
Thermoresponsive behavior and *in vitro* investigations of random copolymers of ethylene oxide and glycidyl methyl ether (rPEGs)

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vorgelegt von

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Hiermit erkläre ich, **Dominik Schulz**, gemäß § 13 Abs. 3 der Promotionsordnung vom 18. Oktober 2021 in der Fassung vom 17. Februar 2025, dass die vorliegende Arbeit mit dem Titel:

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Mir ist bekannt, dass ein Verstoß gegen die genannten Punkte prüfungsrechtliche Konsequenzen hat und insbesondere dazu führen kann, dass die Promotionsleistung als mit „nicht bestanden“ bewertet wird. Die Einschreibung kann für bis zu zwei Jahre widerrufen werden, wenn Studierende zweimal oder häufiger bei Prüfungsleistungen täuschen (§ 69 Abs. 4 und 5 HochSchG).

Mainz, den _____

Dominik Schulz

Use of AI tools

AI tool	Used for	Why	When
ChatGPT (Ver. GPT-4 and GPT-5)	Creation and revision of Matlab2019a scripts; Correction of English grammar and spelling	Matlab scripts were used for data evaluation and visualization; Increasing readability	Ch. 2, 3 and 4 use Matlab scripts for data visualization (SEC, DSC, cloud point measurements, chain simulations) *
Claude (Anthropic)	Revision of Matlab2019a scripts	Matlab scripts were used for data evaluation and visualization	See above

* AI was only used for creation of Matlab scripts for data evaluation performed offline; no data was directly analyzed or plotted using AI tools

Nutzung von KI-Tools

KI-Tool	Verwendet für	Warum	Wann/Wo
ChatGPT (Version GPT-4 und GPT-5)	Verfassen und Überarbeiten von Matlab2019a Skripten; Korrektur von Grammatik und Rechtschreibung im Englischen	Matlab Skripte wurden für Auswertung und Visualisierung von Daten verwendet; Verbesserung der Lesbarkeit	Kapitel 2, 3 und 4 verwenden Matlab Skripte zur Visualisierung von Daten (SEC, DSC, Trübungspunkte, Kettensimulation)*
Claude (Anthropic)	Überarbeiten von Matlab2019a Skripten	Matlab Skripte wurden für Auswertung und Visualisierung von Daten verwendet	Siehe oben

* KI wurde lediglich für die Erstellung von Matlab Skripten zur offline Datenauswertung benutzt; zu keinem Zeitpunkt wurden Daten direkt mit KI-Tools ausgewertet oder Graphen mit KI erstellt

Für die Menschen in meinem Leben

“If there's any real truth, it's that the entire multidimensional infinity of the Universe is almost certainly being run by a bunch of maniacs.”

— Douglas Adams, *The Ultimate Hitchhiker's Guide to the Galaxy*

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[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

Die Zeit mit euch allen war niemals langweilig, gelegentlich chaotisch, manchmal produktiv und immer lustig. Ob Camping am Badensee, Ausflüge zu Konferenzen, Spieleabende, Filmabende, Wanderungen über Weinberge im Rheinland, über eingeschneite Berge im Kleinwalsertal, von Wohnung zu Wohnung mit einem Glühwein in der Hand oder einfach das Führen leidenschaftlicher Diskussionen in der Obstpause – die Erinnerungen sind unersetzlich und werden mich immer begleiten.

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Author Contributions

The results presented in this work have been obtained through collaboration of multiple colleagues of several research groups. In this section, the contributions to the chapters of this Dissertation will be specified.

Chapter 2 was developed as a collaboration between the [REDACTED] group and the [REDACTED] group (Martin-Luther-Universität Halle-Wittenberg). Dominik Schulz covered the synthesis and characterization of the monomer GME and the polymers as well as the measurement of the turbidimetric cloud points. [REDACTED] supported large parts of the characterization of the polymers – especially in the area of MALDI TOF and SEC measurements. [REDACTED] performed the EPR measurements and evaluation of the received data. The manuscript was written as an equal contribution of Dominik Schulz and [REDACTED]. It is currently under review at *Macromolecules*.

Investigations for **Chapter 3** were conducted as a collaboration between the Frey group and the Landfester group (Max-Planck-Institut für Polymerforschung Mainz). Dominik Schulz conducted the synthesis of the polymers, their functionalization with a fluorescent dye and all structural characterizations of the macromolecules. Dr. Mareike Deuker performed the MST measurements and FCS measurements were done by Dr. Kaloian Koynov. Evaluation of the received data of these experiments was done by Dr. Mareike Deuker and the manuscript was written in equal parts by Dominik Schulz and Dr. Mareike Deuker under additional advisement by Dr. Svenja Morsbach. The chapter has been published in *Advanced Science* in 11/2025 ([DOI: 10.1002/adv.202521061](https://doi.org/10.1002/adv.202521061)).

Chapter 4 was also a collaboration project between the [REDACTED] group and the [REDACTED] group. Synthesis of the initiator, the monomer GME and the lipid-polymers as well as the associated analytics were done by Dominik Schulz. Synthesis of the nanocapsules and their functionalization followed by preparation and analysis of the protein corona was performed by [REDACTED]. Additional advisement for analysis of the protein corona was provided by [REDACTED] r. [REDACTED] and Dominik Schulz contributed equally to this work. The manuscript was primarily written by Dominik Schulz with significant contributions from [REDACTED].

Appendix Chapter 1 was conceptualized by the Frey group under project administration by Dr. Philip Dreier and Dr. Rebecca Matthes and provides the foundation of the investigations of this Dissertation. Polymer samples were provided by Dr. Philip Dreier and Fabian Fuß and polymer analytics were performed by Dr. Philip Dreier, Dr. Rebecca Matthes, Fabian Fuß, Julian Schmidt, Dominik Schulz, Dr. Gregor Linden, Dr. Ramona Barent and Dr. Sandra Schüttner. Fabian Fuß and Julian Schmidt also performed cell viability and immunostimulation evaluations as well as competitive ELISA assays of the polymer samples under supervision of Dr. Matthias Bros at the University Medical Centre of the Johannes Gutenberg-Universität Mainz. Hemocompatibility of the samples was assessed by Barry Neun, Edward Cedrone and Dr. Marina Dobrovolskaia at the Frederick National Laboratory for Cancer Research (USA). The original manuscript was written by Dr. Philip Dreier, Dr. Rebecca Matthes, Fabian Fuß, Julian Schmidt, Dominik Schulz and Dr. Gregor Linden while review and editing was done by all contributing scientists. Dr. Philip Dreier, Dr. Rebecca Matthes, Fabian Fuß and Julian Schmidt contributed equally to this work. This manuscript was published in the *Journal of the American Chemical Society* in 06/2025. ([DOI: 10.1021/jacs.5c02716](https://doi.org/10.1021/jacs.5c02716))

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Motivation

Polyethers represent one of the most important and versatile classes of polymers. Among these, poly(ethylene glycol) (PEG) plays a special role in industry application and also for everyday life.¹⁻⁴ Thanks to its backbone structure it is able to create a layer of water molecules around itself by forming hydrogen bonds⁵ and therefore shows excellent water solubility, unlike almost all other polyethers. PEG is synthesized by anionic ring-opening polymerization (AROP) of ethylene oxide (EO) in a living polymerization where no termination or chain transfer reactions occur which allows precise control of the molar mass and dispersity of the polymers.⁴

Other epoxides like propylene oxide^{6,7} or glycidyl methyl ether⁸ can be polymerized by the same technique or by related procedures like double metal cyanide (DMC)^{9,10} catalysis or monomer-activated anionic ring-opening polymerization (MAROP).¹¹ Materials created from monomers like these can have very different properties from PEG and have a wide range of possibilities for application. By copolymerization of different monomers, properties can often be precisely adapted.^{12,13} This can be accomplished by choosing varying molar amounts of the comonomers and by controlling their sequence in the polymer chain. Polymer microstructure depends on the polymerization technique, the reaction conditions and the chemical properties of the comonomers.

PEG in particular has found many applications in day-to-day life. Thanks to its high water solubility and low toxicity it is used as an anti-foaming food additive¹⁴, as a surfactant in cosmetics² and can serve as a laxative (Macrogol) when taken orally.¹⁵ One of the most significant applications, however, is the so-called “PEGylation”, pioneered by Davis and Abuchowski in 1977¹⁶, in which active pharmaceutical ingredients (APIs) or nanomaterials are functionalized with PEG to increase their water solubility, increase their stability and effectively “shield” them from the immune system to enhance their retention time in the blood stream. This phenomenon is commonly referred to as the “stealth effect” of PEG. Gabizon and Martin¹⁷ reported a severely reduced clearance rate of PEGylated liposomal doxorubicin in cancer patients with higher peak concentrations in tumor tissue compared to the free drug. At the same time, distribution of the drug in the reticuloendothelial system (RES) was decreased which prolonged the circulation time in the blood stream¹⁸ and side effects like cardiotoxicity were reduced.¹⁹

Despite these promising characteristics and the original assumption that PEG does not possess any immunogenicity or antigenicity, anti-PEG antibodies (APAs) were already discovered in 1983 by Richter and Åkerblom.²⁰ Initially, only short-lived IgM antibodies were detected in 0.2 % of the general population. Therefore, these findings were considered “of no clinical significance”. Today, the existence of long-lived anti-PEG IgG antibodies is known. While different numbers for their prevalence can be found in literature^{21–26}, a recent study by Deuker *et al* found that anti-PEG IgG antibodies were present in 83 % of the general German population.²⁷ Consequently, PEGylated APIs undergo an accelerated blood clearance (ABC)²⁸ – negating the stealth effect – and can in some cases even cause a hypersensitive pseudo-anaphylactic reaction.^{29,30}

To counteract the increasing occurrence of APAs, other hydrophilic and non-toxic polymers like poly(oxazoline) (POx)^{31,32} and poly(sarcosine) (PSar)^{33,34} are currently investigated as PEG alternatives. Although first results are promising, antibodies against POx and a corresponding ABC effect have already been detected in rats.³⁵ Lai *et al*³⁶ published the structure of the APA in 2020 and found that 16 consecutive EO units in the polymer chain are necessary to induce binding to the Fab-paratope of the antibody *via* Van der Waals interactions, which raises the question of whether antibodies can generally be formed against a regular backbone structure as it is present in many synthetic polymers.

In response to the raising concerns regarding the immunological risk of PEG, we developed the randomized PEG (rPEG), which is a structural isomer of PEG and possesses randomly distributed side chains, which can suppress antibody-binding by interrupting the regular backbone sequence of PEG.³⁷ rPEGs are synthesized by an ideal statistical copolymerization of EO and glycidyl methyl ether (GME). The aim of this work is to investigate the suitability of rPEGs as alternative PEGylation agents.

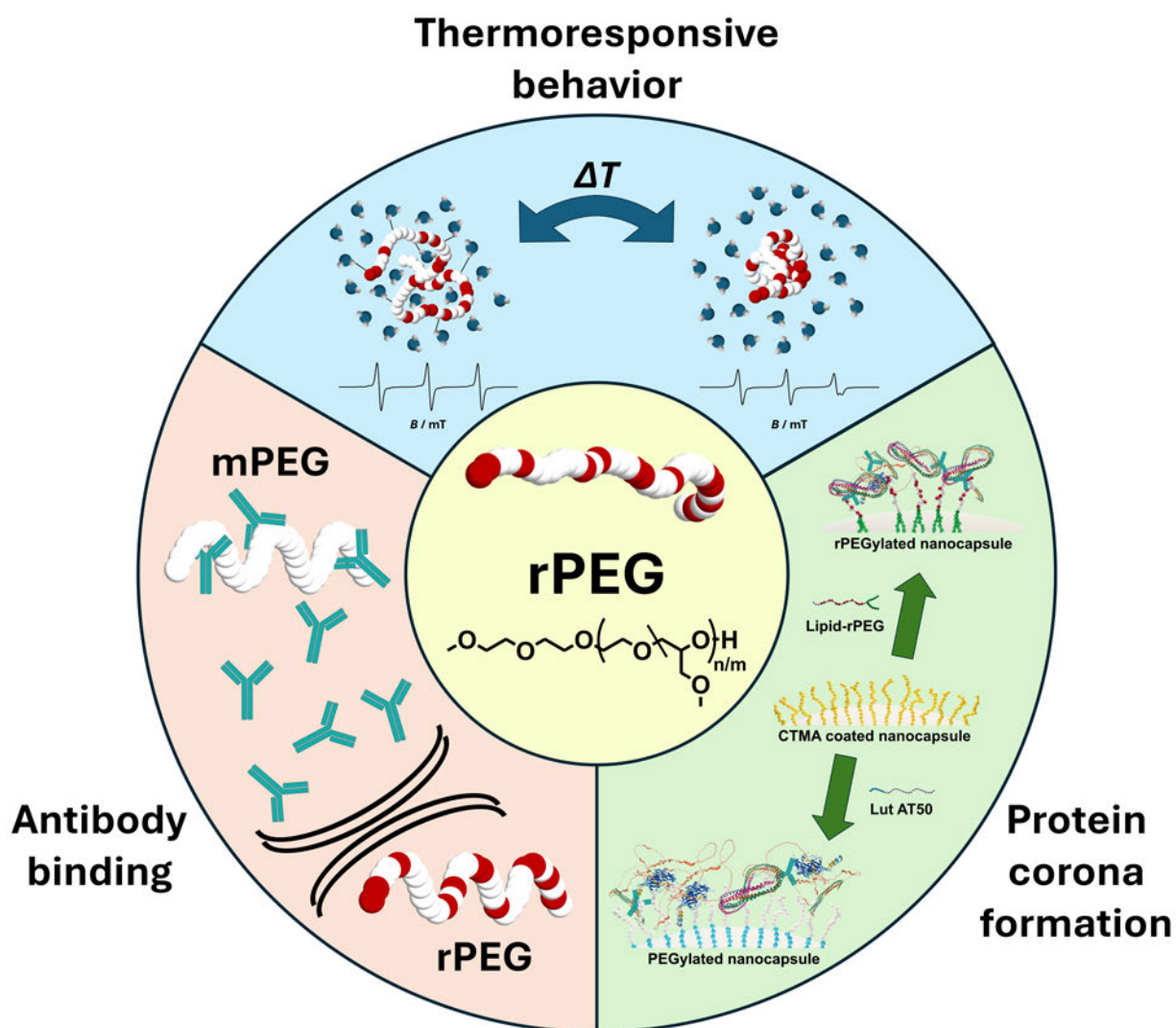
Chapter 2 takes a closer look at the hydrophilicity of rPEGs compared to PEG. Water solubility is a critical property for biomedical applications. Materials for subcutaneous injections have to keep their solubility well above the physiological temperature of 37 °C. No phase separation or precipitation should be observed on contact of the substance with the blood stream. Additionally, formation of a hydration shell dictates interactions with the immune system and therefore can either prolong or reduce retention time in the bloodstream. Evaluation of hydrophilicity was performed

by initial synthesis of ideal statistical copolymers of EO and GME (rPEGs). These were investigated by determining the cloud points for varying molar fractions of GME and by a detailed analysis through electron paramagnetic resonance (EPR) spectroscopy, relying on TEMPO spin-probing.

Chapter 3 explores the reaction of backbone-specific APAs to rPEGs. For this purpose, copolymers of different GME content were functionalized with fluorescein in a multi-step reaction. The functionalized copolymers were then brought into contact with APAs, and their dissociation behavior as well as their hydrodynamic radii were determined *via* fluorescence correlation spectroscopy (FCS) and microscale thermophoresis (MST). By comparing the hydrodynamic radii of free copolymers with the ones exposed to antibodies of different concentrations, the binding of rPEGs to APAs can be quantified by *in vitro* experiments. These data give an indication of possible immune reactions towards rPEGs, although *in vivo* data will still be required to obtain a full evaluation.

In **Chapter 4**, the protein corona of the copolymers was investigated. The protein corona is formed around substances on contact with blood and dictates the behavior of nanomaterials in the bloodstream. Since opsonins and dysopsonins can be incorporated in the protein corona, the interaction of the nanomaterials with the immune system and therefore their elimination half-life is dictated by composition. For a detailed analysis, a lipid initiator was synthesized and used to copolymerize EO and GME to generate lipid-functionalized rPEGs. When coating silica nanocapsules with these Lipid-rPEGs and exposing them to human serum, a protein corona around the nanocapsules is formed. Characterization of opsonins and dysopsonins in the corona can therefore give an idea about the blood clearance behavior of rPEGs compared to PEG.

Appendix Chapter 1 contains our seminal publication on rPEGs, which explores the basic synthesis of the copolymers under AROP conditions as well as comprehensive analytical characterization of the polymer samples. Additionally, the goal of this work was to demonstrate general cell viability and hemocompatibility of the polymers to ensure non-toxicity when applied in the biomedical field. Furthermore, backbone-selective and end group-selective APA ELISAs were performed to show changes in antibody binding between different copolymers depending on the molar GME content, i.e., the concentration of randomly placed side chains.



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Abstract

Polyethers are crucial materials in modern medicine, with polyethylene glycol (PEG) being the most prominent member of this class with a global production of 2.5 million metric tons in 2022. Derived from the polymerization of ethylene oxide (EO) via anionic ring-opening polymerization (AROP), PEG presents exceptional characteristics: it is water soluble, biocompatible and can be excreted through the human renal system up to a molecular weight of 30 kDa. PEGylation – the process of functionalizing active pharmaceutical ingredients (APIs) with PEG – has revolutionized drug delivery research in medical science since its discovery in the 1970s. By enveloping APIs with a corona of PEG molecules, the immune reaction to these compounds is significantly reduced, thanks to the inhibition of opsonin adsorption. This phenomenon, known as the "stealth effect", prolongs the circulation time of PEGylated compounds in the bloodstream significantly compared to their non-modified counterparts. In March 1990 the first PEGylated protein (Adagen® by Enzon Pharmaceuticals) was approved by the food and drug administration (FDA) for application and since then dozens more have entered the market. As of 2023, 38 PEGylated drugs have earned approval by the FDA. However, the discovery of the stealth effect was soon met with the detection of anti-PEG antibodies (APAs). These antibodies can induce chronic adverse reactions to PEG, ranging from accelerated blood clearance (ABC), which counteracts the stealth effect, to hypersensitive reactions similar to anaphylactic symptoms. Recent studies suggest that APAs are present in 83 % of the German general population, likely due to extensive exposure to PEG in everyday household products. This underscores the need for alternatives to PEG that can mitigate immune responses while maintaining its beneficial properties.

To counteract the increasing problem of immunogenic reactions to PEG, extensive research is performed to develop alternatives for PEGylation. We have recently developed a synthesis for random copolymers of EO and glycidyl methyl ether (GME), which have the potential to be a viable alternative for PEG in case of severe immunogenic reactions. These “rPEGs” are structurally isomeric to PEG but possess randomly distributed side chains at the polymer backbone to prevent antibody binding. Previously, copolymers with GME could not be synthesized under GMP-certified conditions, excluding them from biomedical applications. However, we have successfully synthesized random copolymers of EO and GME under the same GMP compliant AROP conditions that are used for the synthesis of PEG, granting the possibility of medical usage.

This thesis covers the synthesis of rPEGs via AROP, functionalization of the copolymers through post-polymerization reactions, investigations of physicochemical properties as well as behavior towards antibodies and proteins under *in vitro* conditions. A theoretical framework for the synthesis, properties and application of polyethers with a focus on PEG is provided in **Chapter 1**. Additionally, general concepts of PEGylation and the phase behavior of polyethers in aqueous solutions are discussed.

Chapter 2 covers the synthesis of the monomer GME and its copolymerization procedure with EO. Several copolymers with constant chain length and varying molar amount of GME were synthesized and characterized via NMR spectroscopy, MALDI TOF mass spectrometry and GPC. The results show a reproducible synthesis for rPEGs of a molar GME content up to 80 mol %. A homopolymer of GME with a chain length of $D_p = 103$ could be synthesized and used for further measurements, but GME homopolymerization still provides limited reaction control compared to the copolymers. Furthermore, the thermoresponsive behavior of rPEGs in aqueous solution was investigated by turbidimetry and ESR. Turbidimetric measurements were performed at concentrations of 5 mg mL⁻¹ and 100 mg mL⁻¹, while ESR measurements were performed at 100 mg mL⁻¹. At a concentration of 5 mg mL⁻¹, cloud points could be measured turbidimetrically for copolymers of molar fractions above 40 mol % and ranged between 96 °C and 70 °C. At concentrations of 100 mg mL⁻¹, cloud points were determined between 86 °C and 68 °C by turbidimetry and show good accordance with the onset of chain collapse and the formation of hydrophobic segments observed in the EPR measurements. Even at high concentrations, all polymers showed cloud points above 60 °C, providing no indication of macroscopic phase separation in the range of physiologically relevant temperatures.

Chapter 3 examines the functionalization of the copolymers with a fluorescent dye, specifically to enable studies via fluorescence correlation spectroscopy (FCS) and microscale thermophoresis, (MST) regarding the binding affinity of monoclonal backbone-specific anti-PEG Immunoglobulin G (IgG) antibodies to rPEGs of constant molar mass and varying molar GME content under *in vitro* conditions. The obtained results showed a steadily decreasing antigenicity of the copolymers with increasing molar content of GME substantiated by a decreasing change in hydrodynamic radius when exposing rPEGs to anti-PEG IgG up to concentrations of 5 µM as well as a strong increase in the dissociation constant K_d for higher molar contents of GME in the copolymer backbone. While reduced binding affinity was observed for copolymers with molar

GME contents up to 42 mol%, a copolymer with a molar GME content of 52 mol% yielded no detectable antibody binding even at high ligand concentration.

The focus of **Chapter 4** is placed on the synthesis of rPEGs with lipid functionalization and analysis of the protein corona formed by particles coated with these copolymers upon exposure to human serum. To achieve full functionalization, a lipid initiator was synthesized, and AROP was performed under adapted conditions to accommodate for reactivity and solubility of this initiator. The prepared lipids of constant chain length and varying molar amounts of GME were coated onto nanocarriers so the effect of the rPEG backbone on the formation of the protein corona could be observed. Although overall protein concentration in the corona was found to be significantly lower for the particles coated with the synthesized samples compared to Lutensol AT50, the relative abundance of anti-PEG IgG in total IgG was higher for all synthesized samples and remained approximately constant for varying molar contents of GME in the copolymers. The lowest total concentration of IgG in the protein corona was found for the sample with the highest molar content of GME. At the same time, this sample showed increased concentrations of opsonins in the corona, while dysopsonins were most abundant in samples with low molar GME content. It was concluded that the unexpectedly low packing density of the synthesized rPEGs on the particle surface had a strong influence on the formation of the protein corona so that only general trends in the composition could be observed while the precise influence of the molar GME content on the composition of the corona could not be conclusively determined.

