083			
0	46.27	101.14	360.23	84.60	0.085	0.083	1.000	
0	41.68	88.00	350.04	74.31	0.081			
24	40.85	89.69	329.60	100.72	0.079			
24	39.62	89.67	321.21	128.50	0.073	0.078	0.940	**94.0**
24	43.81	85.89	361.40	87.49	0.082			

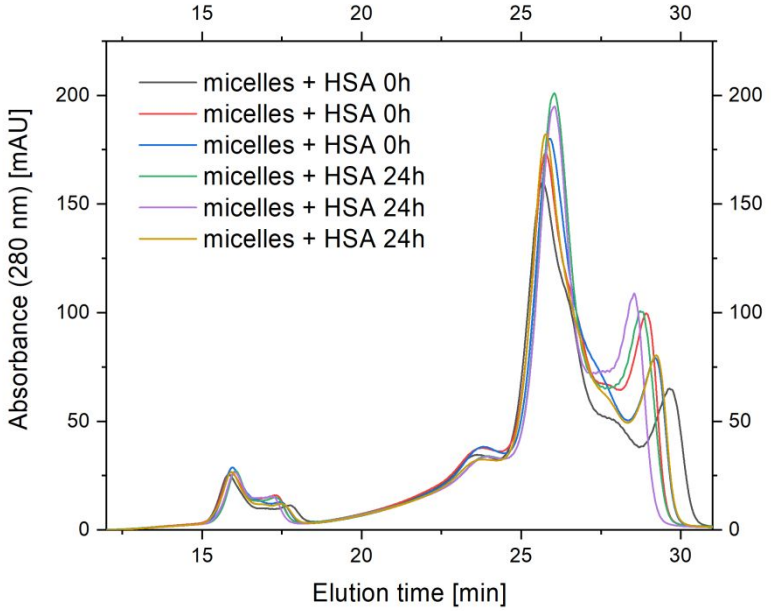


Figure S 24. *FPLC chromatograms at 280 nm of $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles (8 mg/mL) incubated with 45 mg/mL HSA in PBS at 37 °C (micelles: 15 – 18 min; HSA: 18 – 30 min) (n = 3).*

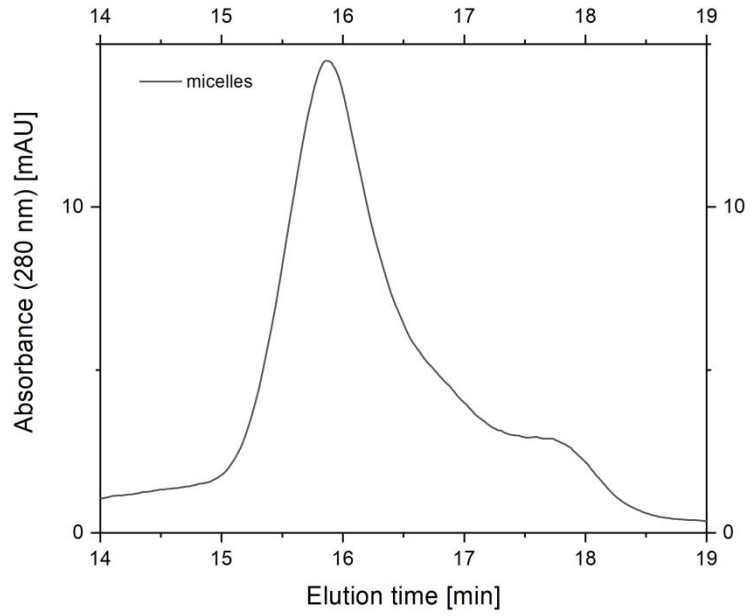


Figure S 25. *SEC traces at 280 nm of $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles (Elution time: 15 – 18 min; concentration 8 mg/mL).*