Appendix Chapter 1 introduces the fundamental concept of rPEGs and provides the basis for the investigations carried out in this work. Successful synthesis of rPEGs under AROP conditions was shown for copolymers up to a GME content of 74 mol% through analysis via ^1H NMR and DOSY NMR spectroscopy, SEC and MALDI TOF. Statistical calculations provided insight into the diminished occurrence of epitopes containing 16 or more consecutive EO units for rPEGs with increasing GME content. First turbidimetric measurements showed no phase separation for any of the polymers in aqueous solution below 70 °C for concentrations of up to 10 mg mL⁻¹. Cell viability and B cell activation as well as hemocompatibility in human blood *in vitro* were demonstrated for the rPEG samples and showed comparable values to mPEG. Competitive backbone-selective and end group-selective APA ELISAs were able to demonstrate a stark reduction in relative antibody affinity to rPEGs with increasing molar GME content compared to mPEG.

Zusammenfassung

Polyether zählen zu den wichtigsten Materialien in der modernen Medizin. Der prominenteste Vertreter dieser Verbindungsklasse ist Poly(ethylenglykol) (PEG) mit einer weltweiten Produktion von 2.5 Millionen Tonnen im Jahr 2022. PEG wird durch die anionische Ringöffnungs-Polymerisation (AROP) von Ethylenoxid (EO) erhalten und zeigt außergewöhnliche Eigenschaften: es ist wasserlöslich, biokompatibel und kann bis zu einem Molekulargewicht von 30 kDA vom Nierensystem des Menschen ausgeschieden werden. PEGylierung – der Prozess der Funktionalisierung von aktiven pharmazeutischen Ingredienzien (APIs) mit PEG – hat die Forschung bezüglich Wirkstofftransport in der medizinischen Wissenschaft seit ihrer Entdeckung in den 1970er Jahren revolutioniert. Indem APIs mit einer Corona aus PEG umhüllt werden, wird die Immunreaktion auf diese Stoffe aufgrund der Inhibition der Opsonin Adsorption deutlich reduziert. Dieses Phänomen, bekannt als „stealth effect“, verlängert die Zirkulationszeit von PEGylierten Verbindungen im Blutkreislauf erheblich verglichen mit unmodifizierten. Im März 1990 wurde das erste PEGylierte Protein (Adagen® von Enzon Pharmaceuticals) von der Food and Drug Administration (FDA) für die Anwendung zugelassen und seitdem wurden Dutzende mehr auf den Markt gebracht. Trotz dieser Fortschritte wurde die Entdeckung des „stealth“ Effekts kurze Zeit später mit dem Nachweis von anti-PEG-Antikörpern (APAs) konfrontiert. Diese Antikörper können chronische negative Reaktionen gegen PEG hervorrufen, die bis zu hypersensitiven Reaktionen ähnlich einem anaphylaktischen Schock reichen können. Eine kürzlich durchgeführte Studie fand ein Auftreten von APAs in bis zu 83 % der deutschen Gesamtbevölkerung. Dies hebt die Notwendigkeit einer Alternative zu PEG hervor, die weiterhin zur Reduktion von Immunreaktionen beiträgt und gleichzeitig dessen vorteilhaften Eigenschaften erhält. Unsere Forschungsgruppe hat vor Kurzem die Synthese eines Copolymers aus EO und Glycidylmethylether (GME) mit statistisch verteilter Monomersequenz entwickelt, die eventuell eine sinnvolle Alternative zu PEG im Falle von schweren Immunreaktionen darstellen kann. Diese „rPEGs“ (random PEGs) sind Strukturisomere zu PEG, besitzen aber zufällig verteilte Seitenketten entlang des Polymerrückgrats, die potenziell die Bindung zu Antikörpern verhindern können. Copolymere mit GME konnten zuvor nicht unter den Bedingungen der „good manufacturing practice“ (GMP) hergestellt werden, wodurch sie von biomedizinischen Anwendungen ausgeschlossen waren. Wir haben diese Copolymere nun erfolgreich unter denselben AROP-

Bedingungen hergestellt, die auch für die Synthese von GMP-zertifiziertem PEG verwendet werden und eröffnen damit die Möglichkeit einer medizinischen Applikation.

Diese Arbeit behandelt die Synthese von rPEGs unter den genannten AROP-Bedingungen, ihre Funktionalisierung durch polymeranaloge Reaktionen, Untersuchungen ihrer physikochemischen Eigenschaften sowie ihr Verhalten gegenüber Antikörpern und Proteinen unter *in vitro*-Bedingungen. **Kapitel 1** umfasst die theoretischen Grundlagen über die Synthese von Polyethern, deren Eigenschaften und ihre Anwendung wobei der Schwerpunkt auf PEG liegt. Zusätzlich werden das generelle Konzept der PEGylierung und das Phasenverhalten von Polyethern in wässriger Lösung diskutiert.

Kapitel 2 beschäftigt sich mit der Synthese des Monomers GME und seinem Copolymerisationsverfahren mit EO. Mehrere Copolymere mit konstanter Kettenlänge und variierenden molaren Anteilen von GME wurden hierfür hergestellt und charakterisiert. Weiterhin wurde das thermoresponsive Verhalten von rPEGs in wässriger Lösung mit Hilfe von Turbidimetrie und Elektronenspinresonanz (electron paramagnetic resonance, EPR) untersucht. Die Resultate beider Methoden zeigten eine deutliche Abhängigkeit der Trübungspunkte vom molaren Gehalt von GME in den Copolymeren. Diese liegen in guter Übereinstimmung mit dem Einsetzen des Kettenkollaps und der Bildung hydrophober Segmente in den Copolymeren, die während der EPR-Messungen beobachtet wurden. Selbst bei hohen Konzentrationen zeigten alle Polymere Trübungspunkte jenseits von 60 °C und zeigen daher keine Anzeichen makroskopischer Phasentrennung im Bereich von physiologisch relevanten Temperaturen.

In **Kapitel 3** wird die Funktionalisierung der Copolymere mit einem fluoreszierenden Farbstoff behandelt. Diese wurde durchgeführt, um Untersuchungen der Bindungsaffinität von monoklonalem Rückgrat-spezifischem anti-PEG-Immunglobulin G (IgG) Antikörpern mit rPEGs von konstanter Molmasse und variierender molarer Anteile von GME mittels Fluoreszenzkorrelationsspektroskopie (FCS) und Microscale Thermophorese (MST) unter *in vitro*-Bedingungen zu ermöglichen. Die Ergebnisse, die mittels FCS und MST erhalten wurden, zeigen einen stetigen Abfall der Antigenität der Copolymere mit einem steigenden molaren Anteil von GME, demonstriert durch abnehmende Änderung des hydrodynamischen Radius bei einer Exposition der rPEGs gegenüber anti-PEG IgG für Konzentrationen bis zu 5 µM, sowie durch eine starke Zunahme der Dissoziationskonstante K_d für steigende molare Anteile von GME im

Polymerrückgrat. Während für Copolymere mit einem molaren GME-Anteil von bis zu 42 mol% eine reduzierte Bindungsaffinität beobachtet werden konnte, so konnte für das Copolymer mit einem molaren GME-Anteil von 52 mol% keine nachweisbare Antikörperbindung mehr nachgewiesen werden.

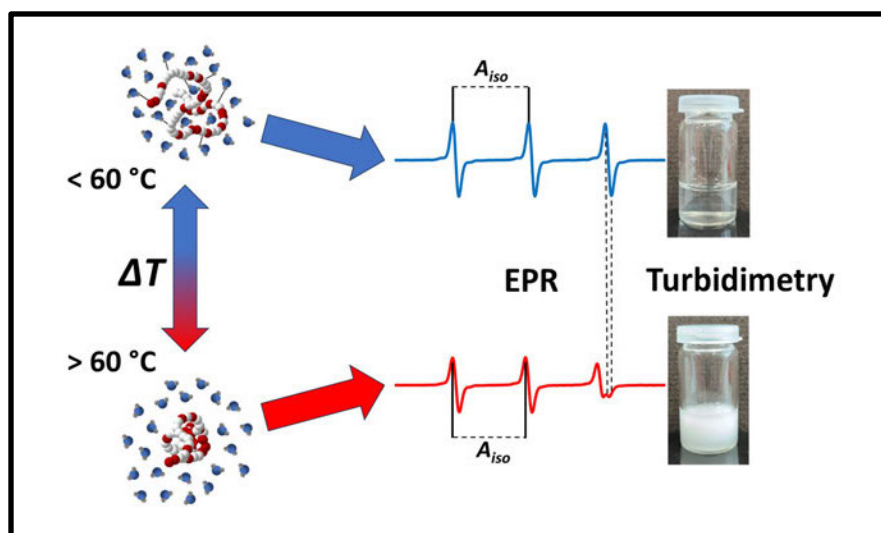
Der Fokus von **Kapitel 4** liegt auf der Synthese einiger Lipid-funktionalisierter rPEGs und der Analyse der Proteincorona, die von Partikeln bei Exposition gegenüber Humanserum gebildet wird, wenn diese mit den Copolymeren beschichtet sind. Mit den synthetisierten Lipiden mit konstanter Kettenlänge und variierendem molaren GME-Anteil wurden Nanocarrier beschichtet, sodass der Effekt des rPEG-Rückgrats auf die Proteincorona analysiert werden konnte. Obwohl die gesamte Konzentration von Proteinen in der Corona deutlich niedriger war für die mit rPEG beschichteten Partikel, wurde die relative Häufigkeit von anti-PEG IgG in der Gesamtmenge IgG für alle synthetisierten Proben höher bestimmt als im Vergleich zu den Lutensol AT50-Proben und blieb annähernd konstant für verschiedene molare Anteile von GME in den Copolymeren. Die niedrigste Konzentration von IgG wurde für die Probe mit dem höchsten molaren Anteil von GME nachgewiesen, welche allerdings erhöhte Mengen an Opsoninen in der Corona aufwies, während Dysopsonine am häufigsten in Proben mit niedrigen GME-Anteilen gefunden wurden. Die Schlussfolgerung wurde gezogen, dass die unerwartet niedrige Beschichtungsdichte der synthetisierten rPEGs auf der Partikeloberfläche einen wesentlichen Einfluss auf die Bildung der Proteincorona hatte, sodass lediglich generelle Trends in der Zusammensetzung beobachtet werden konnten.

Anhang Kapitel 1 gibt eine Einführung in das Konzept von rPEGs und liefert die Grundlage für die Untersuchungen, die in dieser Arbeit vorgenommen wurden. Die erfolgreiche Synthese und Charakterisierung unter AROP-Bedingungen wurde für rPEGs mit einem molaren GME-Anteil von bis zu 74 mol% durchgeführt. Statistische Auswertung bot eine Einsicht in das bei hohen GME-Anteilen verminderte Auftreten von größeren EO-Epitopen. Erste turbidimetrische Messungen zeigten keine Phasenseparation unterhalb von 70 °C für wässrige Lösungen der Polymere bis zu einer Konzentration von 10 mg mL⁻¹. Zellviabilität und B-Zellen-Aktivierung der rPEG-Proben sowie deren *in vitro* Hemokompatibilität in menschlichen Blutproben wurde demonstriert und zeigte vergleichbare Werte zu mPEG. Kompetitive Rückgrat-selektive und Endgruppen-selektive APA ELISAs konnten eine deutliche Reduktion der relativen Antikörper-Affinität zu rPEGs mit steigendem GME-Anteil im Vergleich zu mPEG zeigen.

Graphical Abstract

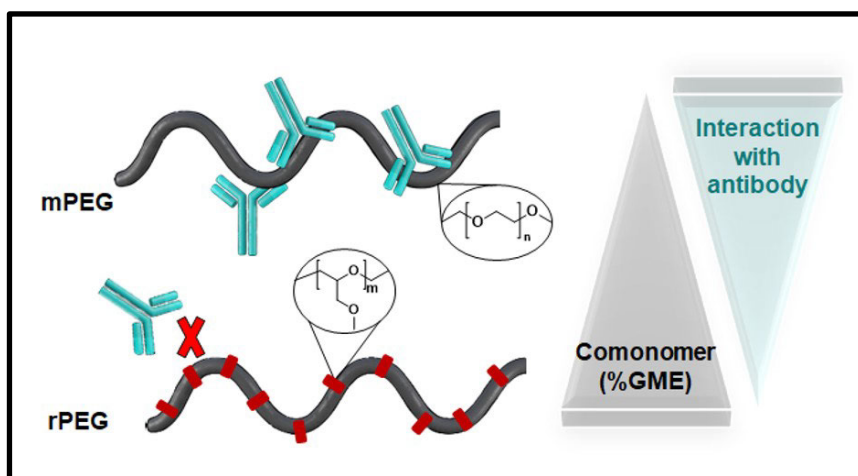
Chapter 2:

Nanososcopic characterization of the thermal phase behavior of random poly(ethylene glycol – glycidyl methyl ether) copolymers (rPEGs)



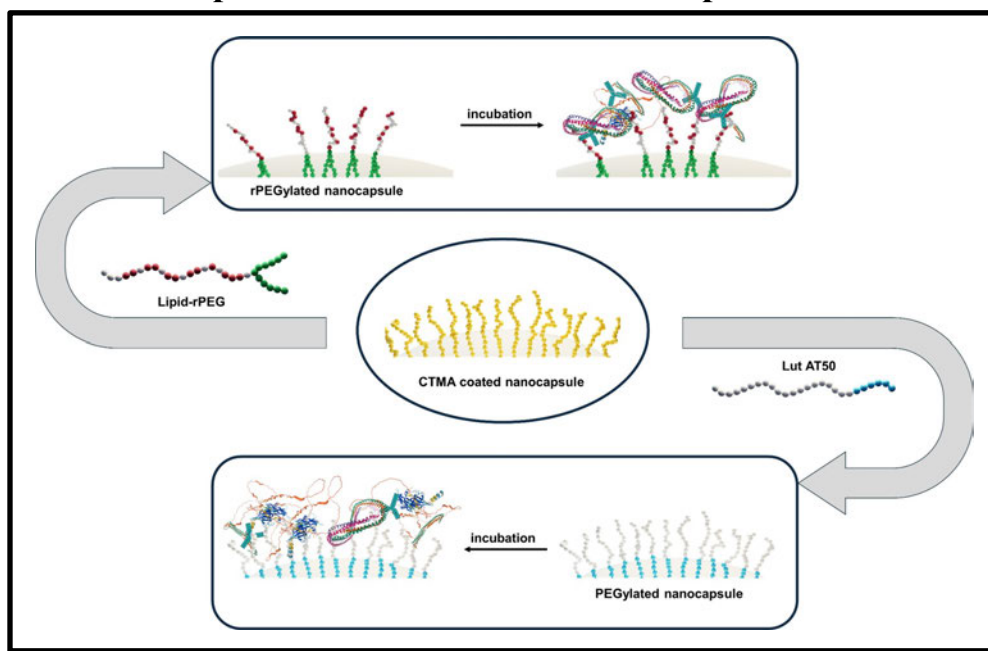
Chapter 3:

Overcoming PEG Antigenicity: Statistical PEG Isomers Reduce Antibody Binding



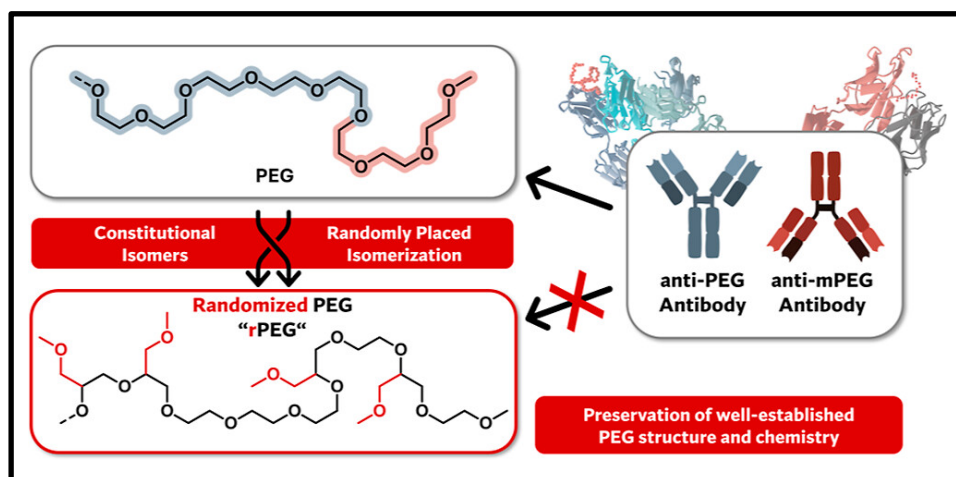
Chapter 4:

Effect of surface functionalization with lipidated random poly(ethylene glycol – glycidyl methyl ether) copolymers (Lipid-rPEGs) on the formation of the protein corona of silica nanocapsules



Appendix Chapter 1:

Isomerization of Poly(ethylene glycol): A Strategy for the Evasion of Anti-PEG Antibody Recognition



Chapter 1

Introduction

Polyethers, Poly(ethylene glycol) (PEG), Anionic ring-opening polymerization (AROP), Copolymerization, PEG conjugation to active pharmaceutical ingredients (APIs), Thermoresponsive behavior in aqueous solution

Introduction

This chapter aims to give a brief overview of the material class of polyethers and will focus on some important representatives of this group. Thereby, emphasis will be placed on **poly(ethylene glycol) (PEG)** as the most prominent polyether. In the first part of the overview the general structure and properties of polyethers will be discussed. The second part will introduce the synthesis of polyethers with a particular focus on the copolymerization of epoxide monomers and the mathematical description thereof. Further discussions about thermoresponsive behavior in aqueous solution as well as medical applications with special attention on PEGylation will also be included in this introduction.

1. Polyethers

Among polymers, polyethers represent one of the most important material classes for applications in academic research and industry.

Structurally, they are built of repeating units which are linked by carbon-oxygen ether-bonds with a C–O bond length of 143 pm and an angle of 112° .¹ Aliphatic polyethers can be described by the general formula $(\mathbf{R-O-R'})_n$ in which R and R' both represent alkyl groups and **n** shows the number of repeating units *i.e.* the degree of polymerization.¹ While most polyethers like poly(butylene oxide) (PBO) or poly(tetrahydrofuran) (PTHF) follow the convention of being named after their respective monomers, the name “poly(ethylene glycol)” is commonly used for the polymer derived from ethylene oxide (EO) because of its structural similarity to ethylene glycol. Mostly, the name is used in biomedical research where PEGs of a molar mass below $30,000 \text{ g mol}^{-1}$ are prevalent. The conventional name poly(ethylene oxide) refers to polymers of EO with a molar mass beyond $30,000 \text{ g mol}^{-1}$ which are mainly used in polymer research.² Likewise, the structurally similar poly(propylene oxide) (PPO), which contains an additional methyl group in every repeating unit, can also be referred to as poly(propylene glycol) (PPG).³ Some examples of polyethers and their corresponding monomers are given in **Figure 1**.

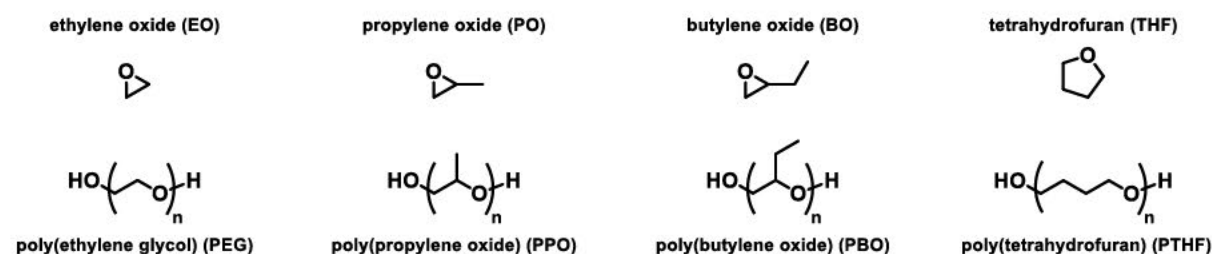


Figure 1. Examples of common epoxide monomers and the polyethers derived from them.

Polyethers have applications in **material science** as preliminary products in the production of polyurethanes^{4,5} and as potential solid electrolyte in lithium-ion batteries.^{6,7} Thanks to its excellent water solubility and low toxicity, PEG is also used in a wide range of **medical and cosmetical applications**. It serves as an emulsifier in cremes⁸ and can be used as a laxative when taken orally (Macrogol).^{9,10} Additionally, copolymers like poloxamers – block copolymers containing a PPG block linked with two PEG blocks on its chain ends – can be used as surfactants¹¹ and show micelle formation in aqueous solution.¹² The annual global production of PEG was 2.5 million metric tons in 2022¹³ and production of PPG was 1.9 million metric tons in the same year.¹⁴ In 2022 the global market size of PEG and PPG were 3.9 billion dollars¹⁵ and 4.4 billion dollars¹⁶ respectively.

2. Poly(ethylene glycol) (PEG)

The **history of PEG** goes back to 1859¹⁷ when Agostinho Vicente Lourenço synthesized the first oligomers of ethylene glycol by heating the compound with ethylene dibromide. A similar reaction was reported at roughly the same time by Charles Adolphe Wurtz, who previously discovered the synthesis of ethylene glycol in 1856¹⁸ and later published the synthesis of ethylene oxide in 1859¹⁹ as well as its polymerization in 1878.²⁰

At low molar masses up to about 600 g mol⁻¹ PEG exists as a viscous liquid at room temperature. At about 1,000 g mol⁻¹ it becomes a wax-like solid by forming crystallized structures in discrete regions of the polymer^{21,22}. With increasing molar mass, the degree of crystallinity increases as well. In its solid state PEG is considered **semicrystalline**.²³ Single crystals of PEG are known for monodisperse PEG₁₆ of very high purity (> 99.6%) and were investigated at – 123 °C where a

closed-packed helical structure was found.²⁴ Other polyethers like PPO can exhibit different microstructures depending on the optical activity of the monomer and the method of polymerization. Using strongly basic initiators to polymerize racemic PO will result in *atactic* PPO which is amorphous at room temperature. If enantiomerically pure PO is used, highly crystalline *isotactic* PPO can be obtained.^{25,26} The same result can be achieved by using metal-based catalysts for stereoselective polymerization of PO.²⁶ Depending on the molar mass, the melting temperature (T_m) of semicrystalline PEG is given between 60 °C and 65 °C and the glass transition temperature (T_g) can be found between – 50 °C and – 77 °C^{27–30}, depending on molecular weight. For PPO, T_g is largely independent from the degree of crystallinity and is found at approximately – 69 °C to – 73 °C.³¹ A melting temperature is only found for *isotactic* PPO and is given in literature at approximately 68°C for PPO with a molar mass of 100,000 g mol⁻¹.³² Since enantiomerically pure PO and catalysts for stereoselective polymerization are very cost intensive, *isotactic* PPO has no commercial application.²⁵

Polyethers are soluble in certain organic solvents like chloroform, dimethylformamide (DMF) and tetrahydrofuran (THF).²² PEG also shows excellent **water solubility** unlike most other polyethers.³³ The unusual water solubility of PEG can be explained by the ether oxygen spacing of 2.88 Å in the PEG chain which corresponds to the distance of oxygen atoms (2.85 Å) in a tetrahedral water lattice. Because of this, the oxygens of PEG can participate in the hydrogen-bonded water network and form a “**hydration shell**” around the polymer.^{34,35} For polyethers with hydrophobic side chains this formation is impeded. Therefore, their water solubility is considerably lower than PEG.³⁴

3. Anionic ring-opening polymerization (AROP)

Polyethers can be synthesized via different routes. While cationic ring-opening polymerization and catalytic polymerization are possible, **anionic ring-opening polymerization (AROP)** gives the best control over the reaction. It was first published in 1929 by Staudinger and Schweitzer³⁶ who achieved molar masses of several thousand g mol⁻¹ by reacting ethylene oxide with metallic potassium. Under strictly controlled conditions AROP can be conducted as a “living polymerization”. The concept was described by Flory³⁷ in the 1940s and early 1950s before Szwarc

introduced the term in 1956³⁸ after demonstrating the “living character” of the polymerization of styrene with metallic sodium.