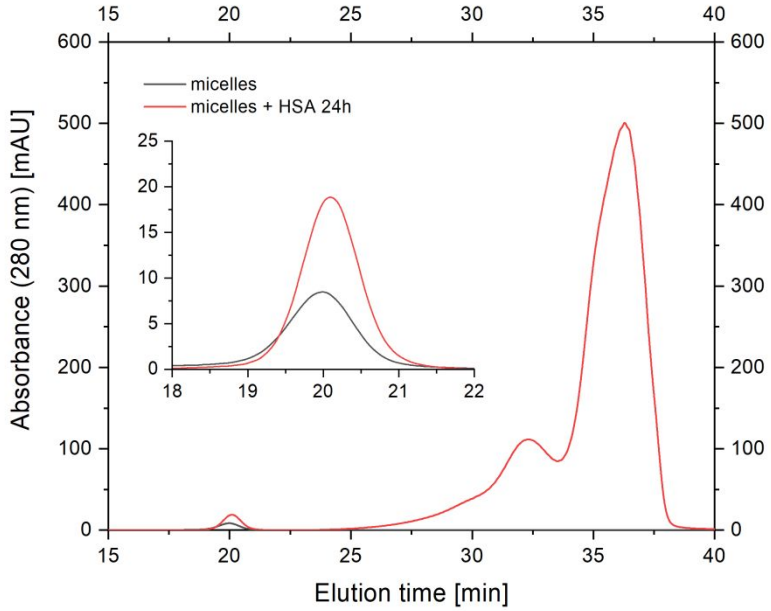


Figure S 26. *SEC traces at 280 nm of $dPGS_{7.8}$-SS-$PCL_{7.8}$ (1 mg/mL) alone and incubated with HSA (10 mg/mL) for 24 h.*

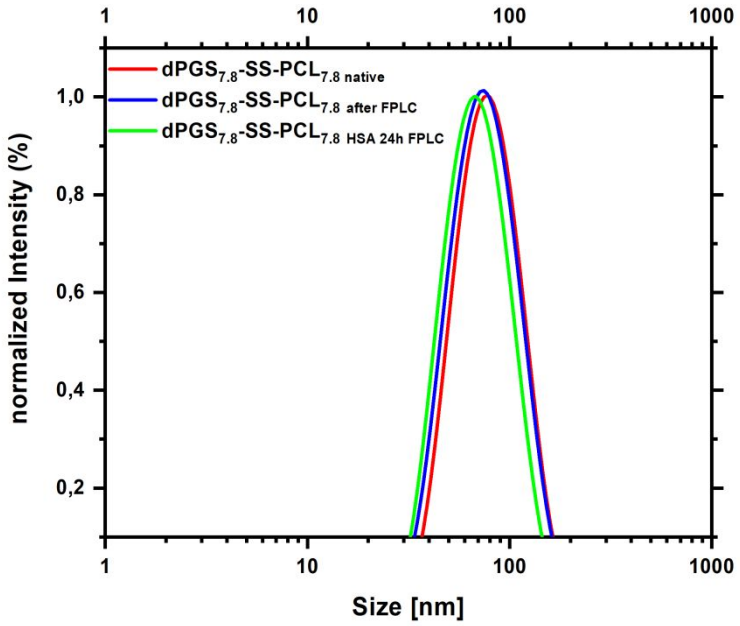


Figure S 27. *the size distribution of $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles before and after SEC separation from HSA shows that micelle size is not affected either by SEC or protein incubation.*

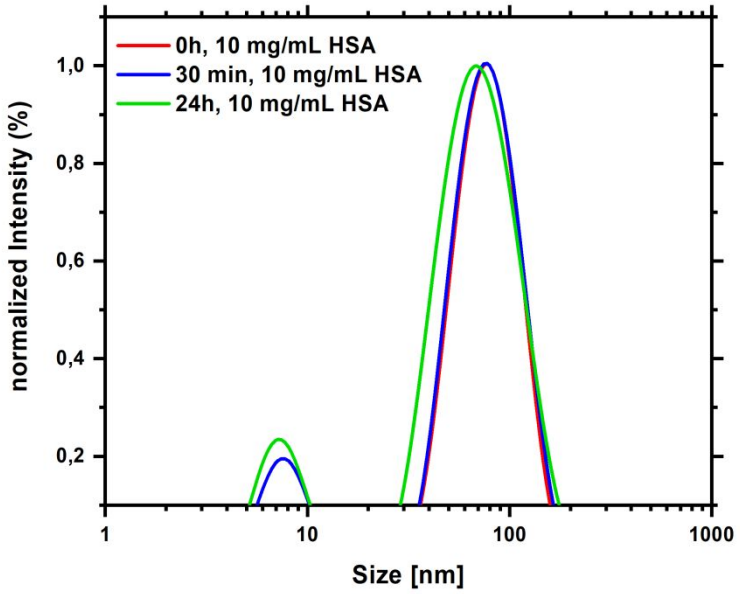


Figure S 28. *the size distribution of $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles incubated with 10 mg/mL HSA in PBS at 37 °C showing no size change of micelles after 24 h indicating no protein interaction with the micellar surface.*

188 **CMC plots dPGS-SS-PCL/PLA/PLGA micelles in PBS at 37 °C by DLS**

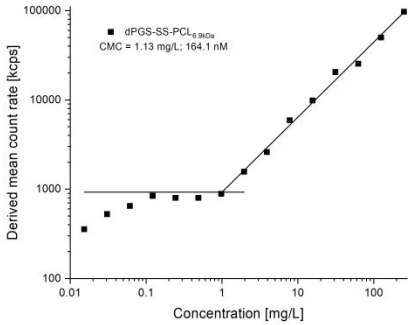


Figure S 29. *CMC plot of $dPGS_{3.0}$-SS-$PCL_{3.8}$.*

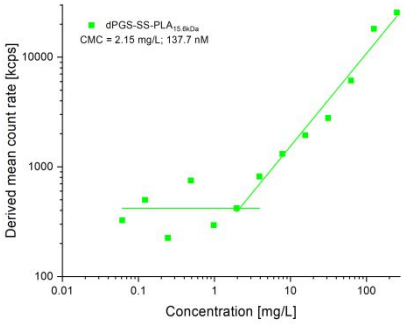


Figure S 30. *CMC plot of $dPGS_{7.8}$-SS-$PLA_{7.8}$.*

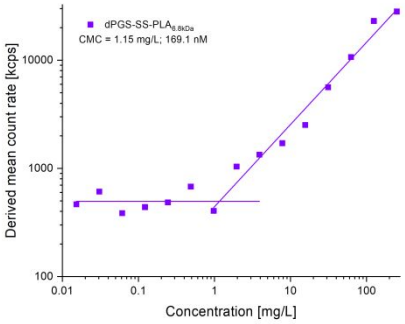


Figure S 31. *CMC plot of $dPGS_{3.0}$-SS-$PLA_{3.8}$.*

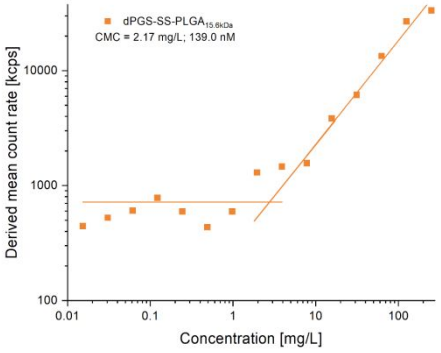


Figure S 32. *CMC plot of $dPGS_{7.8}$-SS-$PLGA_{7.8}$.*

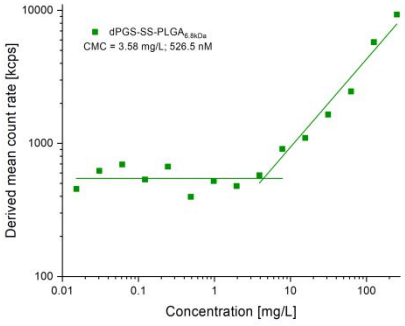


Figure S 33. *CMC plot of $dPGS_{3.0}$-SS-$PLGA_{3.8}$.*