A **living polymerization** is characterized by the absence of permanent termination and chain transfer reactions in a closed system. Since the initiation proceeds at a much higher rate than the propagation, all chains begin their growth at approximately the same time. This leads to a narrow distribution of chain lengths in the polymer.³⁹ The reaction follows an S_N2 mechanism where the epoxide ring is opened by a strong base like an alkoxide. Consequently, the newly formed terminal alkoxide reacts with another epoxide and the chain grows further (**Figure 2**). Termination of the reaction is usually achieved by adding a proton donor like low molecular alcohols (*e.g.* methanol), diluted hydrochloric or acetic acid, or an excess of water.^{40,41}

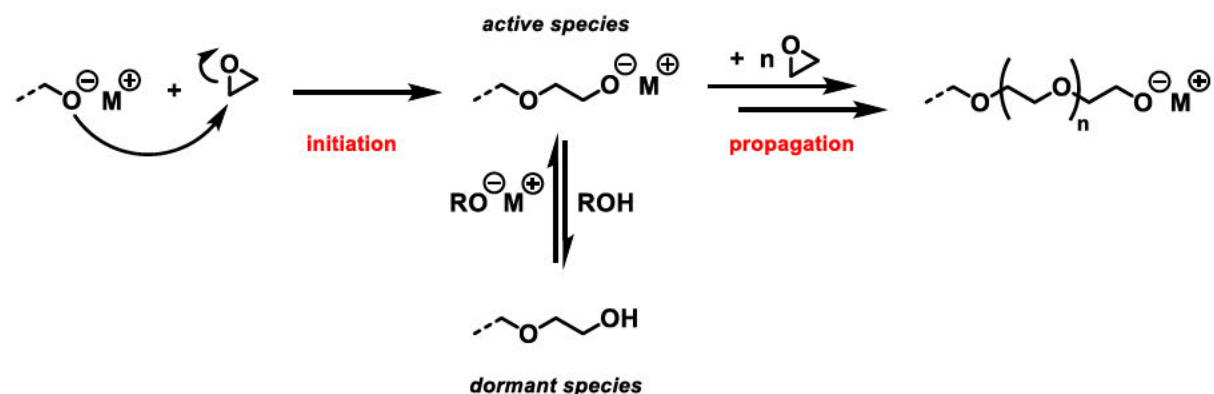


Figure 2. Mechanism of initiation and chain propagation in anionic ring-opening polymerization.

As illustrated in **Figure 2**, partial deprotonation of the alcohol is sufficient for polymerization. **Proton exchange** between active chain ends is very rapid in the equilibrium between the active and dormant species so that the propagation of the chains is not suppressed². Since the proton exchange is reversible, it is not considered a termination or chain transfer reaction. Nevertheless, because of the presence of a dormant species in the reaction mixture, the reaction is in this case no longer considered “living” but instead “controlled”. Although achievable molar masses are limited in the case of controlled anionic polymerizations, low dispersities can still be obtained.

Unlike low molecular compounds, polymers generally show a distribution of different molar masses caused by varying chain lengths obtained during the polymerization. To describe this phenomenon, an average molar mass is given for polymer samples instead of a singular defined molar mass.^{40–42} In the majority of cases, the number average molar mass M_n and the weight average molar mass M_w are used:

$$M_n = \frac{\sum_i n_i M_i}{\sum_i n_i} = \frac{\sum_i m_i}{\sum_i n_i} = \sum_i x_i M_i \quad (\text{eq 1})$$

$$M_w = \frac{\sum_i m_i M_i}{\sum_i m_i} = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} = \sum_i w_i M_i \quad (\text{eq 2})$$

Here, n_i represents the number of polymer chains with the molar mass M_i , while m_i represents the total weight of the polymer chains with the molar mass M_i . Consequently, x_i is the molar fraction and w_i is the weight fraction of the respective chains. To quantify the statistical distribution of the chain lengths, the dispersity D was introduced.

$$D = \frac{M_w}{M_n} = 1 + \frac{1}{P_n} \quad (\text{eq 3})$$

$$\text{with} \quad P_n = \frac{M_n}{M_{ru}} \quad (\text{eq 4})$$

D can be defined as the ratio of the weight average and the number average molar mass and is always greater or equal to 1. This is highlighted through the definition over the number average degree of polymerization P_n . It is calculated as the ratio of M_n and the molar mass of the repeating unit M_{ru} and therefore gives the average number of repeating units in the polymer chains.

For a sample in which all polymer chains have exactly the same degree of polymerization, M_n and M_w would have the same value and thus the dispersity would be equal to 1. In this case the sample would be considered monodisperse. In general, living polymerizations produce polymers with dispersities below 1.10, but monodisperse samples cannot be synthesized because statistical growth of the chains cannot be avoided even under highly controlled conditions. Polymers with a dispersity greater than 1.00 are referred to as polydisperse.

Distribution of chain lengths can be derived as

$$[M_n^-] = [C^-] \frac{(P_n - 1)^{n-1}}{(n - 1)! e^{P_n - 1}} \quad (\text{eq 5})$$

Where $(P_n - 1)$ is often substituted by the kinetic chain length ν

$$P_n - 1 = \nu \quad (\text{eq 6})$$

Here, $[M_n^-]$ is defined as the molar concentration of the n -mere anion while $[C^-]$ is the total concentration of ions in the reaction mixture and remains constant during an ideal living polymerization. Equation (5) follows the general formula of a **Poisson distribution**⁴² which can be observed in polymers synthesized via living polymerizations, for example with size exclusion chromatography (SEC) or matrix-assisted laser desorption ionization coupled with time-of-flight (MALDI TOF) mass spectrometry.

The **rate of the reaction** can be tuned by the conditions chosen for the procedure. Factors like the degree of deprotonation, the counterion $M^\oplus$ of the deprotonated alcohol and the solvent chosen for the polymerization influence the dissociation behavior of the ion pair at the propagating chain end.^{41–43}

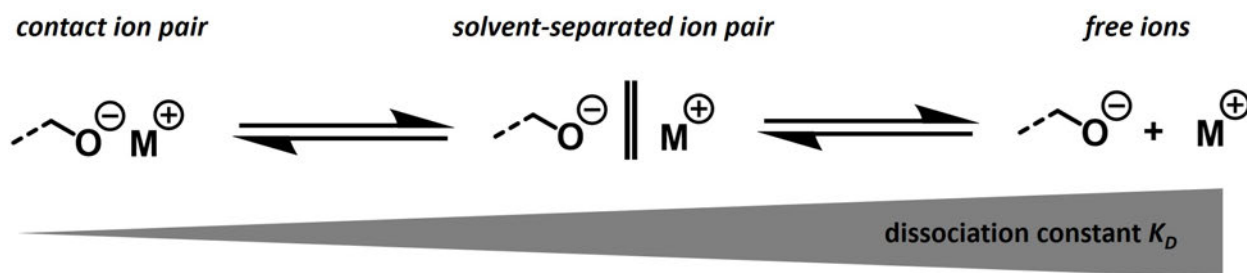


Figure 3. Equilibrium of ion species during anionic polymerizations.

All species of ion pairs appearing during an anionic polymerization are in an equilibrium to each other (**Figure 3**). With increasing dissociation of the ion pair, the reaction rate increases significantly. Free ions are several orders of magnitudes more reactive than ion pairs in anionic polymerizations.⁴¹

Different **solvents** can shift the equilibrium of the ion species. Less polar aprotic solvents like THF or toluene will lower the dissociation constant K_D of the ion pair because the ions cannot be stabilized efficiently by the solvent. The shift towards contact ion pairs and solvent-separated ion pairs will therefore lower the rate of the reaction. On the contrary, a polar solvent like dimethyl sulfoxide (DMSO) or hexamethylphosphoric triamide (HMPTA) will dissolve the ions more effectively and consequently shift the equilibrium towards free ions.⁴⁴

Additionally, the **counterion M^+** will also influence the equilibrium between the species. According to the concept of “hard and soft acids and bases” (HSAB theory) developed by Pearson⁴⁵, hard Lewis acids like lithium ions will form stronger ionic bonds with a hard base like an alcoholate which leads to lower dissociation constants and consequently to a shift of the equilibrium towards a contact ion pair. A soft Lewis acid like cesium on the other hand will only form weak bonds with oxygen and enables a much higher rate of reaction. As a result, lithium salts are usually not applied in oxyanionic polymerizations. Instead, potassium and cesium salts like potassium *tert*-butoxide (KO^tBu) and anhydrous cesium hydroxide are typically used.^{46,47}

To assess the difference between reactivities of ion pairs and free ions, the kinetic of the polymerization has to be evaluated.

For the dissociation



where $[P^{\pm}]$ is the concentration of the ion pair, $[P^{-}]$ is the concentration of the propagating free ion and $[M^{+}]$ is the concentration of the counterion, the dissociation constant K_D can be defined as

$$K_D = \frac{[P^{-}] [M^{+}]}{[P^{\pm}]} \quad (\text{eq 8})$$

and the apparent rate constant of the propagation k_p^{app} is defined as

$$k_p^{app} = \frac{k_p^{-} [P^{-}] + k_p^{\pm} [P^{\pm}]}{[P_t]} \quad (\text{eq 9})$$

with k_p^{-} and $k_p^{\pm}$ as the rate constants of the propagation of the free ions and the ion pairs, respectively, and $[P_t]$ as the total concentration of propagating chain ends.

If there are no other ions introduced to the reaction mixture, $[P^{-}] = [M^{+}]$ can be applied. Since the concentration of free propagating ions is much lower than the concentration of ion pairs in most systems, $[P_t]$ can approximately be assumed to be equal to $[P^{\pm}]$.

Considering equation (8), $[P^{-}]$ can be expressed as

$$[P^{-}] = \sqrt{K_D [P_t]} \quad (\text{eq 10})$$

And therefore $[P^{\pm}]$ is given as

$$[P^{\pm}] = [P_t] - \sqrt{K_D[P_t]} \quad (\text{eq 11})$$

Combining equations (9), (10) and (11) leads to

$$k_p^{app} = k_p^{\pm} + \frac{(k_p^{-} - k_p^{\pm}) \sqrt{K_D}}{\sqrt{[P_t]}} \quad (\text{eq 12})$$

A plot of k_p^{app} versus $\sqrt{[P_t]}^{-1}$ yields $k_p^{\pm}$ as the intercept of a straight line and $(k_p^{-} - k_p^{\pm}) \sqrt{K_D}$ as its slope. $[P_t]$ can be determined by NMR spectroscopy while K_D is available through conductivity measurements. For the AROP of epoxides, values for $k_p^{\pm}$ are typically found to be 1 – 2 orders of magnitude lower than the ones for k_p^{-} , highlighting the high reactivity of free ions compared to ion pairs.^{41,42,48}

The rate of the propagation can furthermore be increased by the addition of **crown ethers or cryptands**⁴⁹ to the reaction mixture. Through complexation of the counterion the ionic bond between the oxygen anion of the propagating chain end and the metallic cation is substantially weakened. The reaction rate therefore approaches values expected for the propagation of a free ion. In the case of epoxide polymerization, the polymer backbone itself resembles a crown ether structure (**Figure 4**). This leads to a higher solvation of the counterion and accordingly to an enhanced reaction rate.⁴³ The effect can be observed through the auto acceleration of the reaction at the onset of the polymerization.

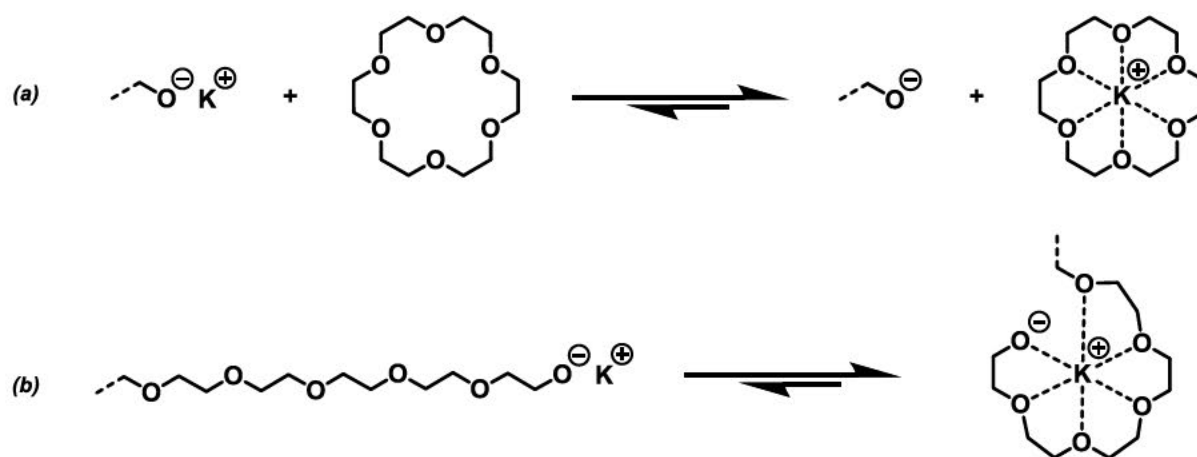


Figure 4. Complexation of a potassium counterion on the propagating chain end in AROP, (a) complexation by addition of 18-crown-6, (b) complexation by the polymer backbone.

Another advantage of crown ethers is the reduction of **chain transfer reactions** in the synthesis of polyethers with aliphatic side chains like PPO. This side reaction occurs when the propagating chain end abstracts a proton from the methyl or methylene group neighboring the epoxide ring instead of reacting directly with the methylene group of the ring. The subsequent reaction creates a new allylic alkoxide which can initiate a new polymer chain. The reaction is given in **Figure 5**.

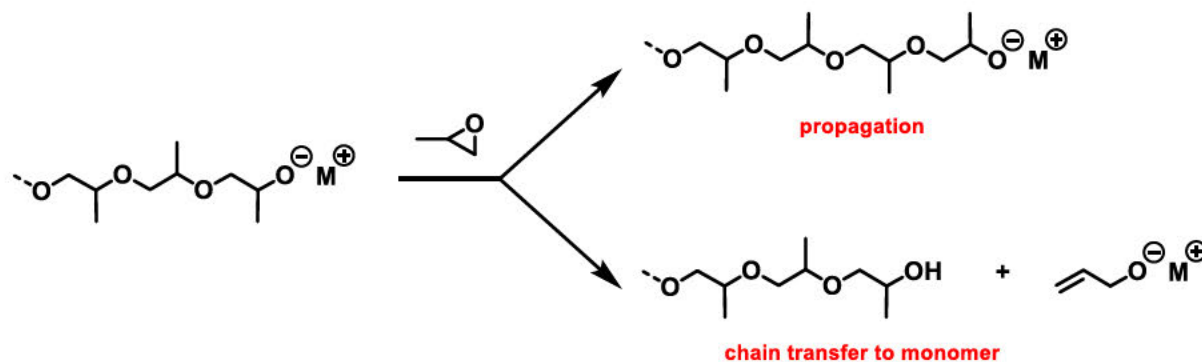


Figure 5. Illustration of the desired propagation reaction compared to the transfer-to-monomer side reaction.

These conditions limit the molar mass of PPO synthesized in bulk to $6,000 \text{ g mol}^{-1}$. The delayed initiation of new chains also leads to broader molecular weight distributions than expected in a living polymerization. Addition of crown ethers increases the rate of the propagation so that PPO with molar masses of $13,000 \text{ g mol}^{-1}$ and relatively low dispersities of 1.18 – 1.23 can be obtained

in bulk.⁵⁰ Other methods to reduce chain transfer reactions include polymerization with a double metal cyanide (DMC) catalyst and a monomer-activated ring-opening polymerization (MAROP).

DMC catalysts are hybrid organic-inorganic materials composed of various combinations of zinc, iron, nickel or cobalt salts. The active site of the catalyst, which contains a zinc atom, substitutes two of its anionic ligands with PO and activates the epoxide ring. Propagation occurs while the chain is coordinated to the zinc atom by insertion of additional monomer. This method effectively suppresses the chain transfer side reaction so that molar masses of more than 40,000 g mol⁻¹ can be achieved for PPO.^{51,52}

Even higher molar masses can be reached with **MAROP**. This method was developed by Carlotti and Deffieux in 2004⁵³ and utilizes a Lewis acid like a trialkyl aluminum species to activate the epoxide ring of the monomer. At the same time, the Lewis acid forms an “ate-complex” with a nucleophile, which can initiate propagation of the activated monomer.⁵⁴

4. Copolymerization

Combination of different monomers in a single polymerization leads to copolymers with distinctive properties different from the respective homopolymers. These properties depend on the physicochemical nature of the monomers used but also on the composition and architecture of the polymers.

Considering linear chain growth, three kinds of copolymer structures can be described: **block copolymers, statistical copolymers and alternating copolymers**. In some cases, statistical copolymers are further divided into (ideal) random copolymers and gradient copolymers.⁴⁰ Examples of these structures are depicted schematically in **Figure 6**.

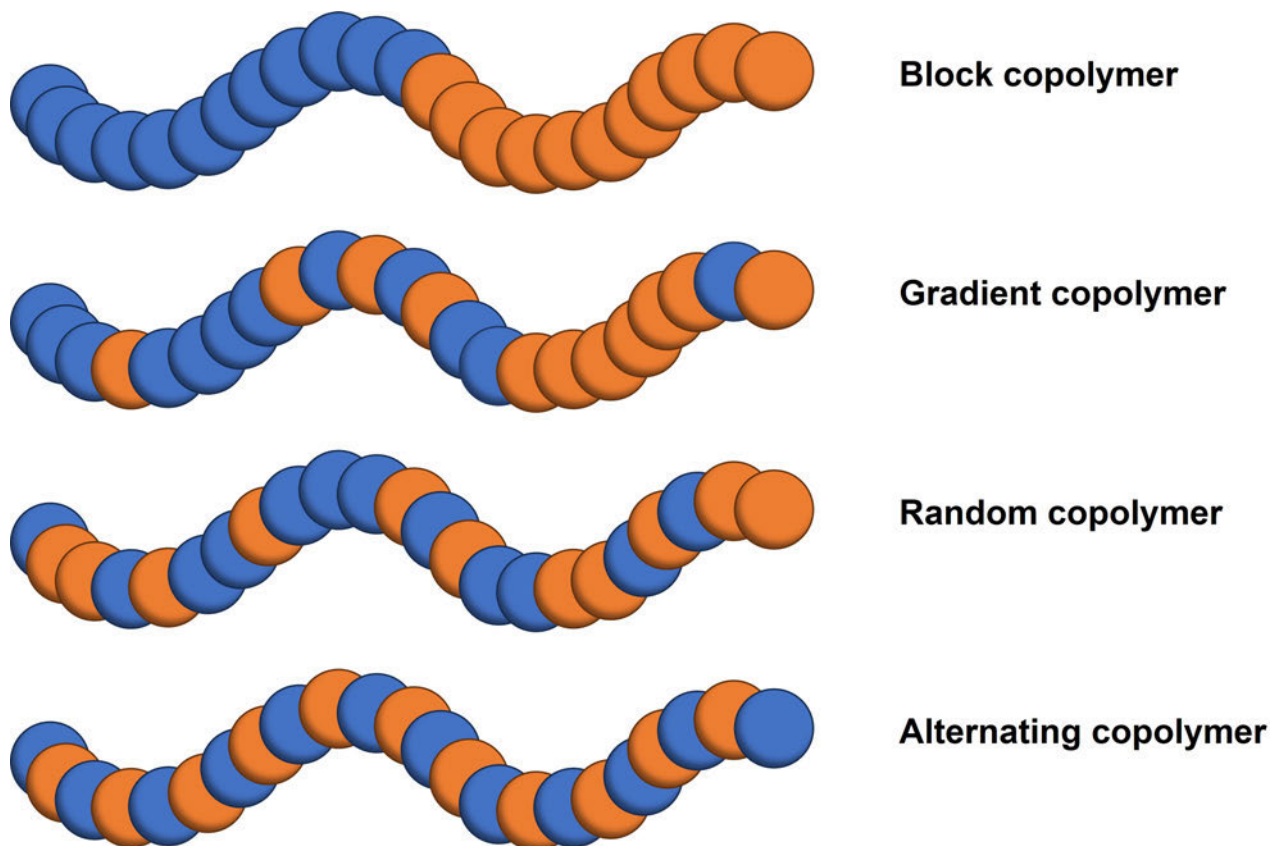


Figure 6. Schematic illustration of possible monomer sequences in copolymers of identical composition.

Occasionally, the terms “statistical” and “random” copolymer are used synonymously while there exists a clear distinction between both notions. Statistical copolymerization dictates that monomers are incorporated into the chain under statistical (Markovian) laws. For a linear chain growth, monomers are therefore added to the chain with a certain probability other than 0 % or 100 % but this probability does not have to be identical for all monomers.

4.1. Polymer composition and reactivity ratios

The **architecture** of the polymer chain depends on the reactivity of the different monomers. It can be determined by various analytical methods, for example MALDI TOF or ^1H - and ^{13}C -NMR spectroscopy.⁴¹ As an alternative to the investigation of the obtained polymers after termination of

the reaction, *in situ* ^1H -NMR kinetics allow for a direct observation of the propagation of the polymer chain and the consumption of the monomers.^{44,55}

To quantify the reactivity of the monomers with themselves or other comonomers, the rate constants of the different propagation reactions must be considered. Schematics of the reactions are given in **Figure 7**.

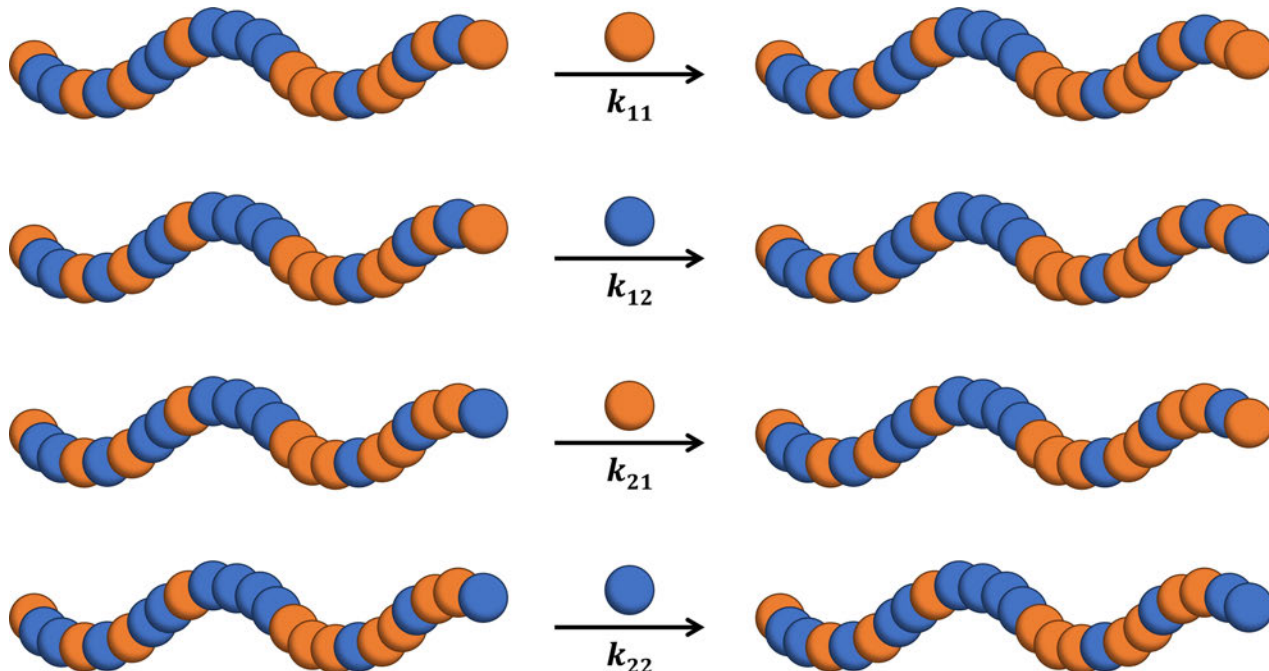


Figure 7. Schematic illustration of all possible propagation reactions in a copolymerization of 2 monomers (orange = monomer 1, blue = monomer 2) including the rate constants k of the different propagation steps.

Here, the **reactivity ratios** r_1 and r_2 are the ratios of the rate constants k of the propagation steps.^{41,56}

$$r_1 = \frac{k_{11}}{k_{12}} \quad (\text{eq 13})$$

$$r_2 = \frac{k_{22}}{k_{21}} \quad (\text{eq 14})$$

It can be deduced from these equations that determination of the reactivity ratios will provide a prediction which polymer architecture is to be expected for different monomer combinations.

If r_1 and r_2 are both much smaller than unity ($r_1 \approx r_2 \approx 0$) the monomers have a very low tendency to react with themselves and rather react with each other. In this case a strictly alternating copolymer is to be expected. For $r_1 < 1$ and $r_2 < 1$ cross-propagation is still preferred but not imperative, meaning that statistical copolymers will be formed. A similar case is found for $r_1 > 1$ and $r_2 > 1$ where homopropagation is preferred. For $r_1 \gg 1$ and $r_2 \gg 1$ block copolymers will be formed because the tendency for the monomers to react with themselves is much greater than the tendency to react with each other. However, since the propagating chain ends stay active indefinitely during a living polymerization, block copolymers are mostly synthesized by sequential monomer addition rather than in a batch polymerization.^{57,58} Alternatively, a macroinitiator can be used as well. For $r_1 > 1$ and $r_2 < 1$ a gradient copolymer will be formed since monomer 1 will be added to the chain at a higher rate than the other, independent from which monomer is currently at the propagating chain end. This monomer will therefore be added preferably until its concentration in the reaction mixture decreases to a point where the probability of addition of monomer 2 surpasses the probability of monomer 1. For the special case in which $r_1 \gg 1$ and $r_2 \ll 1$, two separate homopolymers can be formed because cross-propagation is extremely unlikely. A good example for this can be found in the radical copolymerization of styrene and vinyl acetate ($r_{St} = 55$, $r_{VA} = 0.01$). The vinyl acetate radical is more reactive than the styrene radical by a factor of 1000, while the styrene monomer is 50 times more reactive than the vinyl acetate monomer. Since the more stable styrene radical will not react with the more stable vinyl acetate monomer, only homopolymerization is observed.⁴⁰

A copolymerization is referred to as “ideal” if $r_1 \cdot r_2 = 1$ or $r_1 = 1/r_2$. This is the case when $k_{11} = k_{21}$ and $k_{12} = k_{22}$. Ideal copolymerizations are characterized by the property that each monomer has a clearly defined probability to be added by the propagating chain independent of the adjacent monomer – although this probability does not have to be equal for both monomers. A hypothetical case where $r_1 = 5$ and $r_2 = 0.2$ can be referred to as an “ideal statistical copolymerization” and will result in a gradient copolymer. Only in the case of

$r_1 = r_2 = 1$ the copolymerization will be “ideally random”. Here, both monomers have the same probability to be added to the propagating chain at any given point during the polymerization independently of the adjacent unit. This also means that the composition of the copolymer will solely be determined by the composition of the reaction mixture.

Two distinct types of models have been established to describe copolymerization. Although primarily developed to describe radical copolymerization, these models are also applicable to ionic copolymerization.

4.2. The terminal model for evaluation of the copolymer composition

The terminal model makes the assumption that the rate of chain propagation is solely dependent on the terminal (or ultimate) monomer unit on the propagating chain. Therefore, it can be used to describe non-ideal copolymerizations ($r_1 \neq 1/r_2$). This model was published in 1944 in three different works (Alfrey and Goldfinger⁵⁹, Mayo and Lewis⁶⁰ & Wall⁶¹) and uses a time-independent differential equation to describe the ratio of the rates in which the monomers are built into the chain $d[M_1]/d[M_2]$, *i.e.* the copolymer composition.

$$\frac{d[M_1]}{d[M_2]} = \frac{k_{11}[P_1^*][M_1] + k_{21}[P_2^*][M_1]}{k_{12}[P_1^*][M_2] + k_{22}[P_2^*][M_2]} \quad (\text{eq 15})$$

Here, $[M_1]$ and $[M_2]$ represent the concentrations of the monomers in the comonomer feed and $[P_1^*]$ and $[P_2^*]$ represent the concentrations of the propagating chains with either monomer 1 or monomer 2 as the terminal unit of the propagating chain end. Since in a living polymerization the total number of propagating chains stays constant during the polymerization, a steady state can be assumed in which

$$k_{12}[P_1^*][M_2] = k_{21}[P_2^*][M_1] \quad (\text{eq 16})$$

Using the definitions of the reactivity ratios given in equations (13) and (14) a simplified version of equation (15) can be obtained:

$$\frac{d[M_1]}{d[M_2]} = \frac{[M_1](r_1[M_1] + [M_2])}{[M_2](r_2[M_2] + [M_1])} \quad (\text{eq 17})$$

Equation (17) is commonly referred to as the “**Mayo-Lewis equation**”. The monomer feed concentrations $[M_1]$ and $[M_2]$ are assumed to be constant over the course of the polymerization. However, for non-ideal copolymerizations this is not the case because one monomer is built into the chain at a higher rate than the other. This means that the copolymer composition $d[M_1]/d[M_2]$ will also change during the polymerization. This effect is called “composition drift”.^{41,62} For this reason, the Mayo-Lewis equation is only valid for small conversions where the change in monomer feed concentrations is still negligibly small.

For higher conversions, the integrated form of equation (17) is required, which takes the initial monomer feed concentration and its change over time into account. It is often illustrated using mole fractions to describe the monomer concentrations either in the feed (f_1 and f_2) or in the formed copolymer (F_1 and F_2):

$$f_1 = 1 - f_2 = \frac{[M_1]}{[M_1] + [M_2]} \quad (\text{eq 18})$$

$$F_1 = 1 - F_2 = \frac{d[M_1]}{d[M_1] + d[M_2]} \quad (\text{eq 19})$$

Combining equations (17), (18) and (19) leads to⁴²:

$$\begin{aligned}
 F_1 &= \frac{r_1 f_1^2 + f_1 f_2}{r_1 f_1^2 + 2f_1 f_2 + r_2 f_2^2} \\
 &= \frac{r_1 f_1^2 + f_1(1-f_1)}{r_1 f_1^2 + 2f_1(1-f_1) + r_2(1-f_1)^2} \quad (\text{eq 20})
 \end{aligned}$$

For some special cases of copolymerization, composition of the polymers can easily be deduced by equation (20). For $r_1 = r_2 = 0$ the molar fraction of the copolymer will be $F_1 = 0.5$, indicating a perfectly alternating copolymer. If $r_1 = r_2 = 1$, the molar fraction of the copolymer F_1 will be equal to the molar fraction of the monomer feed f_1 – as expected in an ideal random copolymerization. In case that r_1 has a much greater value than r_2 , all terms not containing r_1 become increasingly negligible and thus, F_1 will converge towards unity. This means that a homopolymer will be formed if there is a large disparity between r_1 and r_2 . Other cases require more complex calculations, many of which were discussed by Wall in 1941.⁶³

A variation of this equation was used by **Fineman and Ross**⁶⁴ in 1950 to develop a method for determination of the reactivity ratios. By rearranging equation (20) into a linear equation, r_1 and r_2 can be calculated as slope and axis intercept of a straight line:

$$Y = X \cdot r_1 - r_2 \quad (\text{eq 21})$$

with
$$Y = \frac{f_1(2F_1 - 1)}{F_1 \cdot f_2} \quad \text{and} \quad X = \frac{f_1^2(1 - F_1)}{F_1 \cdot f_2^2}$$

This method was refined by **Kelen and Tüdös**⁶⁵ in 1975 by introducing an additional parameter $\alpha = \sqrt{X_{min} \cdot X_{max}}$. Here, X_{min} is the smallest value of X calculated by the experimental data while X_{max} is the maximum value for X . These adaptations lead to:

$$\eta = \xi \cdot \left(r_1 + \frac{r_2}{\alpha} \right) - \frac{r_2}{\alpha} \quad (\text{eq 22})$$

with $\eta = \frac{Y}{\alpha + X}$ and $\xi = \frac{X}{\alpha + X}$

The parameter α distributes the weight given to all data points more evenly and therefore reduces the influence of data points derived from error-prone measurement ranges.

Skeist⁶⁶ derived a conversion-composition equation in 1946 to calculate the expected change in composition of the monomer feed and the copolymer:

$$\ln \frac{M}{M_0} = \int_{f_{1,0}}^{f_1} \left(\frac{1}{F_1 - f_1} \right) df_1 \quad (\text{eq 23})$$

By substitution of equation (20) into (23), Meyer and Lowry⁶⁷ were able to expand Skeists theory and develop a model to accurately describe the monomer conversion M/M_0 dependent on its molar fraction f_1 :

$$\frac{M}{M_0} = \left(\frac{f_1}{f_{1,0}} \right)^\alpha \left(\frac{f_2}{f_{2,0}} \right)^\beta \left(\frac{f_{1,0} - \delta}{f_1 - \delta} \right)^\gamma \quad (\text{eq 24})$$

with $\alpha = \frac{r_2}{1 - r_2}$; $\beta = \frac{r_1}{1 - r_1}$; $\gamma = \frac{1 - r_1 r_2}{(1 - r_1)(1 - r_2)}$; $\delta = \frac{1 - r_2}{2 - r_1 - r_2}$

Equation (24) is called “**Meyer-Lowry equation**”. It is valid if $r_1 \neq 1$ and $r_2 \neq 1$ and therefore describes composition of monomer feed and copolymer even at high conversions for non-ideal random copolymerizations if the initial feed composition $f_{1,0}$ as well as the reactivity ratios r_1 and r_2 are known.

4.3. The non-terminal model for ideal copolymerizations

Contrary to the terminal model, the non-terminal model assumes that chain growth is independent of the terminal monomer unit of the propagating chain. Therefore, it is used to describe ideal polymerizations where chain propagation is indiscriminatory for both monomers ($r_1 = 1/r_2$). As explained in chapter 4.1, both monomers are added with a constant but distinct probability to the propagating chain throughout the entire polymerization. Only in the case of an ideal random copolymerization are the reactivity ratios for both monomers equal ($r_1 = r_2 = 1$).

The non-terminal model was first introduced by **Wall** in 1941⁶³ in the analysis of the copolymerization of vinyl monomers. He proposed a differential equation to describe the composition of the copolymer by using the polymerization rates of the monomers $d[M_1]$ and $d[M_2]$:

$$\frac{d[M_1]}{d[M_2]} = \frac{k_{11}[M_1]}{k_{12}[M_2]} = r_1 \frac{[M_1]}{[M_2]} \quad (\text{eq 25})$$

In 1972 **Jaacks**⁶⁸ showed that the terminal model by Mayo and Lewis could be used to determine the reactivity ratios of non-terminal copolymerizations if one monomer is present in a large excess in the monomer feed. This becomes apparent when equation (17) is rearranged to:

$$\frac{d[M_1]}{d[M_2]} = \frac{r_1 \frac{[M_1]}{[M_2]} + 1}{r_2 \frac{[M_2]}{[M_1]} + 1} \quad (\text{eq 26})$$

If $r_1 ([M_1]/[M_2]) \gg 1$ and $r_2 ([M_2]/[M_1]) \ll 1$, equation (26) can be approximated by equation (25). Jaacks also showed that reactivity ratios of non-terminal copolymerizations could be determined by the integrated form of equation (25) for any comonomer ratio.

Beckingham, Sanoja and Lynd⁶⁹ developed a method based on the non-terminal model in 2015 (BSL model). Their work specifically aims at describing the ionic copolymerization which generally exhibits ideal behavior since the rate of monomer incorporation is largely independent on the chain terminus and much rather dependent on the reactivity of the monomer. The BSL model puts the total conversion p_{12} of both monomers in relation to the conversions p_1 and p_2 of each monomer:

$$p_{12}(p_1) = 1 - f_{1,0}(1 - p_1) - (1 - f_{1,0})(1 - p_1)^{r_2} \quad (\text{eq 27})$$

$$p_{12}(p_2) = 1 - f_{1,0}(1 - p_2)^{r_1} - (1 - f_{1,0})(1 - p_2) \quad (\text{eq 28})$$

By tracking conversions during the polymerization by $^1\text{H-NMR}$ and plotting p_{12} against p_1 and p_2 individually, the reactivity ratios can be determined.

In 2019 the **Frey group**⁷⁰ showed that the copolymerization of EO and PO could accurately be described with the Meyer-Lowry equation (24) by adapting it to ideal behavior. For $r_1 = 1/r_2$, the Meyer-Lowry equation can be simplified to:

$$\frac{M}{M_0} = \left(\frac{f_1}{f_{1,0}} \right)^\alpha \left(\frac{1 - f_1}{1 - f_{1,0}} \right)^\beta \quad (\text{eq 29})$$

with $\alpha = \frac{1}{r_1 - 1}$ and $\beta = \frac{r_1}{1 - r_1}$

Since the initial feed concentration $f_{1,0}$ is known, plotting (M/M_0) against f_1 yields the reactivity ratio r_1 of monomer 1 from which r_2 can be calculated under ideal conditions.

Furthermore, by simulating systematic errors in the data they demonstrated that the non-terminal model could obtain reactivity ratios with much smaller deviations than the terminal model for EO-PO copolymerization.

5. PEG conjugation to active pharmaceutical ingredients (APIs)

The approval of recombinant DNA produced insulin in 1982 marked a historical point for the **application of biopharmaceuticals**.⁷¹ In the last four decades, hundreds of biopharmaceuticals have been approved for treatment. In the period between 2018 and 2022 alone, 180 active biopharmaceutical ingredients have entered the market.⁷² Despite this great success, biopharmaceuticals suffer from a lot of setbacks in their application. Poor stability and solubility, short circulation half-lives, potential for immunogenicity and low absorption decreased the utility of first-generation ingredients and necessitated a high administration frequency.^{73–76} To address this issue, several strategies for increasing the efficacy of biopharmaceuticals have been developed.

Protein engineering allows the modification of peptides and proteins by alternation of distinct amino acids in the sequence of these molecules.⁷⁷ Native insulin, for example, tends to form dimeric or hexameric structures, impeding its therapeutic effect after injection. By altering amino acids B23 to B28 in the insulin B chain, self association and accordingly the delay of the therapeutic effect could be reduced.^{78,79} Furthermore, using protein fusion, properties of different proteins can be combined into one molecule.⁸⁰ Limitations of these approaches arise because prediction of the secondary structure of a protein based on the sequence of amino acids is still challenging⁸¹ and can alter effect and biocompatibility of an API.⁸²

Nanocarrier technology has also been at the forefront of drug delivery research for the last decades. Liposomes were among the first discovered nanoparticles. They were first described in 1964 by Bangham and Horne.⁸³ Liposomes are vesicles composed of a lipid bilayer which is formed by amphiphilic molecules. These molecules contain a hydrophobic chain and a hydrophilic head and have a high tendency to form membrane structures in aqueous solution.⁸⁴ Often, phospholipids or cholesterol derivatives are used for this purpose.^{84–87} Thanks to the lipid bilayer, these particles are able to encapsulate hydrophilic as well as hydrophobic components (**Figure 8**) and deliver active ingredients beyond cell membranes⁸⁸ while stabilizing them against degradation.⁸⁹ Furthermore, in case of toxic ingredients like Doxorubicin, which is used for chemotherapy, encapsulation can reduce side effects by releasing the nanocarrier content preferably in targeted tissue.^{85,90} Liposomes also form the base structure of the lipid nanoparticles that were used as delivery systems for the mRNA vaccine against the SARS-CoV-2 coronavirus.⁹¹

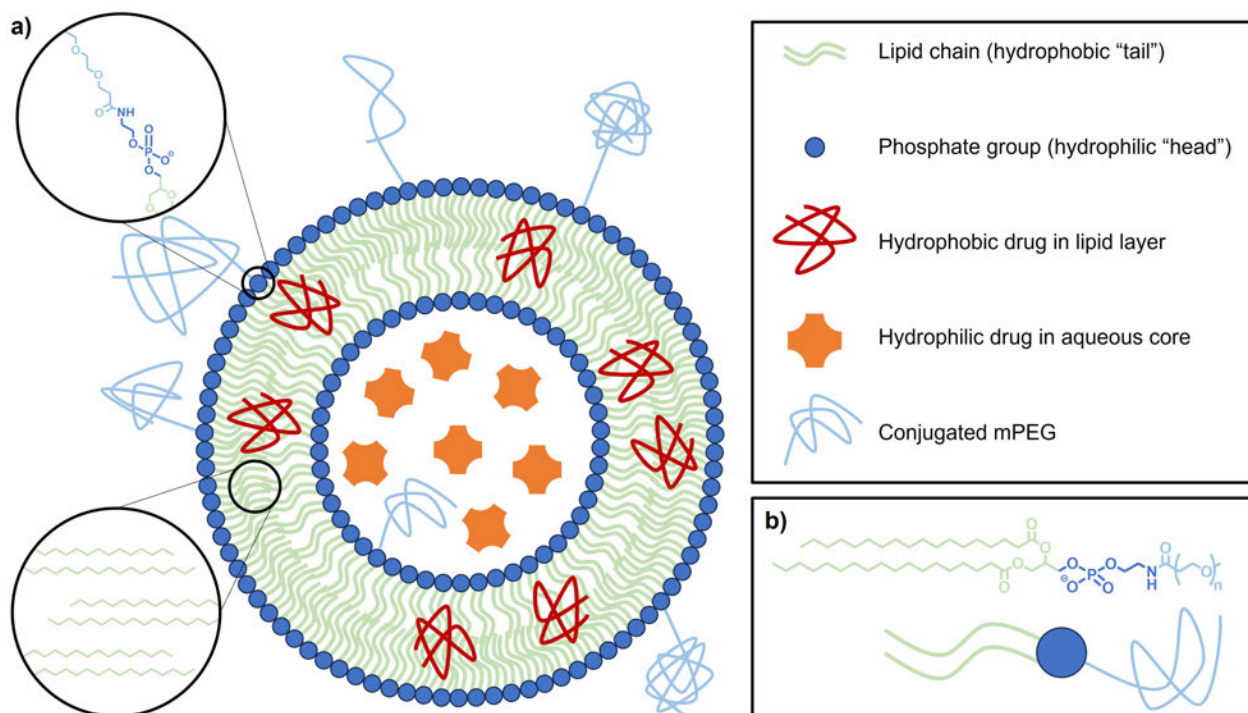


Figure 8. a) schematic composition of a liposome; b) chemical structure of a PEGylated phospholipid, here: 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[methoxy-(polyethyleneglycol)] (DSPE-PEG).

Additionally, several metal-based nanoparticles made from iron oxide, titanium dioxide, gold or silver have been approved for clinical trials in recent years.⁹² Despite these improvements, unfunctionalized nanocarriers suffer from some of the same disadvantages as non-encapsulated APIs, i.e. limited solubility in an aqueous environment and possible activation of immunogenic reactions.

5.1. PEGylation

The PEGylation technique was developed by Abuchowski and Davis in 1977.⁹³ It involves **covalent attachment of a PEG chain** onto an active ingredient with the goal of increasing its circulation time in the bloodstream and can be applied to peptides/proteins and nanocarriers. By the formation of hydrogen bonds PEG binds approximately 3 molecules of water per monomer unit and forms a hydration shell around its backbone. This property is responsible for the high hydrophilicity of PEG, as discussed in chapter 2, and therefore also increases the water solubility

of components attached to it.⁷⁵ Moreover, functionalization with PEG impedes immunogenic recognition and increases circulation half-life of active ingredients significantly.^{94,95} PEGylated liposomal Doxorubicin, for example, was found to increase its circulation half-life by a factor of 60 compared to free Doxorubicin.⁹⁶ Alleviation of immune recognition is caused by steric hindrance of the reaction with immune cells.⁹⁷ The hydration shell provides further shielding of the active ingredient. Binding of water molecules increases the “effective” molecular weight of the PEGylated agent and can reduce glomerular filtration by the kidneys.⁷⁵ Additionally, reduction of surface hydrophobicity decreases protein adsorption on nanocarriers which hinders uptake of the mononuclear phagocyte system (MPS).⁹⁸ Overall combination of these phenomena is commonly known as the “**stealth effect**” of PEG. Higher retention time enables better control of blood levels of active ingredients and reduces the necessary frequency of administration.^{89,97}

Commonly, **methoxy-PEG (mPEG)** is used for conjugation on APIs by chemically activating the free terminal hydroxy group. Although functionalization on the C-terminus of proteins is possible by protein splicing⁹⁹, conjugation is usually carried out on the N-terminus or on free amino groups in the amino acids of the protein.^{73,100} As it is one of the most abundant amino acids, conjugation is typically performed on lysine.^{74,100} Abuchowski and Davis utilized cyanuric chloride for attachment on mPEG and subsequently to bovine serum albumin (BSA).⁹³ Since both remaining chlorides could react with free amino groups, crosslinking of proteins was observed. To solve this issue, Inada *et al*¹⁰¹ synthesized bi-functionalized cyanuric chloride which possesses only one active site for protein conjugation. Second-generation PEGylation processes utilize more selective reagents like *N*-hydroxysuccinimide esters (NHS-esters) for protein functionalization.^{95,100} These reactions are shown in **Figure 9**.