Size distributions of dPGS-SS-PCL/PLA/PLGA micelles: DLS plot

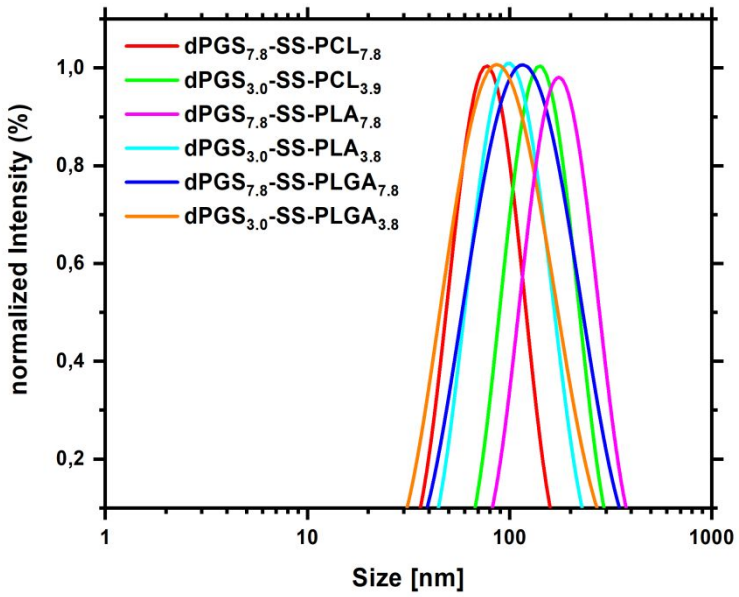


Figure S 34. *the size distribution of dPGS-SS-PCL/PLA/PLGA micelles in PBS at 1 mg/mL.*

Degradation in the presence of 10 mM GSH in PBS at 37 °C

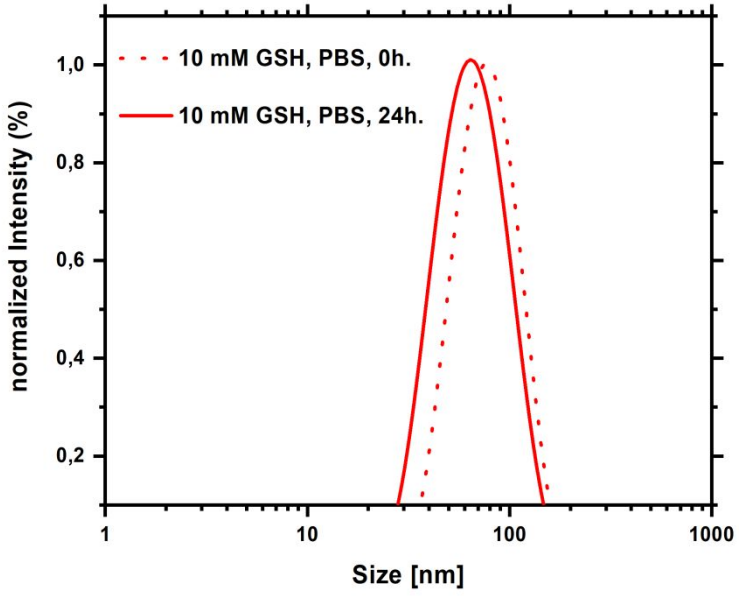


Figure S 35. *the size distribution of $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles in the presence of 10 mM GSH after 24 h at 37 °C.*

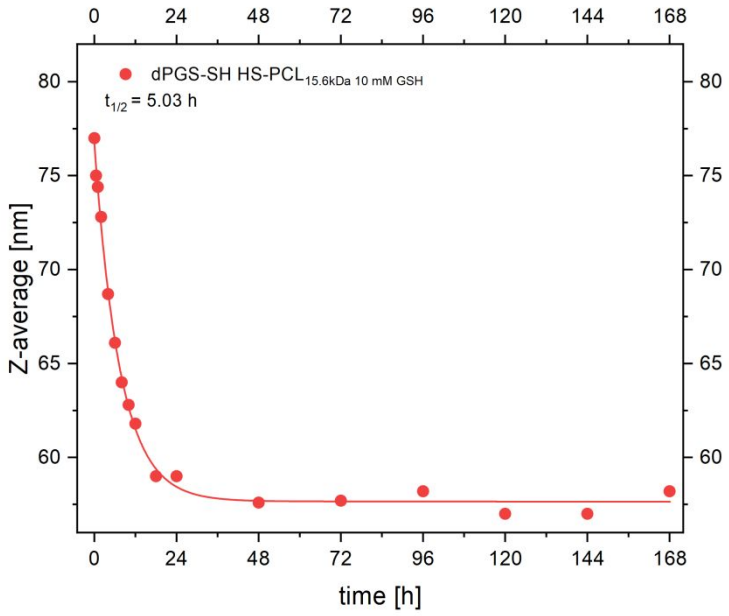


Figure S 36. *time-depended size evolution of $dPGS_{7.8}$-SS-$PCL_{7.8}$ in the presence of 10 mM GSH.*