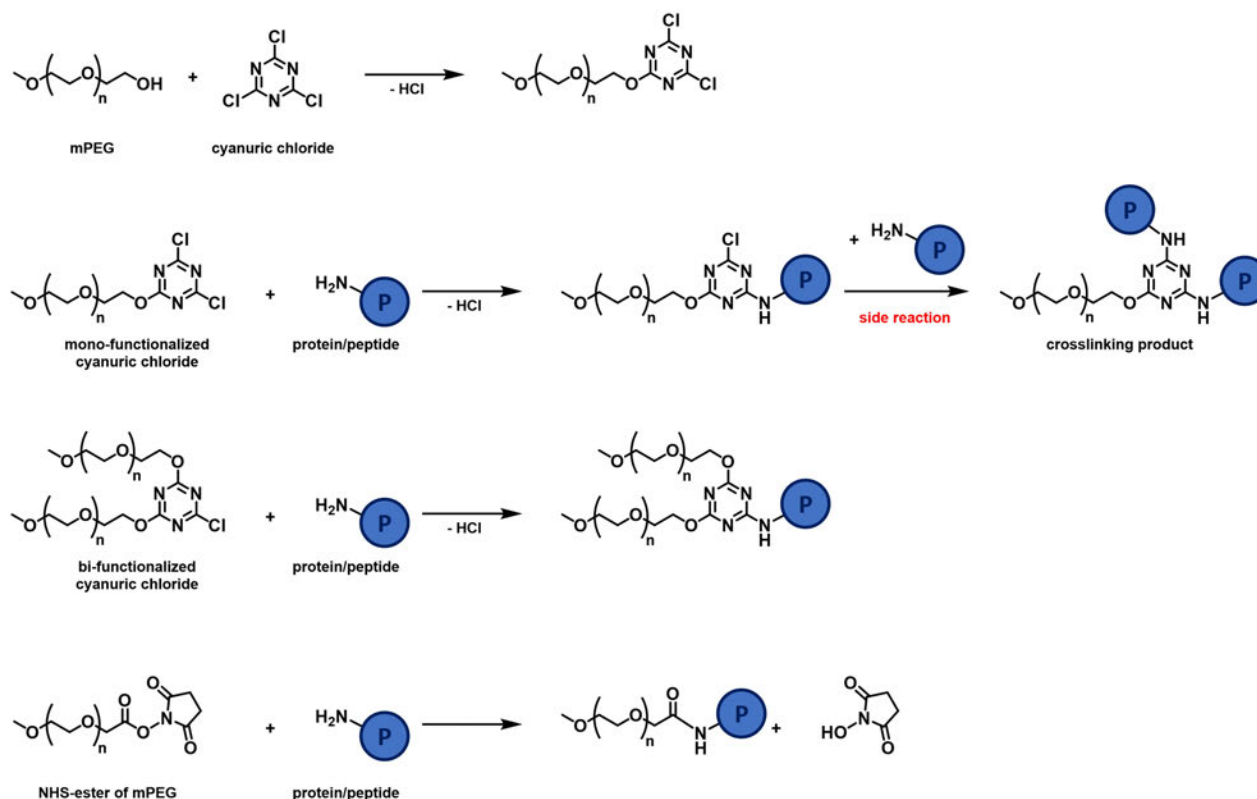


Figure 9. Various protein functionalization reactions of activated mPEG.

5.2. Anti-PEG antibodies

Despite the initial belief that PEG itself could not induce immune reactions, **anti-PEG antibodies (APAs)** were found in animal blood just a few years after the publication of PEGylation by Davis and Abuchowski. In 1983, Richter and Åkerblom published the discovery of specific antibodies to PEG used to functionalize proteins in animal trials.¹⁰² Shortly after, APAs were also found in the human bloodstream – again by Richter and Åkerblom¹⁰³ – but originally dismissed as “of no clinical significance”. In this first publication, mentionable titers of APAs were only found of the Immunoglobulin M (IgM) isotype. Therefore, it was believed that APAs were only generated as an acute reaction in hypersensitive patients and no long-term immunization was expected.

However, in the last 25 years the occurrence of persistent anti-PEG Immunoglobulin G (IgG) in healthy donors has been shown repeatedly^{104,105}, proving that recognition of PEG is “memorized” by the immune system.

While the original publication¹⁰² found anti-PEG IgM in only 0.2% of healthy donors, later studies have found a much higher abundance.^{106–108} A recent study by the Landfester group showed the occurrence of either anti-PEG IgM or anti-PEG IgG in 83 % of healthy donors in the German general population.¹⁰⁹ This abundance of APAs is hypothesized to be caused by frequent contact to PEG in cosmetics or food.^{110–112} Furthermore, large scale vaccinations during the COVID19 pandemic exposed a large majority of the population to subcutaneous injections with PEGylated liposomes, potentially increasing the formation of APAs.^{113,114}

The consequence of immune reactions to PEG is generally an **accelerated blood clearance (ABC)**.¹¹⁵ Opsonization by anti-PEG IgG increases recognition by the MPS and causes a rapid clearance of PEGylated APIs from circulation.¹⁰⁵ Thus, the stealth effect is largely counteracted, and efficacy of the APIs is reduced drastically.¹¹⁶ In more severe cases, complement activation-related pseudoallergy (CARPA)^{117,118} can be observed, on rare occasions even pseudoanaphylaxis.^{116,119}

To address this issue, alternative PEGylation agents have been developed. New hydrophilic polymers have been considered to potentially replace PEG in medical applications.¹¹⁹ **Poly(oxazoline) (POx)** is a highly hydrophilic polymer with adaptable thermodynamic behavior.¹²⁰ It can be attached to nanocarriers¹²¹ and provides a stealth effect towards the immune system.¹²² Like PEG, it has been shown to possess very low toxicity and clinical trials for FDA clearance are currently ongoing.^{123,124} **Poly(sarcosine) (PSar)** possesses similar hydrophilicity. It is derived from the endogenous amino acid sarcosine and has been shown to increase circulation time¹²⁵ and to provide a stealth effect similar to PEG.¹²⁶ Both of these polymer classes can be synthesized under highly controlled conditions. Several other polymers like Poly(vinylpyrrolidone) (PVP)¹²⁷ and Poly(glycerol) (PG)¹²⁸ are also considered for substitution of PEG. In spite of these advances, antibodies against poly(2-methyl-2-oxazoline) were observed in biodistribution experiments in rats.¹²⁹ Therefore, the question remains whether the immune system is capable of adapting to other polymers with regular backbone structures.

5.3. The protein corona

As soon as nanomaterials come into contact with the human bloodstream, proteins bind to the surface of these materials.^{130–134} The adsorbed proteins are commonly referred to as the “**protein corona**”. Because the surface proteins are what is perceived by the cells, the corona dictates the interaction of nanomaterials with cells and therefore also controls their biodistribution and recognition by the immune system.^{135,136} Since biological environments are very complex and the human blood contains thousands of proteins which can even vary between different individuals¹³⁷, the composition of this corona is impossible to predict accurately and has to be investigated by incubation of the materials in blood samples through *in vitro* or *in vivo* experiments.

When nanomaterials enter the bloodstream, abundant proteins like human serum albumin bind to them in the span of a few seconds.¹³⁸ Over the course of several minutes to a few hours, the adsorption kinetics change and less abundant proteins with higher affinity displace the rapidly formed original protein layer.¹³⁹ These high affinity proteins bind to the materials for multiple hours and are called the “**hard corona**”.^{132,135} Occurrence of this competitive adsorption is referred to as the “Vroman effect”.^{140,141} At the same time, a more dynamic outer layer of proteins – the “**soft corona**” – remains in exchange with the biological environment.¹⁴² Additionally, since protein composition varies in different parts of the body, the corona will have different compositions, depending on the route in which the nanomaterials enter the body – be it through inhalation, ingestion or intravenously.¹³⁴

Furthermore, there are several other factors that dictate how the protein corona is formed. A schematic graphical illustration of these factors can be found in **Figure 10**.

The **morphology** of the particle surface can have an effect on the adsorption of proteins. Gold nanoparticles have been shown to adsorb less bovine serum albumin (BSA) when the particle surface was rough, highlighting the influence of morphology on adsorption behavior.¹⁴³ Moreover, a higher degree of denaturation was observed for BSA on a rough particle surface. A similar effect was observed for polystyrene microspheres, where the amount of adsorbed proteins as well as the composition of the protein corona was influenced by etching the surface of the particles.¹⁴⁴

Variation of **particle size and shape** also led to different results during protein corona formation. Larger particles were found to adsorb more proteins overall¹⁴⁵ and can form multiple layers of a protein corona.¹⁴⁶ Smaller particles have been shown to possess a higher density of proteins, because the higher curvature provides more available volume for proteins close to the surface.¹⁴⁷ However, through the ability to form multilayers, the overall amount of adsorbed proteins is higher for larger particles.¹⁴⁶ Meanwhile, particles with different shapes showed different affinities to certain proteins. Investigation of mesoporous silica nanoparticles showed a four times higher amount of overall adsorbed proteins for rods compared to spheres¹⁴⁸, while the concentration of immunoglobulins in the protein corona of rods was found to be higher than in the corona of spheres. In addition, mesoporous silica particles have shown to cause different degrees of denaturation in human serum albumin and fibrinogen depending on their shape.¹⁴⁹

Moreover, **hydrophobicity** of the particle surface plays an important role in protein corona formation. In a study by Yu *et al* investigating the adsorption of BSA on gold nanoparticles functionalized with varying amounts of hydrophilic and hydrophobic ligands, a higher affinity of BSA to hydrophobic surfaces was demonstrated.¹⁵⁰ At the same time, certain proteins like vitronectin were found to slightly prefer hydrophilic surfaces, while the concentration of Apolipoproteins remained approximately constant for all surface functionalizations. These results confirm that several different forces influence the binding behavior of proteins on such particles.^{151,152} In many cases, PEGylation can be used to inhibit the formation of a protein corona on nanomaterials.^{153–157} Using a similar functionalization approach, adsorption of BSA could even be suppressed completely by surface coating of gold nanoparticles with a zwitterionic ligand, after which the hydrodynamic radius of the particles showed no increase after incubation in a BSA-buffer solution.¹⁵⁸

Next to the properties of the nanomaterial itself, its environment also dictates which proteins can be adsorbed.

Adsorption of lysozyme on silica nanoparticles was observed to be influenced by the **pH value** of the protein solution.¹⁵⁹ At an acidic pH value of 5, lysozyme made up a lower percentage of the total protein adsorbed on the particles. The effect was found to be more pronounced for larger

particle sizes. Notably, electrostatic attraction between protein and nanoparticle led to a protein-mediated aggregation of the particles to form clusters at pH 5.

Temperature was also found to have an effect on the composition of the protein corona of polystyrene nanoparticles stabilized with different Lutensol moieties.¹⁶⁰ At temperatures of 4 °C, 25 °C and 37 °C, changes in concentration of clusterin, certain apolipoproteins and fibrinogen could be determined. Additionally, for particles stabilized with Lut-COOH, a significant decrease in coagulation proteins could be seen for increasing temperature. Similarly, adsorption of HSA and Apo-Transferrin on iron nanoparticles was found to be dependent on incubation temperature between 9 °C and 43 °C.¹⁶¹

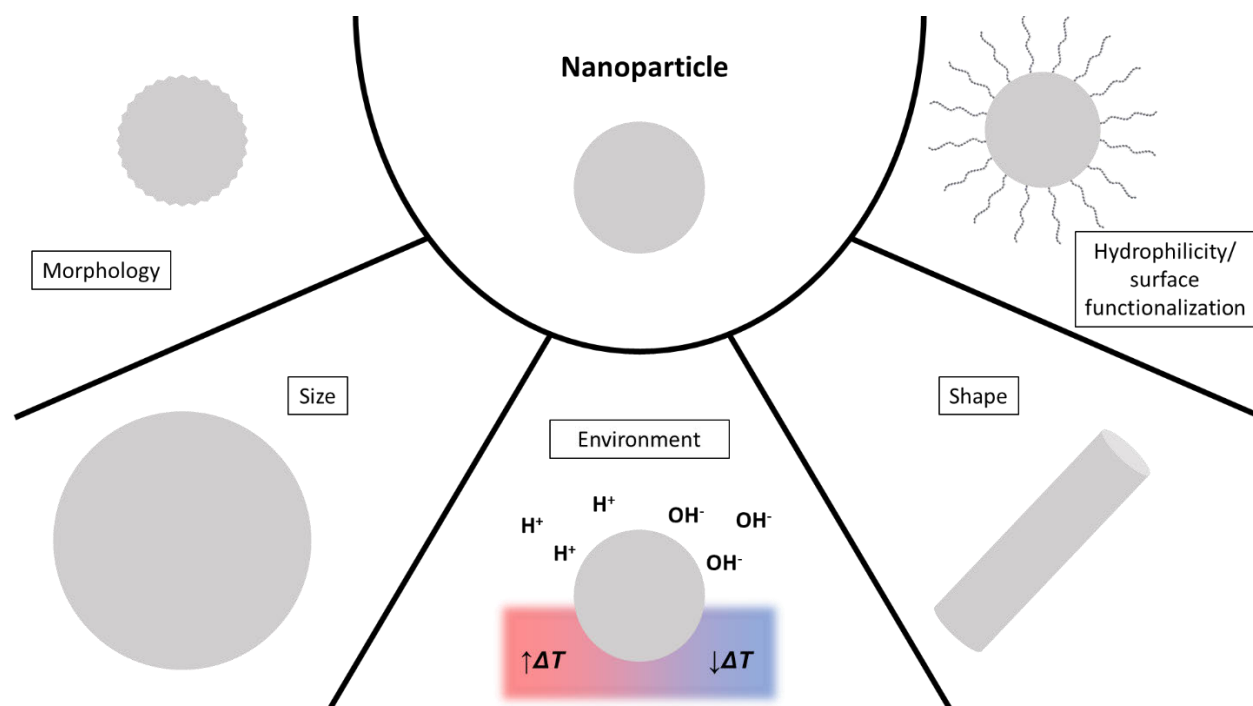


Figure 10. Graphical illustration of different factors that influence protein corona formation on nanoparticles.

Ultimately, understanding how the protein corona is formed and which factors determine its composition enables the design of nanomaterials with reduced interactions with the immune system and therefore enhanced circulation time in the bloodstream. Moreover, targeted cell uptake can be achieved. A strategy to accomplish this goal can be to reduce the adsorption of **opsonins**, which

“mark” nanomaterials for phagocytosis, while possibly amplifying the adsorption of **dysopsonins**, which inhibit rapid clearance mechanisms.^{134,162,163} As a different approach, overall reduction of the amount of adsorbed proteins has been discussed as a stealth mechanism.^{164,165} In both cases, PEGylation or functionalization of the nanomaterials with other hydrophilic polymers has been shown to be a valid approach to reach this goal.

6. Thermoresponsive behavior in aqueous solution

Many polymers are known to exhibit thermoresponsive behavior in solution.^{166–169} This can be expressed by an apparent precipitation of the polymer from the solution or a separation into a polymer saturated solvent phase and a polymer depleted solvent phase. In both cases, phase separation is induced by a change of temperature. The group of polymers that exhibit such behavior is divided into two different kinds. One shows an **upper critical solution temperature (UCST)** and one a **lower critical solution temperature (LCST)**. In the case of an UCST a decrease in temperature leads to a collapse of the polymer in a “coil to globule transition” which causes the polymer to become less miscible or immiscible with the solvent. For polymers with an LCST the same collapse of the coil is induced by an increase in temperature.¹⁶⁹ In both cases this leads to a miscibility gap in the phase diagram. On a macroscopic scale the phase separation can be observed as clouding of the solution. Therefore, both critical solution temperatures can be determined turbidimetrically by measuring the transmittance of visible light dependent on the temperature of a polymer solution. Alternatively, determination by dynamic light scattering and ¹H-NMR are also possible.¹⁷⁰ For a specific concentration of the polymer, the temperature at which turbidity can be observed is defined as the cloud point temperature (T_{cp}). Determining cloud point temperatures for different molar ratios of polymer and solvent will result in a binodal curve in the phase diagram. An upper bound of the binodal represents the UCST, while a lower bound represents the LCST. Therefore, the LCST is the lowest possible temperature where phase separation occurs in a polymer/solvent-mixture and the UCST is the highest possible temperature for a phase separation to take place. Thus, below the LCST and above the UCST, the polymer and the solvent are miscible at any given concentration.^{169,171} In the phase diagram the UCST and LCST are represented by the maximum and respectively minimum of the binodals as can be seen in **Figure 11**. Since for

pharmacological applications only the behavior of the polymers in aqueous solution is relevant, this case will be discussed further in this work.

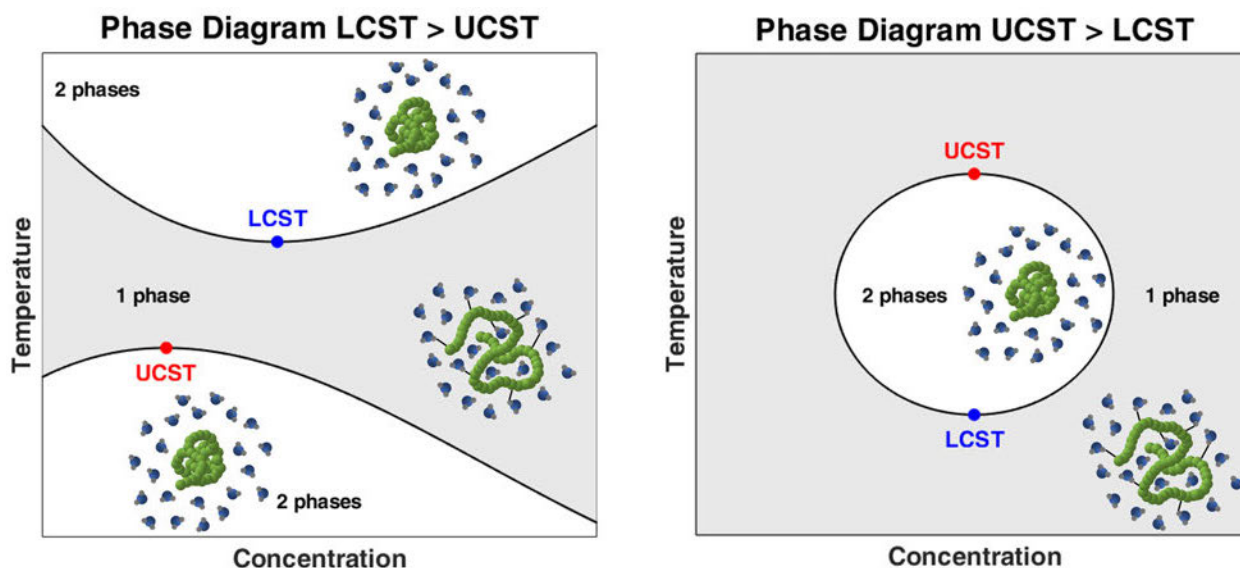


Figure 11. Phase diagrams of binary mixtures of polymer (green) and water (blue) showing the LCST (blue) and the UCST (red) as the minimum and maximum of the binodals, respectively.

Thermodynamically, critical solution behavior can be described by the Gibbs-Helmholtz equation:

$$\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix} \quad (\text{eq 30})$$

For a polymer to dissolve in water the Gibbs free energy of mixing ΔG_{mix} must be negative. For hydration of polymers both enthalpy of mixing ΔH_{mix} and entropy of mixing ΔS_{mix} possess negative values. This can be explained by the formation of hydrogen bonds between the polymer and water, which is enthalpically favored but entropically disfavored.¹⁷² In the case of an LCST transition, the enthalpic term will outbalance the entropic term for low temperatures. With an increase in temperature, the hydrogen bonds between the polymer and water will be weakened and the value of ΔH_{mix} will decrease. At the same time, the entropic term $T\Delta S_{mix}$ will increase in value, ultimately leading to a positive ΔG_{mix} and a phase separation. This case is considered an **entropy-driven phase transition**.¹⁶⁹

Polymers that undergo an UCST transition generally show strong supramolecular interaction which decreases the absolute value of ΔH_{mix} , therefore resulting in a positive ΔG_{mix} . With increasing temperature, the supramolecular interaction weakens resulting in a higher absolute value for ΔH_{mix} until the enthalpic term becomes dominant and mixing of the phases occurs. This phase transition is considered **enthalpy-driven**.¹⁶⁹

Thermoresponsive behavior in solution does not solely depend on the type of polymer but can also be influenced by the solvent, its salt content and its pH, size of the polymer chains, PDI and concentration of the polymer. Determined cloud point temperatures can also differ based on the wavelength of the light and the type of glass cuvette used for measurement.¹⁷⁰ Consequently, it is often difficult to compare the cloud points of different publications because there is no general consent about some of these factors. Furthermore, there is no standardized convention at which transmittance value the cloud point temperature should be measured. Therefore, all these variables have to be documented and published to receive comparable results.

Many polymers show both an LCST and an UCST but often only one of these appears at ambient temperatures. PEG has been shown to possess an LCST at 120 °C and an UCST at 296 °C in water (volume fraction = 0.1, MW = 15 kg mol⁻¹) rendering both temperatures impractical for application¹⁷³ but highlighting the excellent water solubility of PEG at temperatures below 100 °C. Thermoresponsive behavior of polyethers can be tuned by incorporation of **hydrophobic side chains**. By impeding the formation of hydrogen bonds between water and the polymer the value of ΔH_{mix} is decreased compared to PEG. Consequently, lower temperatures are necessary for phase separation. Polypropylene oxide (PPO) for example shows a significantly lower solubility in water than PEG thanks to the additional methyl groups in the chain. PPO is only soluble in water up to a molar mass of 400 g mol⁻¹ while PEG remains water-soluble even at molar masses of up to 10⁶ g mol⁻¹.^{174,175} To achieve a tunable thermoresponsive behavior, EO can be copolymerized with other monomers to produce less hydrophilic polyethers. A common example is PEG-PPO-PEG block copolymers, which are often referred to as **Poloxamers** or by their trade name Pluronic[®], developed and marketed by BASF. These polymers possess a hydrophobic PPO middle block surrounded by two hydrophilic PEG blocks. Cloud points of these polymers can be adapted by

varying the length of the PPO middle block or the surrounding PEG blocks or by varying the length of the polymer as a whole. The influence of the total length of the polymer on the cloud point can be seen between Pluronics[®] L43 and P123 which have approximately the same molar content of PO of 64 FPEG%. While Pluronic[®] L43 with a molar mass of 1,850 g mol⁻¹ possesses a cloud point of 42 °C, for Pluronic[®] P123 with a molar mass of 5,750 g mol⁻¹ a cloud point of 90 °C can be found. Moreover, for Pluronic[®] L35, which has approximately the same molar mass as Pluronic[®] L43 but a lower molar PO content of 43%, a cloud point of 73 °C is detected (all T_{cp} measured at 1 mg mL⁻¹).¹² The adaptable cloud points and their property to form micellar structures in aqueous solution make Pluronics[®] suitable for various applications as surfactants and biomaterials.