Table S 6. *pH dependency of shell-shedding of micelles; neutral pH shows a higher count rate indication higher particle concentration.*

Compound	pH	Count rate (kcps)
10 mM GSH, PBS	7.4	33920
10 mM GSH, PBS	5.5	25140

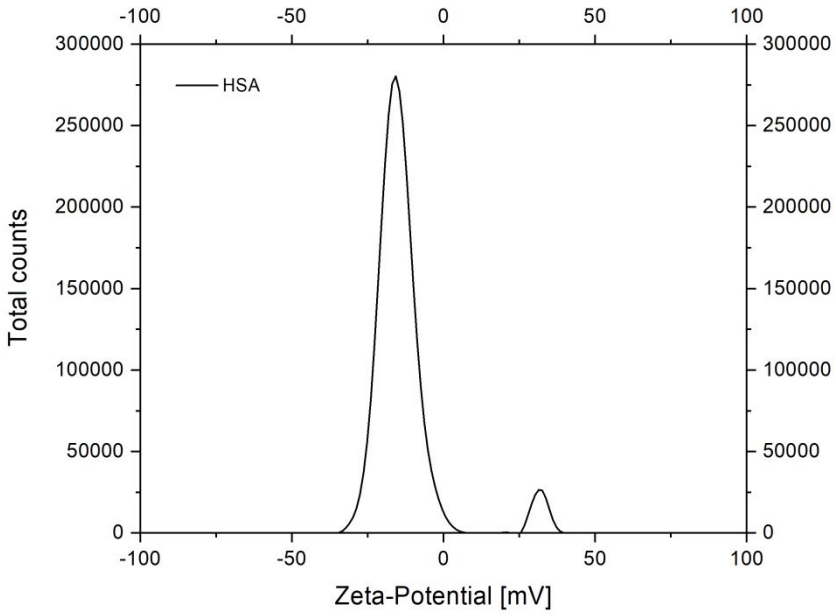


Figure S 37. *Zeta-potential of HSA (c = 10 mg/mL) in PB buffer pH 7.4.*

Drug-Loading: Sunitinib-loaded micelles purified with SEC column (photography)

Figure S 38. *SEC of dPGS-SS.micelles to separate non-encapsulated drugs (Sunitinib) in full view; Sunitinib-loaded micelles were loaded on the column and the yellow band was collected.*

Cellular Uptake of free Sunitinib and loaded in $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles on HeLa cells

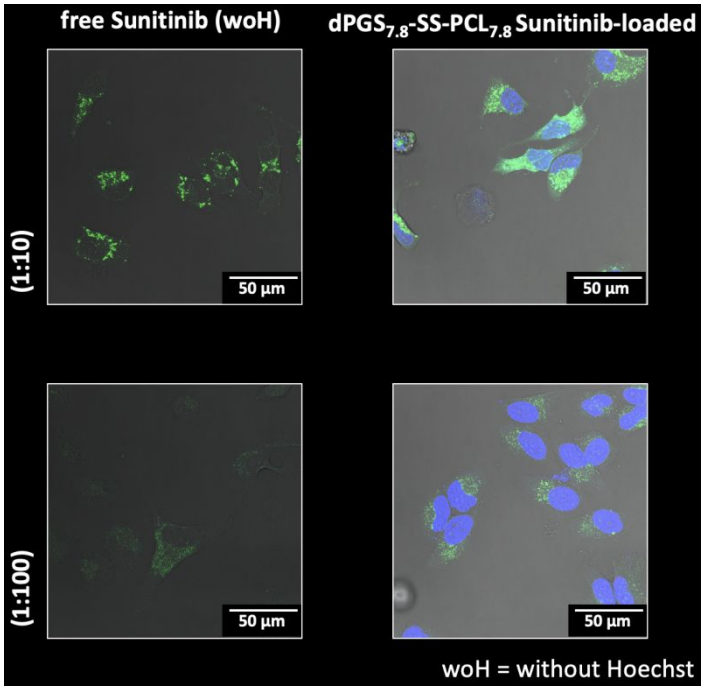


Figure S39. *Cellular uptake on HeLa cells of free Sunitinib and Sunitinib-loaded $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles with Hoechst staining after 24 h incubation.*