Generally, biocompatible polymers with cloud points close to body temperature can be used for drug delivery and gene delivery¹⁷⁶. Poly-(*N*-isopropylacrylamide) (PNIPAM) for example shows a T_{cp} of 33 °C (at 5 mg mL⁻¹) which can be tuned to more than 40 °C by copolymerization with acrylamide¹⁷⁷. PNIPAM has been studied as a drug delivery agent in recent years and several patents have been published so far.¹⁷⁸ Further applications include the synthesis of thermoresponsive hydrogels¹⁷⁹ and the preparation of smart materials for water collection.¹⁸⁰ For PEGylation cloud points significantly above body temperature are required. Since the formation of a hydration shell is crucial for the stealth effect, collapse of the PEGylation agent must be prevented.^{75,181}

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Chapter 2

Nanoscopic characterization of the thermal phase behavior of random poly(ethylene glycol – glycidyl methyl ether) copolymers (rPEGs)

Nanoscopic characterization of the thermal phase behavior of random poly(ethylene glycol – glycidyl methyl ether) copolymers (rPEGs)

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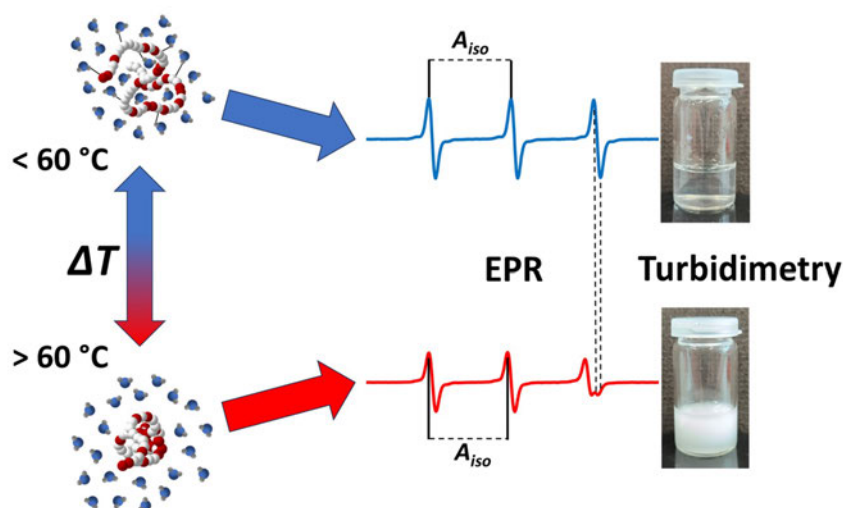
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TOC graphic



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ABSTRACT

PEGylation – linking of poly(ethylene glycol) (PEG) to a nanocarrier or active pharmaceutical ingredient (API) to achieve stealth properties – is a key technology of nanomedicine. However, PEG has been shown to induce the formation of anti-PEG antibodies, motivating the search for alternative polymers for conjugation with nanocarriers. Recently the concept of randomized PEG (rPEG), random copolymers of ethylene oxide (EO) and glycidyl methyl ether (GME) was introduced. These polymers are structural isomers of PEG with potential for manifold biomedical applications. To investigate their phase behavior in aqueous solution, rPEGs with systematically varied monomer ratios were synthesized. Turbidimetric determination of the cloud point temperatures (T_{cp}) was applied to study macroscopic LCST behavior. Paralleling these measurements, local nanophase separation was investigated by electron paramagnetic resonance spectroscopy (EPR) using amphiphilic spin probes to address two key questions: (i) polymer hydration at nanoscopic level and (ii) to determine the temperature at which the onset of the collapse of the polymer chains occurs. The results reveal no phase separation below 96 °C for copolymers of a GME content of 35 mol% and less (26 and 0 mol% GME), while for GME content exceeding 40 mol%, cloud points of 70 °C – 96 °C were observed. Comparison of both methods shows good accordance between the cloud points determined by turbidimetry and EPR with the exception of poly (glycidyl methyl ether) (PGME), for which EPR showed a lower T_{cp} by 5 – 10 °C. Combining the results with literature data, a model could be established that gives insight into the nanoscopic processes and allows for good approximations of the macroscopic cloud point temperatures in dependence of GME content. Excellent aqueous solubility of all samples could be demonstrated in the physiological temperature range, satisfying the requirements for biomedical applications.

Introduction

When aiming at pharmaceutical or medical applications, the behavior of polymers in aqueous solution is of particular importance.^{1,2} Consequently, key polymers like poly(ethylene glycol) that possess excellent aqueous solubility are of special interest for the medical field.^{3,4} This feature together with its very low toxicity have led to an immense variety of uses for PEG in the fields of pharmaceutical technology and nanomedicine^{5–7}, cosmetics⁸ and also as a food additive.⁹ The high aqueous solubility of PEG is due to the position of the oxygen atoms in the polymer backbone. With 2.88 Å the oxygen-oxygen distance is very similar to the distance of the hydrogen atoms in liquid water at 25 °C (2.85 Å).¹⁰ Therefore, PEG can be integrated into a water lattice at room temperature by forming a hydration shell around its backbone.^{10,11}

However, this behavior does not hold true for all polyethers. Introduction of hydrophobic side chains into the backbone of the polyether, for instance by copolymerization of propylene oxide, leads to significantly reduced solubility in aqueous solution, because the formation of the hydration shell of the polymer chain is sterically disabled.¹¹ The solubility of polyethers in aqueous solution can therefore be tailored either by variation of the epoxide monomer side chain¹² or copolymerization of hydrophobic and hydrophilic monomers.^{13,14}

Most commonly, the aqueous solubility of polymers is quantified by observing the dependence of their solubility on the temperature of the solution.^{15,16} The formation of the hydration shell of a polyether is enthalpically favored but entropically disfavored. For low temperatures, the enthalpic term of the Gibbs-Helmholtz equation will outbalance the entropic term, leading to negative Gibbs free energy ΔG_{mix} and therefore a solution of the polymer in water. With increasing temperature, the entropic term $T\Delta S_{mix}$ will increase in value until ΔG_{mix} becomes positive, and the polymer undergoes a coil to globule transition. This leads to a phase separation caused by “shedding” of the hydration shell and eventually results in precipitation of the polymer.^{17,18} Macroscopically, this can be observed by turbidity of the mixture. For a defined concentration of the polymer in a solvent like water, a cloud point temperature T_{cp} can be determined by measuring the transmittance of a polymer/solvent mixture depending on its temperature. The lowest possible cloud point that can be achieved when varying the molar ratio of polymer and solvent in the mixture, is called the lower

critical solution temperature (LCST). Below this temperature, polymer and solvent are completely miscible at any given ratio.¹⁹

The turbidity of a solution, however, is a macroscopically observable effect. To observe the coil to globule transition on a molecular level, other methods such as NMR spectroscopy and light scattering (DLS) can be employed. Valuable information at nanoscopic level can be obtained via electron paramagnetic resonance (EPR) spectroscopy. This method affords comprehensive data regarding the hydration state of a polymer and local density of the polymer-rich phase. Furthermore, transition temperatures well below the macroscopically detected values are perceptible that mark the onset of the transition. The strength of this technique in elucidating such aspects has been shown in several publications by the authors and other researchers^{20–28} (**Figure 1**). TEMPO (2,2,6,6)-tetramethylpiperidinyloxyl is a stable aminoxyl radical that is widely used in EPR studies. Due to its moderate solubility in water and high solubility in various organic solvents ($P_{ow} \sim 90$)^{29,30} it serves as an amphiphilic spin probe, suitable for monitoring both water-rich (hydrophilic) and polymer-rich (hydrophobic) nanoscopic regions. Moreover, rotational dynamics of the spin probe can be monitored, through the defined correlation time as a single measure (τ_c , in nano to picosecond, ns – ps, regime) which provides another hint regarding the presence of a spin probe in different media (like collapsed or aggregated).

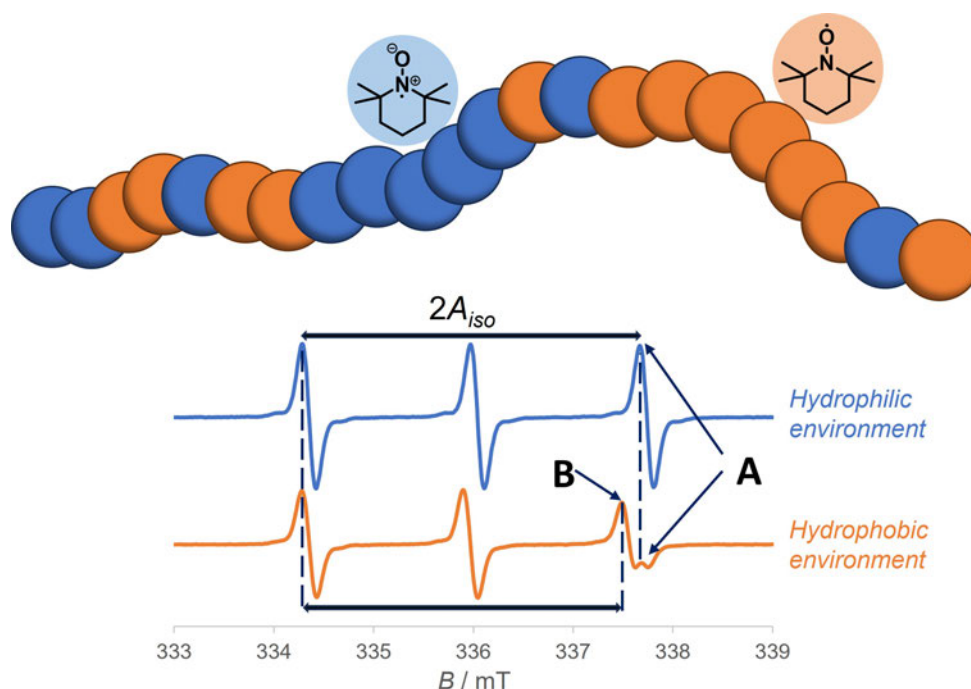


Figure 1. Polymer chain with more polar/hydrophilic (blue) and less polar/more hydrophobic (orange) regions and the mesomeric structures of TEMPO molecules predominantly located in those regions. The change in the polarity of the surrounding medium is best reflected in the high field peak position (~ 337.5 mT), where the two species are clearly discernible.

The electronic structure of TEMPO can be described between two mesomeric structures (**Figure 1**). While located in a polar, water-rich environment, the slightly more prevalent resonance structure has a higher spin density located on the nitrogen atom. If TEMPO is present in a water-depleted, more polymer-rich region, the other resonance structure is more populated, in which the oxygen atom bears higher spin densities. This change in spin density localization can be observed in two ways: either as a variation of the g_{xx} element of g -tensor to higher values, or as a shift in the so-called ^{14}N -hyperfine coupling constant (A_{iso}), a measure of which for a nitroxide is the hyperfine splitting that can be read out of from isotropic spectra as roughly half distance between two outermost lines (see **Figure 1**).^{23,28,31} Therefore, collapse of the hydration shell in little hydrated regions of the copolymers can be spectroscopically observed by the impact on interacting spin probes. While the scattering of visible light for turbidity measurements requires particle sizes of several hundred nanometers, spin probes can already detect localized chain collapse behavior in the range of a few nanometers.^{22,28,32}

We recently developed the concept of randomized PEG (rPEG).³³ rPEGs are random copolymers of ethylene oxide (EO) and glycidyl methyl ether (GME) synthesized via statistical anionic ring-opening copolymerization (AROP). GME possesses a methyl(methoxy) side chain, making it a structural isomer of two EO molecules. Consequently, this property translates to the rPEGs, which are structural isomers of PEG, regardless of their EO/GME composition (**Figure 2**). The methyl(methoxy) side chains of GME-units lower the overall hydrophilicity of the copolymer compared to PEG to a certain extent. Thanks to the ideally random nature of this copolymerization the side chains are distributed randomly at the polyether backbone. By avoiding the regularity of the monomer sequence in the backbone of PEG, rPEGs can evade immune reactions caused by anti-PEG antibodies. We recently reported an *in vitro* study³⁴ (see also **Chapter 3** of this dissertation), in which we investigated antibody binding to rPEGs with varying molar GME content. We observed that a GME content above 25 mol% significantly reduces binding of backbone-specific anti-PEG antibodies. At approximately 50 mol% GME content, no antibody binding could be observed anymore, qualifying rPEGs as a potential PEGylation alternative to address the rising concerns regarding the prevalence of anti-PEG antibodies in the population. Furthermore, since rPEGs with a molar content of more than 26 mol% GME are amorphous at room temperature – contrary to the semicrystalline PEG – these polymers can be used to create unprecedented polyether materials for which crystallinity is undesired but a PEG-like structure is mandatory, e. g., in areas such as membrane technology³⁵ or battery development.³⁶ Since excellent aqueous solubility is a crucial requirement for any medical application, the aim of this work was to investigate, if the incorporation of GME into the polyether backbone would lead to a change in the nanoscopic chain collapse behavior and subsequent significant decrease in the cloud point temperatures of the copolymers. Furthermore, the temperature dependence of local hydration on a nanometer scale has been studied.

Results and Discussion

Synthesis and characterization of rPEG copolymers

rPEGs of varying composition have recently been shown to possess low cell toxicity and high aqueous solubility at physiological temperatures.³³ The aim of this investigation is to gain detailed insight into the thermoresponsive behavior of rPEGs in aqueous solution, from the nanoscale up to the macroscale. For this purpose, turbidimetry measurements were performed to assess the cloud point temperatures of rPEGs with respect to molar GME content. The macroscopic cloud point was compared to EPR spin probe investigations, intrigued by the question whether localized hydrophobic regions of the copolymers can collapse before the phase separation can be detected by clouding of the solution. Understanding this behavior will give further insight into the hydrophilicity of rPEGs and shed light on the temperature dependence of the nanoscopic hydration shell.

Monomer synthesis

GME is a common epoxide monomer, which is commercially available from several vendors. Large scale synthesis as well as laboratory synthesis are based on the methoxylation of epichlorohydrin (ECH).^{37,38} A significant disadvantage of this method is that in all commercial GME batches, residuals of unreacted ECH are still present (see **Supporting Information, Figure S1**) which causes undesired chain termination in anionic ring-opening polymerization. Therefore, in several works PGME could only be synthesized with low molecular weights and broad molecular weight distributions via classic AROP.³⁹ However, using specialized techniques like the monomer activated anionic ring-opening polymerization (MAROP), high molecular weights were achieved.⁴⁰ Since MAROP requires toxic additives that are not approved by the food and drug administration (FDA) and residues of ECH lead to undefined polymer compositions, these polymers would not be considered for biomedical applications. As reported by our group, high purity GME without ECH contamination permits conventional AROP as used for pharma-grade PEG, allowing for higher molar masses and narrow molecular weight distributions of the resulting polymers.³³

Polymer synthesis

For an accurate comparison, we aimed at copolymers of the same chain length, which translates to the same degree of polymerization, systematically varying molar fractions of GME in the backbone. The targeted chain length for all rPEGs was set to 113 monomer units, which is equivalent to a common PEG of 5,000 g mol⁻¹. As a reference point, mPEG of similar chain length was synthesized under the same conditions. The degree of polymerization and composition for all synthesized polymers are given in **Table 1**. In the following sections, the copolymer composition is described with $\text{rPEG}_{D_p}^f$ where f is the molar fraction of GME and D_p is the degree of polymerization of the sample. For mPEG and poly (glycidyl methyl ether) (PGME) homopolymer, only the D_p is given.

Table 1. Characterization data of rPEGs of varying monomer compositions.

Polymer	<i>mol% GME</i> (target)	<i>mol% GME</i> ^a	<i>M_n</i> (target)	<i>M_n</i> ^b	<i>M_n</i> ^c	<i>D_p</i> (target)	<i>D_p</i> ^b	<i>D</i> ^c
mPEG ₁₀₆	0	0	5,010	4,724	4,060	113	106	1.06
rPEG ₁₁₁ ^{0.26}	25	26	6,243	6,217	4,860	113	111	1.05
rPEG ₁₁₁ ^{0.35}	33	35	6,640	6,617	5,500	113	111	1.07
rPEG ₁₀₆ ^{0.41}	40	41	6,948	6,629	4,990	113	106	1.07
rPEG ₁₁₀ ^{0.51}	50	51	7,477	7,384	5,710	113	110	1.08
rPEG ₁₀₅ ^{0.64}	60	64	7,962	7,593	5,820	113	105	1.06
rPEG ₁₀₅ ^{0.73}	70	73	8,446	8,007	5,850	113	105	1.06
rPEG ₁₁₅ ^{0.82}	80	82	8,931	9,292	7,160	113	115	1.06
PGME ₁₀₃	100	100	9,900	9,085	6,520	113	103	1.05

^a calculated by ¹H NMR spectroscopy

^b calculated by MALDI TOF mass spectrometry

^c calculated by SEC

Random incorporation of the comonomers during copolymerization is essential to obtain an ideally random monomer sequence in the backbone. In a previous study we demonstrated that copolymerization of EO and GME in dimethyl sulfoxide (DMSO) indeed leads to a perfectly random incorporation of both comonomers.³³ *In situ* $^1\text{H-NMR}$ kinetics in several different aprotic solvents showed reactivity ratios of $r_{\text{EO}} \approx r_{\text{GME}} \approx 1$ in $\text{DMSO-}d_6$.³³ Therefore, all polymerizations were performed at 30 °C in DMSO. Based on these reactivity ratios, the composition of the copolymers is determined by the composition of the monomer feed. The polymerization reaction and basic characterization data are depicted in **Figure 2** and **Figure 3**. Further details regarding polymer synthesis and full characterization data can be found in the **Supporting Information**.

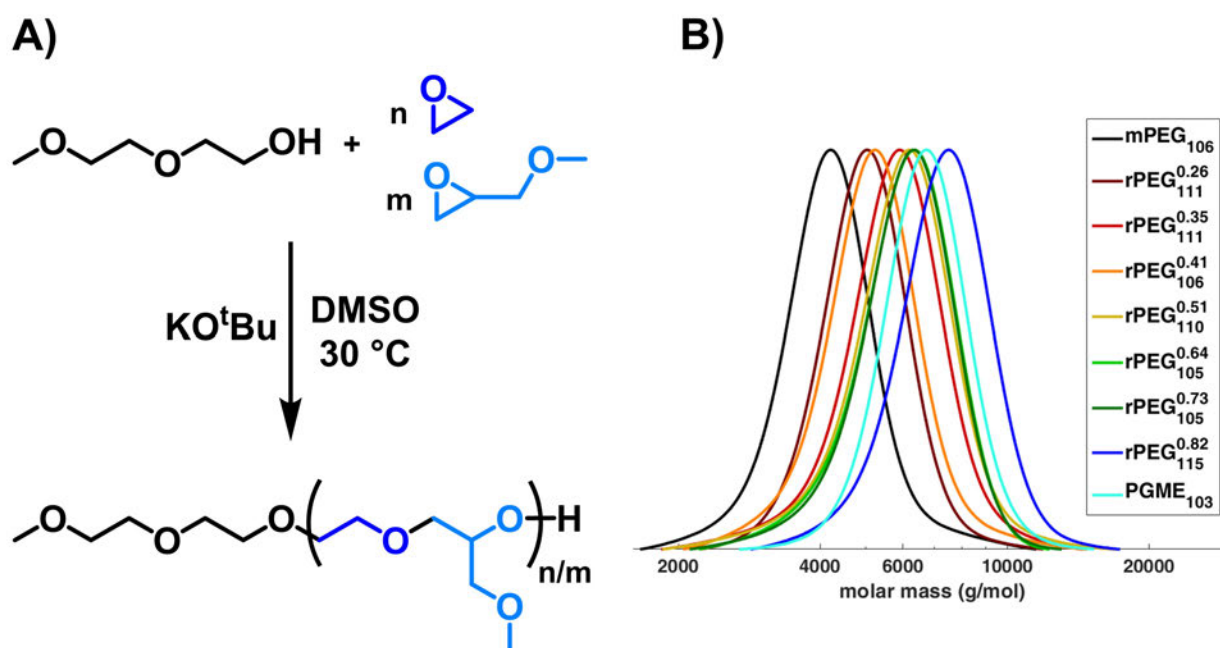


Figure 2. A) General reaction scheme for the copolymerization of EO (dark blue) and GME (light blue). Diethylene glycol monomethyl ether (black) was used as an initiator in all (co)polymerizations. B) SEC curves of the synthesized copolymers.

Most copolymers were prepared using a single monomer addition step. For **rPEG**₁₁₅^{0.82} and **PGME**₁₀₃, two monomer additions were necessary. However, the synthesis of PGME with a degree of polymerization exceeding 100 proved challenging. Several attempts were required to obtain a PGME sample with a degree of polymerization close to the targeted 113 with narrow molecular weight distribution. A detailed discussion regarding the issues encountered is given in the Supp. Inf. **PGME**₁₀₃ was found to be an outlier in the regular synthesis of PGME, which was performed with potassium *t*-butoxide as a base in DMSO at 30 °C using two monomer additions 24 h apart – the same conditions used for the successful synthesis of **rPEG**₁₁₅^{0.82}. Other GME homopolymers of the same series synthesized under the same conditions showed a maximum of $D_p \approx 85$. Further improvements can potentially be made by varying the solvent composition or using a stronger base like sodium naphthalenide to deprotonate the PGME-macroinitiator.