Cell viability on A549 cell line $dPGS_{7.8}$-SS-$PCL_{7.8}$

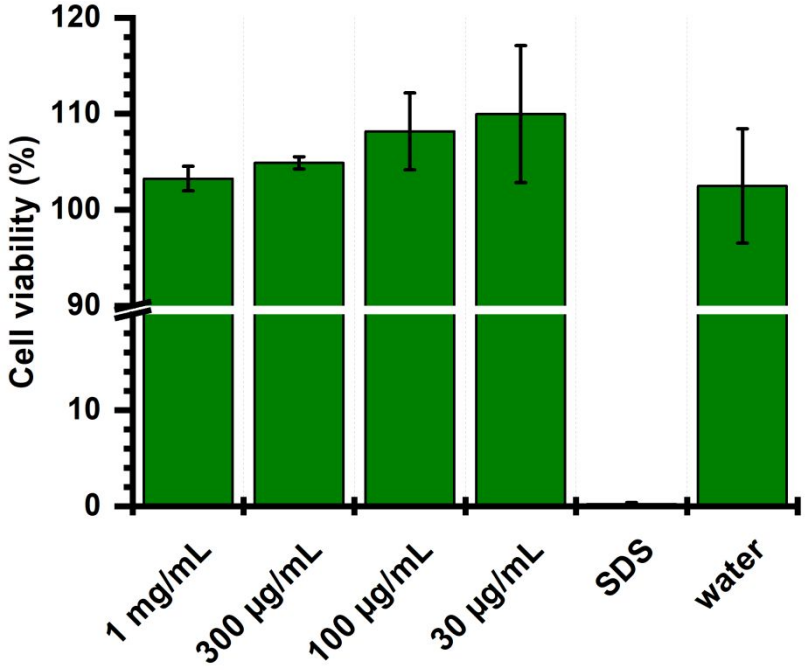


Figure S 40*. CCK-8 assay on A549 cell lines for cell combability of empty $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles (48 h, n=3).*

Cell viability on Vero E6 cell line $dPGS_{7.8}$-SS-$PCL_{7.8}$

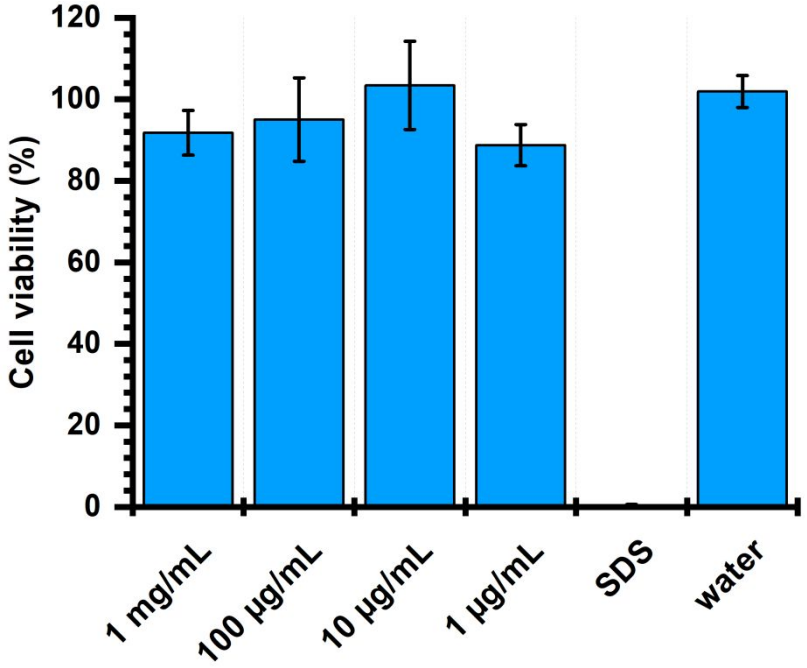


Figure S 41. *CCK-8 assay on Vero E6 cell lines for cell combability of empty $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles (24 h, n=3).*

Cell viability on HBE cell line $dPGS_{7.8}$-SS-$PCL_{7.8}$

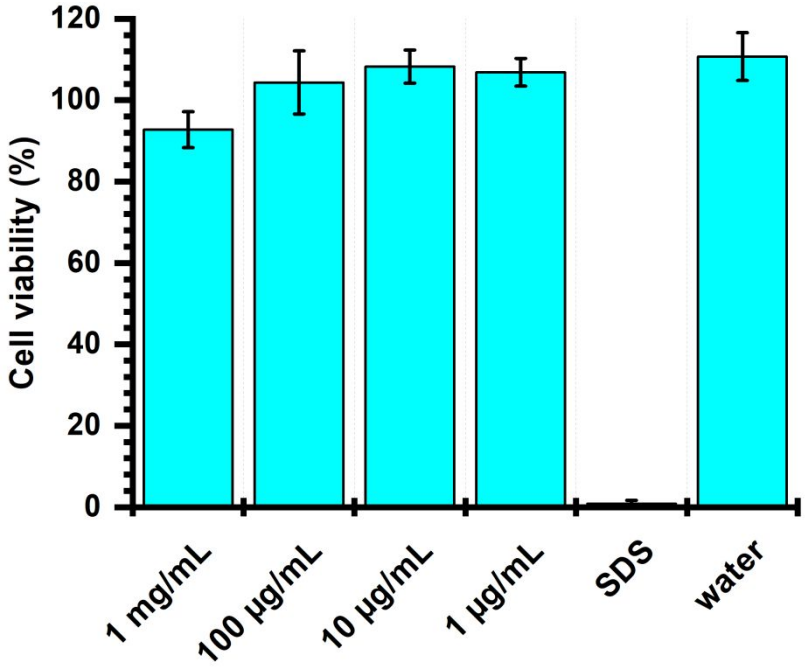


Figure S 42. *CCK-8 assay on HBE cell lines for cell combability of empty $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles (24 h, n=3).*

Cell viability on L929 cell line $dPGS_{7.8}$-SS-$PCL_{7.8}$

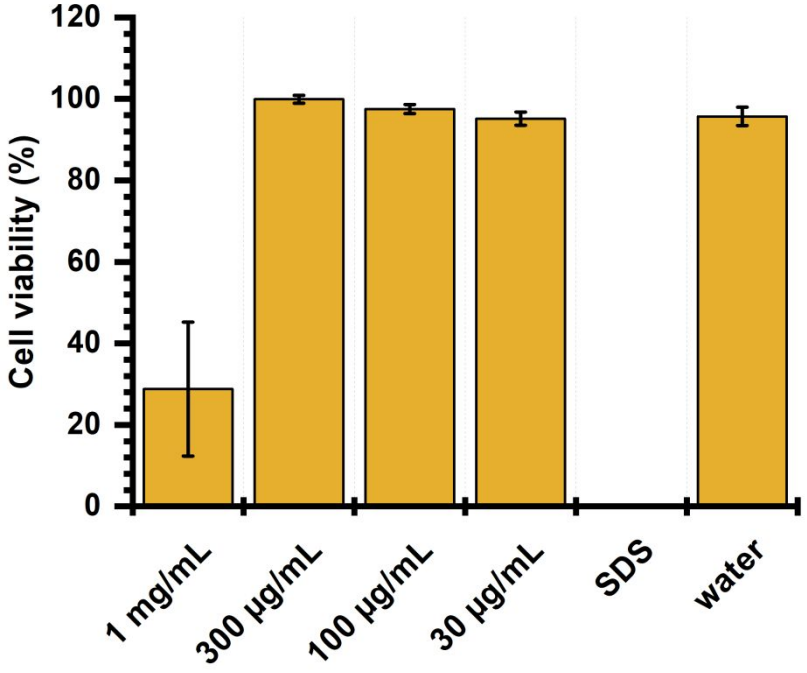


***Figure S 43**. CCK-8 assay on L929 cell lines for cell combability of empty $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles (48 h, n=3).*

Cell viability on HUVEC cell line $dPGS_{7.8}$-SS-$PCL_{7.8}$ (Anti-Angiogenesis Assay)