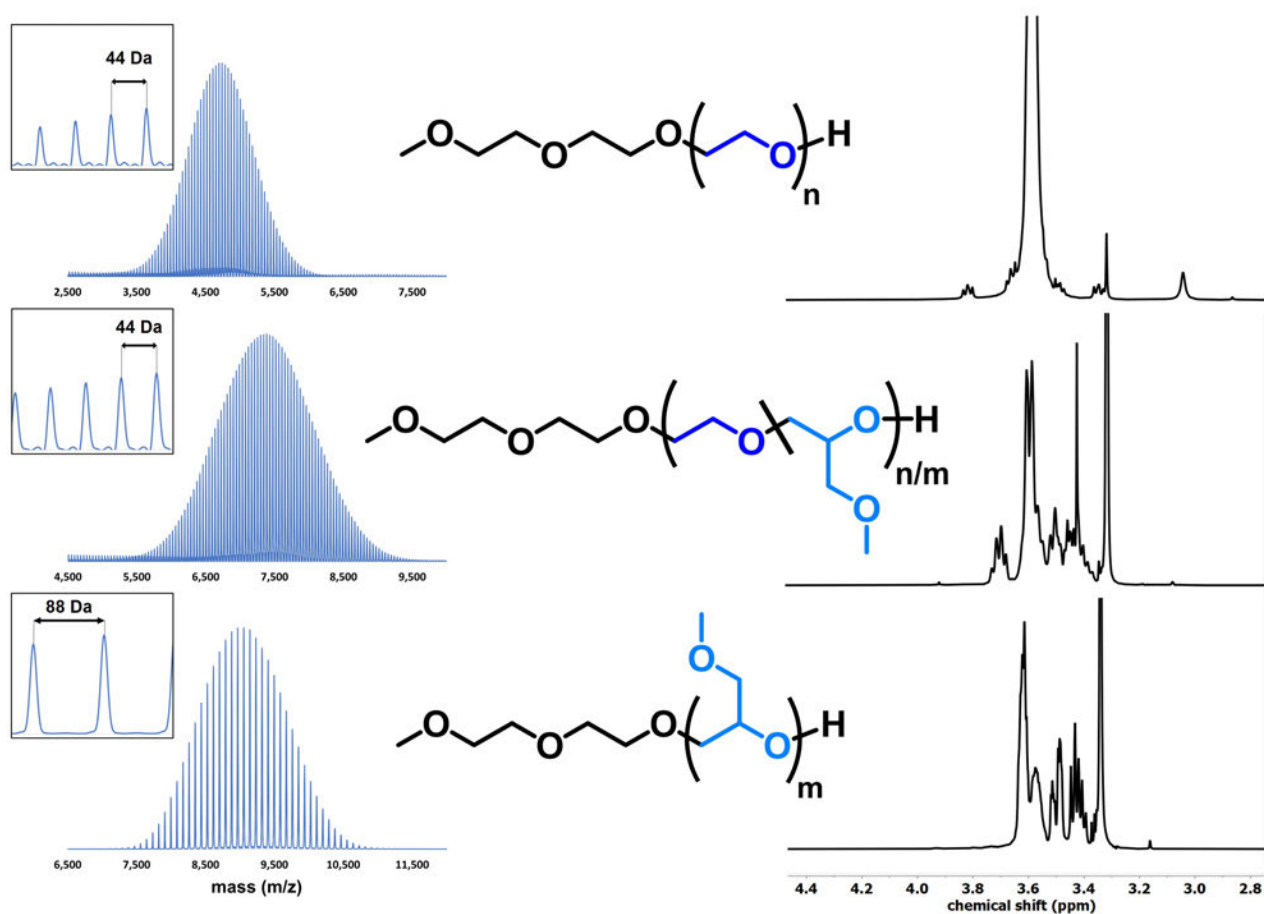


Figure 3. Comparison of characterization data recorded for **mPEG**₁₀₆ (top), **rPEG**₁₁₀^{0.51} (middle) and **PGME**₁₀₃ (bottom). Typical MALDI TOF spectra of the polymers are shown on the left.

For comprehensive characterization of the rPEG samples, a combination of ^1H NMR, SEC and MALDI TOF was employed. SEC shows monomodal distributions and low values for the dispersity $\mathcal{D}$ for all rPEG samples. The molar mass of the polymers shows systematically lower values than the MALDI TOF mass spectra. Since SEC measurements are based on a PEG calibration, it can be deduced that the hydrodynamic radius of the polymers does not change linearly with increasing molar ratio of GME in the backbone, while the molecular weight does. Since GME is isomeric to exactly two EO molecules, it possesses precisely twice the molar mass of EO. For this reason, the relative molar mass increase of the polymers is equal to the molar ratio of GME in the backbone. An rPEG sample which contains 25 mol% GME will, for example, have a 25 % higher molar mass than mPEG of the same chain length, i.e., degree of polymerization. Analysis of the data from **Table 1** shows an average underestimation of the molar mass of 23 % when comparing SEC and MALDI TOF of the copolymers and PGME.

For accurate values of the molar masses of the polymers, MALDI TOF mass spectra were recorded for all samples. Because of the aforementioned isomeric nature of EO and GME, the mass signals of the distinct polymer chains overlap for every possible monomer ratio, and the mass signals of rPEGs therefore appear to show the same mass of the monomer unit as mPEG (**Figure 3** top and middle). As expected, in the mass spectrum of **PGME₁₀₃** only the monomer mass of 88 Da can be observed (**Figure 3** bottom).

The average molar percentage of GME in every polymer sample was determined from ^1H NMR spectra. By calculating the ratio of the methoxy signal at 3.35 ppm and the backbone signal at 3.36 – 3.95 ppm, the ratio of GME units and EO units in the backbone can be obtained. Since none of the signals can be used as an absolute reference, only the ratio of GME and EO can be calculated from the ^1H NMR spectra. A detailed calculation of the EO-GME ratios of all copolymers is given in the **Supporting Information**.

Turbidity measurements and cloud points

rPEGs show composition-dependent thermoresponsive behavior in aqueous solution. Their cloud points can be correlated to the molar amount of GME. Incorporation of fewer hydrophilic side chains at the polymer backbone impedes the formation of the hydration shell and therefore decreases the absolute value of ΔH_{mix} compared to PEG. Since rPEGs are structural isomers of PEG, the effect is considerably less pronounced than in the industrially relevant EO/PO copolymers.⁴¹ A statistical copolymer of 50 mol% EO and PO at 5,100 g mol⁻¹ shows a cloud point of 55 °C⁴², whereas a statistical copolymer of 20 mol% EO and 80 mol% PO shows a cloud point of 18 °C at a concentration of 30 wt%.⁴³ Poly(ethyl glycidyl ether) (PEGE), despite only one additional methylene group in each side chain in comparison to PGME, shows cloud points below 15 °C even at a low molecular weight of 3,000 g mol⁻¹ (measured at 1 mg mL⁻¹), resembling poly(propylene oxide) in its solubility behavior.⁴⁴ These observations show that incorporation of less hydrophilic side groups at an aliphatic polyether backbone can drastically reduce the aqueous solubility of the respective copolymers compared to PEG.

To assess the hydrophilicity of rPEGs, two different analytical methods were utilized. Macroscopically, cloud points were determined by turbidimetry, observing clouding of polymer solutions in water by monitoring the transmission of visible light. At the nanoscopic level, polymer solutions were analyzed by cw-EPR spectroscopy using TEMPO as a spin probe, enabling to observe the onset of chain collapse with increasing temperature on a nanometer level.

For the sake of comparison with turbidimetric data from previous publications, cloud point measurements were performed at a concentration of 5 mg mL⁻¹ in Millipore water. Furthermore, several selected samples were also measured at high concentrations of 100 mg mL⁻¹ (10 wt%) to be comparable with those obtained by EPR. All cloud point temperatures measured by optical spectroscopy were determined at 50 % transmittance. The determined cloud point temperatures and the heating curves of these measurements for both concentrations are given in **Table 2** and **Figure 4**.

Table 2. Cloud point temperatures of the synthesized copolymers for concentrations of 5 mg mL⁻¹ and 100 mg mL⁻¹. At high concentration, only three copolymers were measured.

Polymer	T_{cp} (for conc. = 5 mg mL ⁻¹)	T_{cp} (for conc. = 100 mg mL ⁻¹)
	/ °C	/ °C
rPEG₁₀₆^{0.41}	96.0	85.6
rPEG₁₁₀^{0.51}	85.8	--
rPEG₁₀₅^{0.64}	83.2	71.6
rPEG₁₀₅^{0.73}	78.6	--
rPEG₁₁₅^{0.82}	73.2	68.4
PGME₁₀₃	69.9	--

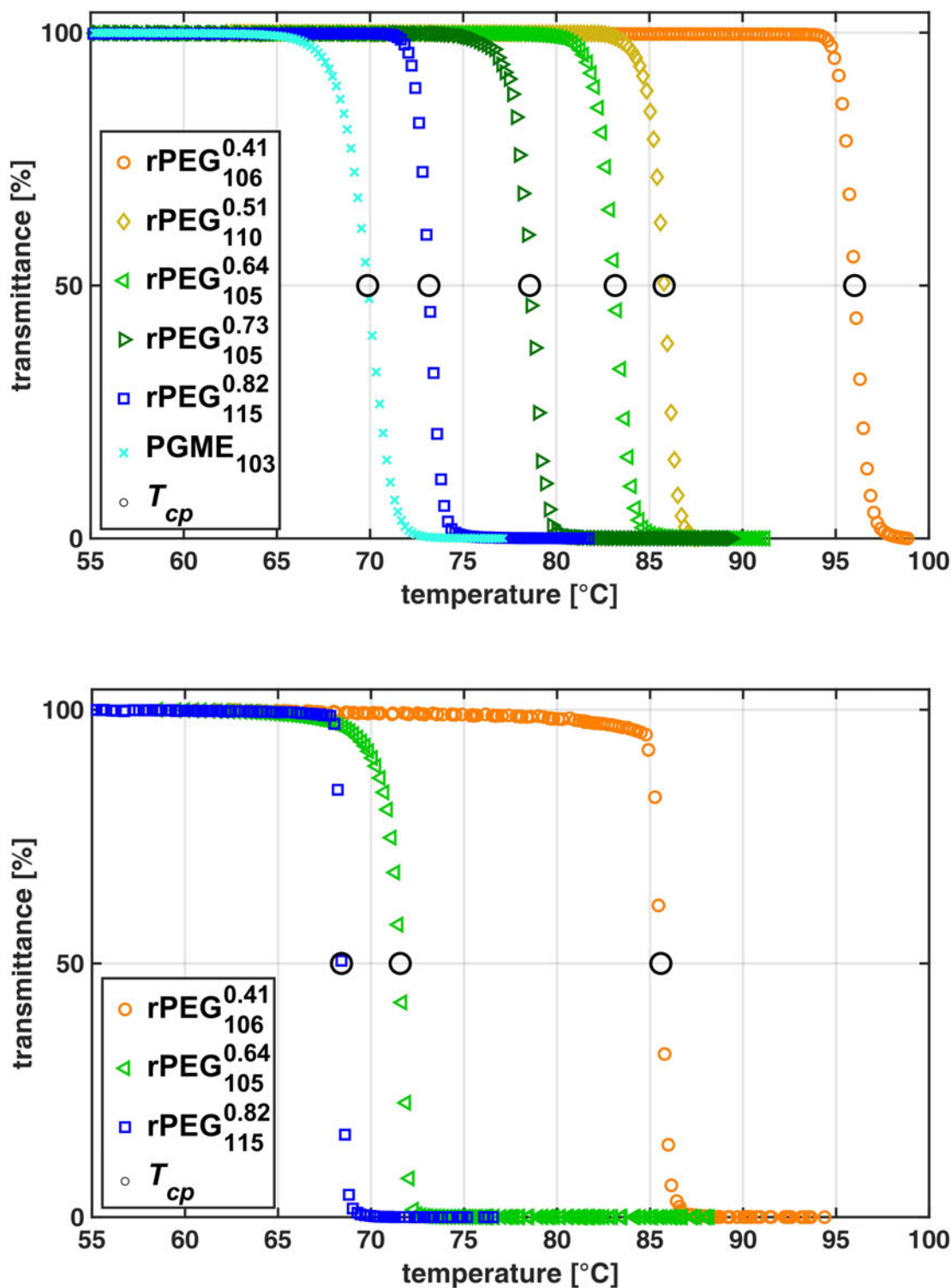


Figure 4. Heating curves of the cloud point measurements of rPEG samples. The respective data is summarized in **Table 2**. Measurements were performed at a concentration of 5 mg mL⁻¹ (top) and at a concentration of 100 mg mL⁻¹ (bottom) in Millipore water. Below a molar GME fraction of 41 mol%, no cloud points were observed. Data points are min-max normalized between 0 and 100 % transmittance.

From the measurements it can be deduced that an increase in the molar fraction of GME in the polymer backbone leads to a decrease in hydrophilicity. Between a GME fraction of 41 mol% and 100 mol%, a constant decrease in T_{cp} is observed. For a GME content below 41 mol% no T_{cp} could be observed. Remarkably, the T_{cp} of **PGME**₁₀₃ was observed at 69.9 °C while previously published results found cloud point temperatures between 55 °C and 66 °C.^{45,46} The discrepancy in the values is most likely explained by the materials used, the polymerization methods by which PGME was synthesized, and the methods used for data collection.

As mentioned above, commercially available GME contains traces of ECH that are challenging to remove (see **Supporting Information**). Under AROP conditions, the presence of ECH leads to chain termination reactions, broadening the molar mass distribution and limiting the achievable molar mass of the polyethers. Several publications relied on commercially available GME with a guaranteed purity of > 85 % for PGME synthesis^{39,45}, suggesting considerable amounts of ECH contaminant in the monomer. While all publications purify GME before use, even small amounts of ECH present in the polymers will significantly alter the properties of the copolymers like cloud point temperatures.

In a previous work⁴⁵, the copolymerization of EO and GME by monomer activated ring-opening polymerization (MAROP) was reported, and a T_{cp} of 55 °C for a PGME of 5,200 g mol⁻¹ was determined. Polymers in this earlier work were synthesized using tetraoctylammonium bromide and triisobutylaluminum as initiator and catalyst, respectively. Residues of these substances could have altered solution behavior of the polymers by influencing the formation of the hydration shell, causing an earlier onset of precipitation. Furthermore, since potentially remaining ECH can be copolymerized with EO and GME by MAROP, the resulting polymer most likely shows decreased hydrophilicity because of statistically distributed ECH units that will impede the formation of the hydration shell. Besides PGME, copolymers of EO and GME were also synthesized via MAROP, exhibiting cloud point temperatures several degrees below the values found in our recent experiments³³ and in this work. This can also be explained by the polymerization method. Since EO is incorporated preferably under MAROP conditions, gradient polymers with EO-rich and GME-rich sequences are obtained. These GME-rich sequences can potentially collapse at lower temperatures, decreasing the overall values of T_{cp} for the copolymers.

Isono *et al*⁴⁶ observed a cloud point of 65.5 °C for a similar PGME-sample of 4,940 g mol⁻¹ (measured at 1 wt%), which was also synthesized by MAROP. Watanabe *et al*³⁹ found a T_{cp} of 57.7 °C for a PGME of 3,000 g mol⁻¹ in a previous work. Measurements of cloud point temperatures were here also performed with 1 wt% solutions of the polymers. According to their report, ECH was removed completely before polymerization. According to our experience, distillation alone is not sufficient to effectively remove all traces of ECH, so impurities cannot be ruled out. Additionally, polymerization in this work was performed at 110 °C, which could lead to an increased amount of side reactions, which in our hands were already observed at considerably lower reaction temperatures of 50 °C.

In **Figure 5** the measured cloud point temperatures are plotted against the molar fraction of GME in the copolymers. Extrapolating from the measured data points listed for a concentration of 5 mg mL⁻¹ in **Table 3** (blue), cloud point temperatures for rPEGs with low GME content can be predicted (orange). Following a linear trend, the T_{cp} of mPEG was predicted to be 110.4 °C (**Supporting Information, Figure S35**). This is in contrast to previous publications where the T_{cp} of mPEG of similar molar mass and concentration was found at approximately 130 °C.⁴⁷ Measurements were performed in a sealed sample tube in this case to keep water from evaporating. Adding the comparable value (green) of $T_{cp} = 132.2$ °C for mPEG ($M_w = 8,000$ g mol⁻¹, $\bar{D} = 1.6$, weight fraction = 0.012) from Prausnitz *et al*⁴⁷, a polynomial trend was found to be more accurate. Based on this polynomial fit, cloud point temperatures for **rPEG₁₁₁^{0.35}** ($T_{cp} = 99.2$ °C) and **rPEG₁₁₁^{0.26}** ($T_{cp} = 106.5$ °C) could be made.

Table 3. Predicted cloud point temperatures (orange) based on a polynomial fit of measured values (blue) at conc. = 5 mg mL⁻¹. The T_{cp} of mPEG₁₀₆ was taken from a comparable sample described in literature⁴⁷ (green).

Polymer	T_{cp} (for conc. = 5 mg mL ⁻¹) / °C
mPEG ₁₀₆	132.2
rPEG ₁₁₁ ^{0.26}	106.5
rPEG ₁₁₁ ^{0.35}	99.2
rPEG ₁₀₆ ^{0.41}	96.0
rPEG ₁₁₀ ^{0.51}	85.8
rPEG ₁₀₅ ^{0.64}	83.2
rPEG ₁₀₅ ^{0.73}	78.6
rPEG ₁₁₅ ^{0.82}	73.2
PGME ₁₀₃	69.9

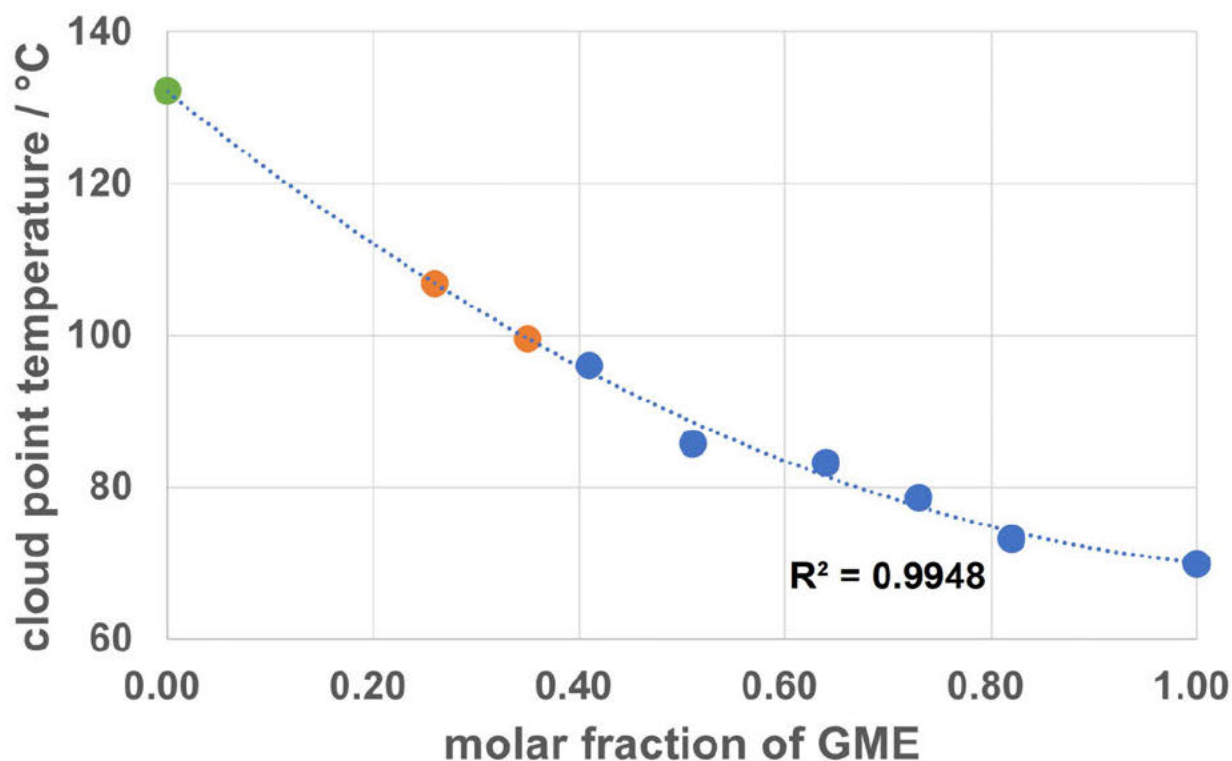


Figure 5. Molar fraction of GME in all rPEGs plotted against cloud point temperatures for conc. = 5 mg mL⁻¹ (blue = measured values; orange = predicted values based on fit; green = literature value taken from Prausnitz *et al*⁴⁷); polynomial fit of measured cloud points including literature value for mPEG and predicted values based on this fit.

The polynomial fit equation of cloud points T_{cp} depending on molar fraction of GME (f_{GME}) measured at 5 mg mL⁻¹ was calculated to be:

$$T_{cp}(f_{GME}) = 47.44 \cdot f_{GME}^2 - 109.53 \cdot f_{GME} + 132.10 \quad (\text{eq 2})$$

Concentration effect on turbidity measurements

As can be seen in the work of Prausnitz *et al*⁴⁷, cloud points of PEG solutions generally decrease with increasing polymer concentrations for weight fractions up to 100 mg mL⁻¹ (10 wt%). This behavior is also known for other hydrophilic polymers like poly(*N*-isopropylacrylamide) (PNIPAM)⁴⁸ and various poly(oxazoline)s (POx).⁴⁹ Therefore, similar behavior was expected for rPEG. To investigate the effect of polymer concentration on the cloud points and to gain better comparability to the EPR data, cloud point temperatures of some exemplary samples were measured at higher concentrations, as used for EPR (100 mg mL⁻¹). The heating curves of these measurements are also depicted in **Figure 4** (bottom). Again, a complementary $T_{cp} = 124.6$ °C ($M_w = 8,000$ g mol⁻¹, $\bar{D} = 1.6$, weight fraction = 0.100) from Prausnitz *et al*⁴⁷ was included in the fit. A polynomial fit was applied here as well. The results can be seen in **Table 4** and **Figure 6**. Even though a similar curve for both concentrations is obtained, further assessment will be necessary to verify the fit for high concentration predictions, since only three data points and one literature value were available for calculations. Nevertheless, similar thermoresponsive behavior of low and high polymer concentrations can be deduced from the available data.

Table 4. Predicted cloud point temperatures (orange) based on a polynomial fit of measured values (blue) at conc. = 100 mg mL⁻¹. The T_{cp} of mPEG₁₀₆ was taken from a comparable sample described in literature⁴⁷ (green).