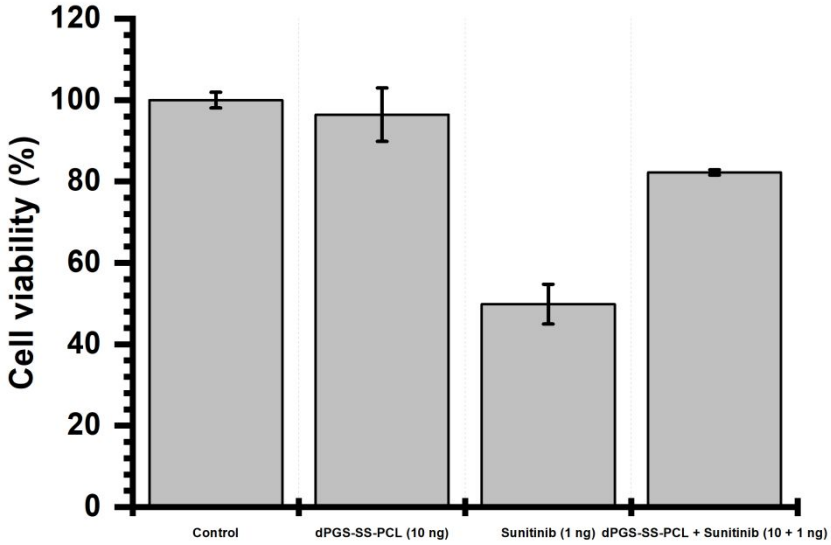


***Figure S 44**. CCK-8 assay on HUVEC cell lines for cell combability of empty $dPGS_{7.8}$-SS-$PCL_{7.8}$ micelles, free Sunitinib and Sunitinib-loaded micelles (4 h, triplicate).*

References

1. Tanzi, M. C.; Verderio, P.; Lampugnani, M. G.; Resnati, M.; Dejana, E.; Sturani, E., Cytotoxicity of some catalysts commonly used in the synthesis of copolymers for biomedical use. *J. Mater. Sci.: Mater. Med.* **1994,** *5* (6), 393-396.
2. Dzienia, A.; Maksym, P.; Hachuła, B.; Tarnacka, M.; Biela, T.; Golba, S.; Zięba, A.; Chorążewski, M.; Kaminski, K.; Paluch, M., Studying the catalytic activity of DBU and TBD upon water-initiated ROP of ε-caprolactone under different thermodynamic conditions. *Polym. Chem.* **2019,** *10* (44), 6047-6061.
3. Kamber, N. E.; Jeong, W.; Waymouth, R. M.; Pratt, R. C.; Lohmeijer, B. G. G.; Hedrick, J. L., Organocatalytic Ring-Opening Polymerization. *Chem. Rev.* **2007,** *107* (12), 5813-5840.
4. Dove, A. P., Organic Catalysis for Ring-Opening Polymerization. *ACS Macro Lett.* **2012,** *1* (12), 1409-1412.
5. Lohmeijer, B. G. G.; Pratt, R. C.; Leibfarth, F.; Logan, J. W.; Long, D. A.; Dove, A. P.; Nederberg, F.; Choi, J.; Wade, C.; Waymouth, R. M.; Hedrick, J. L., Guanidine and Amidine Organocatalysts for Ring-Opening Polymerization of Cyclic Esters. *Macromolecules* **2006,** *39* (25), 8574-8583.
6. Qian, H.; Wohl, A. R.; Crow, J. T.; Macosko, C. W.; Hoye, T. R., A Strategy for Control of "Random" Copolymerization of Lactide and Glycolide: Application to Synthesis of PEG-b-PLGA Block Polymers Having Narrow Dispersity. *Macromolecules* **2011,** *44* (18), 7132-7140.
7. Wilms, D.; Stiriba, S.-E.; Frey, H., Hyperbranched Polyglycerols: From the Controlled Synthesis of Biocompatible Polyether Polyols to Multipurpose Applications. *Acc. Chem. Res.* **2010,** *43* (1), 129-141.
8. Sunder, A.; Hanselmann, R.; Frey, H.; Mülhaupt, R., Controlled Synthesis of Hyperbranched Polyglycerols by Ring-Opening Multibranching Polymerization. *Macromolecules* **1999,** *32* (13), 4240-4246.