Polymer	T_{cp} (for conc. = 100 mg mL ⁻¹) / °C
mPEG ₁₀₆	124.6
rPEG ₁₁₁ ^{0.26}	96.8
rPEG ₁₁₁ ^{0.35}	89.3
rPEG ₁₀₆ ^{0.41}	85.6
rPEG ₁₁₀ ^{0.51}	78.7
rPEG ₁₀₅ ^{0.64}	71.6
rPEG ₁₀₅ ^{0.73}	69.7
rPEG ₁₁₅ ^{0.82}	68.4
PGME ₁₀₃	67.7

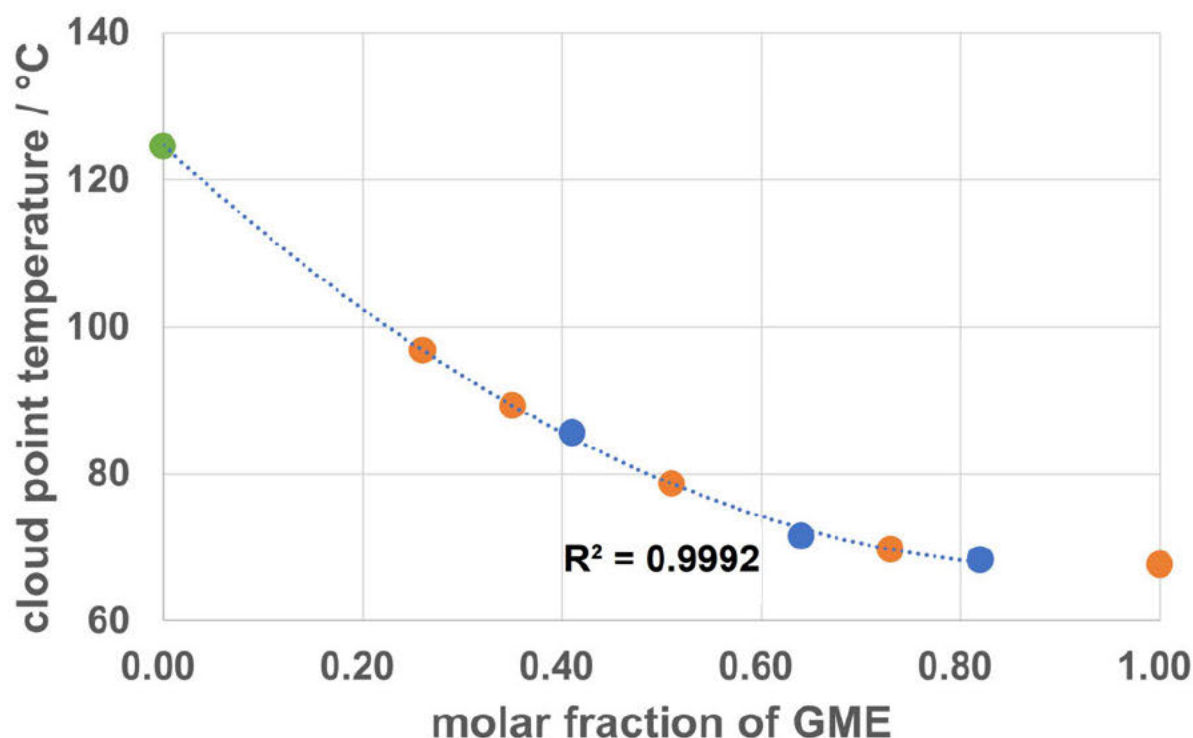


Figure 6. Molar fraction of GME in rPEGs plotted against cloud point temperatures for conc. = 100 mg mL⁻¹ (blue = measured values; orange = predicted values based on fit; green = literature value⁴⁷); polynomial fit of measured cloud points including literature value for mPEG and predicted values based on this fit.

The polynomial equation calculated for this fit for polymer concentrations of 100 mg mL⁻¹ is:

$$T_{cp}(f_{GME}) = 67.97 \cdot f_{GME}^2 - 124.95 \cdot f_{GME} + 124.72 \quad (\text{eq 3})$$

EPR observation of cloud points

EPR measurements on thermoresponsive polymer materials have been reported for relative polymer concentrations ranging from 1 to 15 wt%.^{25,27,32,50} This is based on the fact that the amount of polymeric nanoparticles used in pharmaceutical applications is typically higher than 10 wt%.^{51,52} Therefore, we selected a polymer content of 10 wt% to enable direct comparison of our results with previous studies on thermoresponsive polymers and still be able to model the potential applications of the newly synthesized rPEGs in therapeutic contexts such as drug delivery.

All measured EPR spectra and their corresponding spectral simulations are summarized in **Figures S37-S46**. The representative spectra at temperatures below, at and above the transition temperature were chosen for spectral simulations to get a better view of variation in hydration and possible

trends. In the following we describe variation in transition temperature within three classes of polymers with low, medium and high GME content.

Polymer samples with low content GME (0 – 35 mol%: mPEG₁₀₆ , rPEG₁₁₁^{0.26} and rPEG₁₁₁^{0.35})

As expected, the **mPEG₁₀₆** homopolymer sample showed no change in terms of polarity of surroundings or dynamics of the spin probe (A_{iso} or τ_c , see **Table S3** and **S4**) TEMPO during warm-up or cool-down cycles. Variation of TEMPO concentrations from 0.5 to 1 mM, did not result in any significant changes either, indicating that transition temperature of this sample is not detectable below or at 90 °C, as expected.^{39,53} Copolymerization of 26 mol% GME (**rPEG₁₁₁^{0.26}**) leads to line broadening and consequently damping of the spectral intensity that indicates slightly less hydrated polymer regions as seen from the point of view of our amphiphilic probe molecules. Spectral simulations show the presence of two components experiencing slightly different polarity ($\Delta A_{iso} \approx 1$ MHz, see **Table S5**), starting from 70 °C up to 90 °C. However, these hidden features beneath broadened spectral lines are not strong enough to be observable as splittings in the spectra. During cool-down of the sample, the presence of two components (from 90 to 60 °C) was still detectable. Comparing two thermal cycles, no hysteresis was observed in warm-up/cool-down spectra. Further increase of the molar amount of GME to 35 mol% (**rPEG₁₁₁^{0.35}**) did not lower the cloud point. Spectral simulations (see **Table S6**) reveal a slightly dehydrated, rather polymer-rich component (25 %) in the cool-down cycle at around 80 °C. It shows the existence of a spectral species in lower polarity, indicating formation of a minor hydrophobic component of the polymeric system at this temperature. This is shown in **Figure 7 (C)** where the line broadening of the high-field line is visible. The system stays water-rich (hydrophilic, $A_{iso} \approx 48$ MHz) almost throughout the entire cool-down process to 10 °C. For the sake of clarity, the measured spectra at selected temperatures and the corresponding simulations are given in **Figures S38 to S40**.

Polymer samples with medium GME content (~ 40 – 60 mol%: rPEG₁₀₆^{0.41}, rPEG₁₁₀^{0.51} and rPEG₁₀₅^{0.64})

Even at a GME content of 41 mol%, no clear splitting of the spectra can be observed. However, spectral simulations depict a clearly separated two-component system ($\Delta A_{iso} \approx 2$ MHz, see **Figure S41, Table S7**) at 86 °C, denoting this temperature as the EPR transition point. At this temperature, only 10 % of the sample is dehydrated (more hydrophobic, water-poor component). By heating up to 88 °C, the fraction of the hydrophobic (partially dehydrated) component grows to about 40 % without changing the spectral characterizations A_{iso} or g_{iso} . Increasing temperature to 90 °C, there is a clear distinction between the water-rich and water-poor areas, reflected in a 1.5 Hz difference in A_{iso} , but even better in the 4 MHz difference of $2A'_{zz}$ and 10 times slower tumbling rate of the polymer-rich component. This is an indication that the spin probe is located between dehydrated (aggregates/collapsed polymer) and hydrated polymer areas. The polymer system is mainly hydrophilic (~ 95 %) while cooling down to 88 °C and remains completely water-soluble down to 10 °C. No sign of thermal hysteresis could be observed when comparing the heating and cooling cycle for this sample in terms of A_{iso} (polarity of surrounding medium) or dynamics (tumbling rates).

Increasing the GME content to 51 mol% lowered the cloud point by about 4 °C compared to the polymer with 41 mol% GME (see **Table S8**). Not only is a shift in the position of the high-field peak toward lower fields visible but also a clear splitting of the high-field line that gradually grows with increasing temperature. A gradual decrease in the line intensity that starts at 60 °C and continues up to at 82 °C can be observed. As found by spectral simulations (**Figure S42, Table S8**), in this temperature range the system is dominated by a water-rich component (3 MHz difference to the hydrophobic component) which tumbles three times faster than the polymer-rich component. Further temperature increase to 86 °C makes the polymer-rich component the dominant species (with a spectral share of about 60 %). At 90 °C, the two components arrive at an equilibrium (1:1 ratio) and are completely distinguishable via difference in their polarity and tumbling rates. The same trend is seen for cooling down to 82 °C and subsequently down to 10 °C, the polymer stays water-soluble. No thermal hysteresis could be observed during heating or cooling cycles.

At 64 mol% GME content, a gradual increase in hydrophobicity of the medium is observed during the heating cycle, indicated by smaller values of A_{iso} (see **Figure S43, Table S9**, and selected spectra in **Figure 7 (B)**). Inspection of the spectra shows an intensity drop of about 50 % from 70 °C to 72 °C which marks this temperature as the onset of the cloud point. This trend continues with a further decrease in intensity and gradual separation between hydrated and dehydrated components at around 86 °C. The A_{iso} values of the two components show a difference of approximately 1 MHz and the hydrophilic component (which makes up ~ 41 % of spectral contribution) tumbles two times slower. Elevating the temperature to 90 °C shifts the equilibrium between two components toward a fully dehydrated one at 90 °C. By cooling down to 86 °C and further to 80 °C, a two-component spectrum with gradually dominant water-rich components (48 % and 73 %) can be seen. The spin probe TEMPO stays in water-rich areas while cooling down, again reaching an A_{iso} of about 48 MHz, indicative of water-soluble polymer or poor polymer-polymer interactions.

Polymer samples with high GME content (~ 70 – 100 mol%: rPEG₁₀₅^{0.73}, rPEG₁₁₅^{0.82} and PGME₁₀₃)

For the sample composed of 73 mol% GME, the first change appears at around 68 °C. At this temperature an intensity drop of the high-field line of the spectrum can be observed, along with line broadening that occurs at 70 °C. Elevating the temperature further up to 74 °C, there are clearly two species detectable (apparent from a difference in A_{iso} of approximately 3 MHz and change of g_{iso} from 2.0059 to 2.0057, see **Figure S44, Table S10**). The more hydrophobic component (43 % spectral fraction) has clearly 10 times slower rotational dynamics, which together indicate the start of the collapse process, the restricted motion of TEMPO and its location mostly in polymer-rich areas. The temperature range for the presence of such partly dehydrated polymer extends to 90 °C and also while cooling down to 74 °C. By cooling further down, the dominance of a water-rich one-component system is observed again.

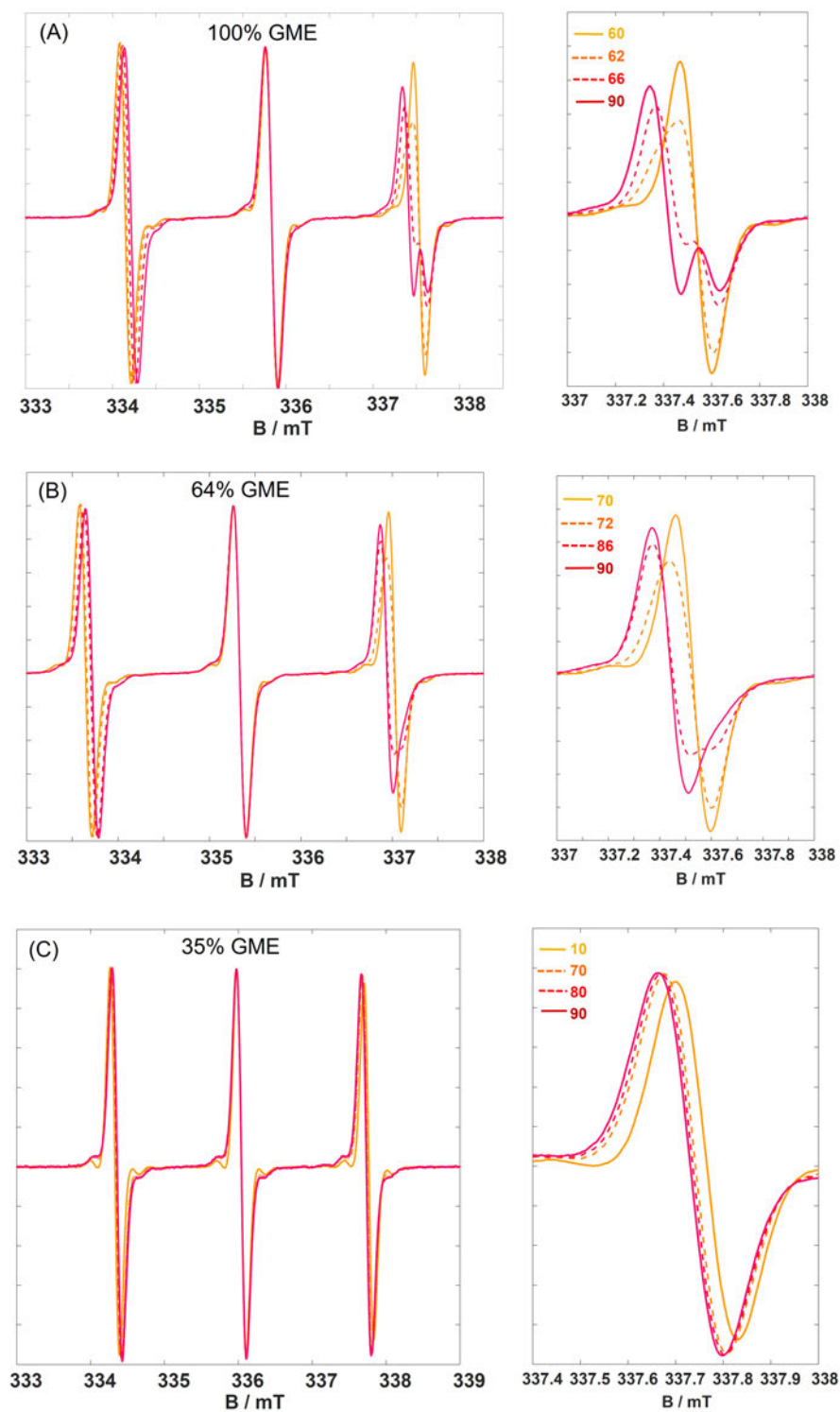


Figure 7. Representative EPR spectra of polymer samples with (A) high, (B) medium and (C) low content GME (100, 64 and 35 %). For simplicity, only temperatures with profound changes in intensity or splitting of high field line are shown. Temperatures before onset of cloud points are shown in solid line. The onset temperatures and those during transition are shown by dashed lines. Final heating temperature for all examples is given as well (90 °C, solid line).

At 82 mol% GME content, the line broadening along with a sharp drop of high-field intensity becomes even more pronounced at a temperature of 68 °C. The polymer remains partially dehydrated (about 60 – 63 %). By elevating the temperature from 68 °C to 90 °C, the spectra show gradual increase in polymer-rich component, while the collapsed polymer regions remain partially hydrated. The presence of a dehydrated component becomes much more evident from a difference of ~ 3 MHz in A_{iso} and its spectral contribution of about 60 – 63 % (see **Figure S45**, **Table S11**). By cooling down, the sample gets partially hydrated down to 72 °C and upon further cooling, the sample is fully hydrated again.

In case of the GME homopolymer **PGME**₁₀₃, first changes in terms of signal broadening and damping of high-field line intensity are observable already at 60 °C (in accordance with macroscopic turbidimetry measurements in literature^{39,54}). However, the turbidimetric cloud point here was determined at 69.9 °C. As also observable in **Figure 7 (A)** the simulation reveals the dominance of the hydrophilic component (92 %) and fast tumbling of the spin probe (two times faster, compared to the hydrophobic component) which shows that the sample is still completely dissolved in water at 60 °C. By stepwise heating the sample from 60 °C to 90 °C, the fraction of the polymer-rich component (dehydrated) grows to about 60 % (from 8 to 68 %, see **Figure S46**, **Table S12**), while the data show a gradual lowering in its A_{iso} (~ 2 MHz). Together with change in A_{iso} between two components at elevated temperatures, the polymer-rich component is fully separated from the hydrated component by two times slower dynamics as well.

Spectral simulations provide detailed insights into the distribution of TEMPO in water- and polymer-rich regions within the samples. Plotting the fraction of the water-rich component of each sample as a function of temperature offers a clear representation of sample solubility behavior. As an example, the change in local hydration obtained by spectral simulation and consequently change in solubility of two polymer samples **rPEG**₁₀₆^{0.41} and **PGME**₁₀₃ with temperature variation is depicted in **Figure 8**. The temperature marking the onset of the transition (transition point) on the EPR-base nanoscale is observable as the start of separating A_{iso} of the two components with different polarity.

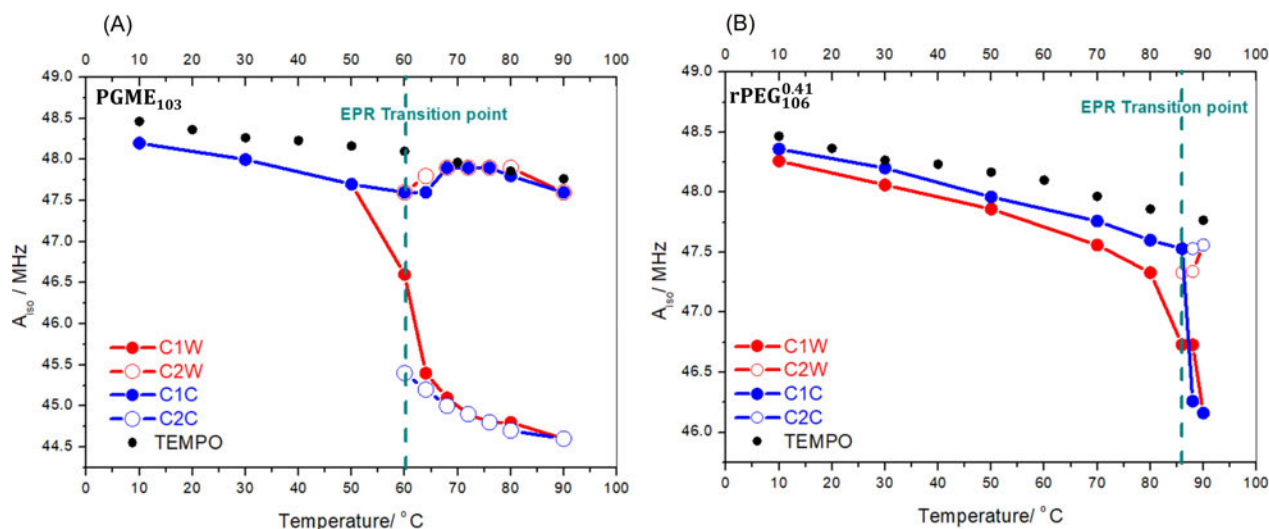


Figure 8. Change in local hydration (A_{iso} / MHz) of polymer samples (A) PGME_{103} and (B) $\text{rPEG}_{106}^{0.41}$ due to temperature variation, as detected by EPR spectroscopy. The first and second components are abbreviated as C1 and C2. Simulated data for warming up (w) and cooling down (c) cycles are shown in red and blue curves. For the sake of comparison, isotropic hyperfine coupling of the spin probe (TEMPO) is shown as black points. EPR based transition temperatures are indicated by dashed lines.

To sum up, samples with a GME content of 0 – 35 mol% remain soluble across the entire temperature range. Signs of reduced solubility begin to emerge when the GME content reaches approximately 41 mol%. Even at elevated temperatures (90 °C), these samples remain predominantly soluble, which means that water-rich areas comprise approximately 60 % of the material. Although increasing the molar fraction of GME leads to a corresponding increase in less hydrophilic regions, samples containing 51 mol% and 64 mol% GME exhibit only a moderate decrease in solubility ($\sim 10\%$), and this occurs primarily at temperatures above 80 °C. A pronounced decrease in solubility is observed when the GME content approaches $\sim 70\%$, occurring within a relatively narrow temperature range of 70 – 78 °C. Beyond this point, the samples exhibit predominantly hydrophobic characteristics, even with further increases in temperature. This decline in solubility can be attributed to synergy of the two main forces; alteration in local polarity and structure of aggregates formed after transition temperatures.

GME repeat units become dominant at 70 mol%, constituting more than two thirds of the whole structure. As a result, the proportion of the hydrophobic hydration^{55,56} of methyl groups present in GME is increased. During the hydrophobic hydration process, water molecules assemble into cage-like structures around a hydrophobic molecule, forming hydrogen bonds between these aqueous

domains. Around the transition temperature, these structures are disrupted and the hydrophobic nature of the solvated molecule is more pronounced. Therefore, a decrease in the water-rich component fraction and consequently an increase in hydrophobicity is observed for samples with > 70 mol% GME. This dehydration pattern is clearly observable also in EPR spectra (see **Figure 8**). The obvious splitting in high field line (see **Figure 9 (A-2)** ~ 337.6 mT) at around the transition temperatures and higher, indicates two media with different hydrations, quantitatively described by a difference of ~ 3 MHz in their A_{iso} (see **Figure 9 (B)**).

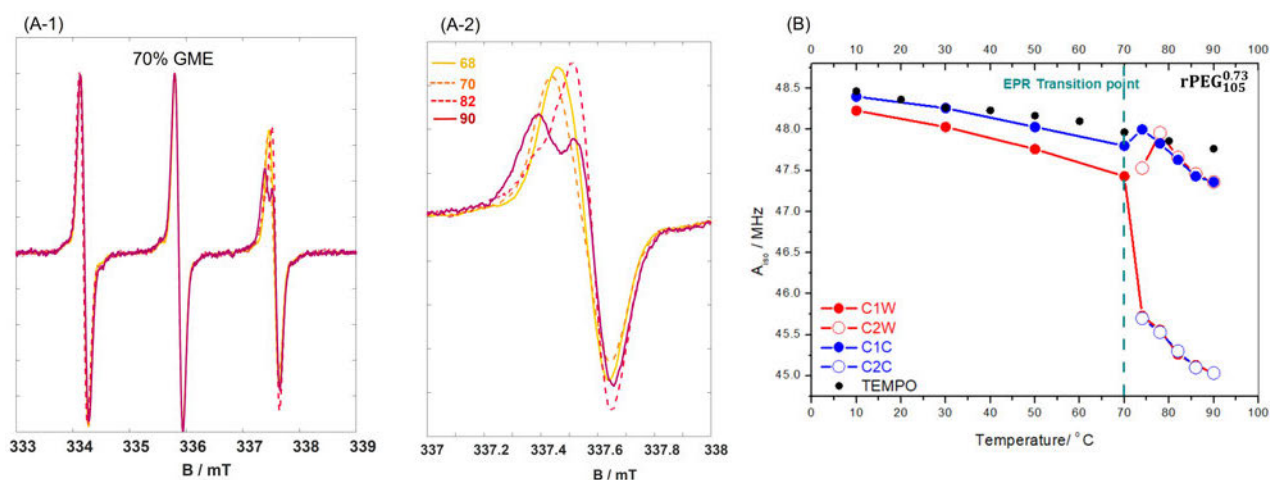


Figure 9 EPR spectra of polymer with 70 mol% GME at temperatures below, at and above the thermal transition (A 1-2). The change in local polarity during heating and cooling cycles is shown in (B). Descriptions as in **Figure 8**.

Interestingly, at higher GME contents (82 mol% and 100 mol%), an increase in solubility is observed (~ 50 %). Considering that the GME structure prevents the formation of ordered structures as observed for regular PEGs, one might consider the increase of steric hindrance through GME's methyl methoxy groups to aid in alteration of the polymer packing and solution conformation. Our observations may be interpreted in terms of the ratio between the hydrophobic and hydrophilic components of the polymer. In the case of an elastin-like polypeptide (ELP)-based block copolymer, it has been shown that the length of the hydrophilic block determines the structure of the aggregates formed above the transition temperature at specific hydrophilic-to-hydrophobic weight ratios.⁵⁷

Similarities in aggregate formation above the transition temperature were observed between the block copolymer and the random polymer sample, although the trends occurred in opposite directions. When the overall weight fraction of the hydrophilic and flexible PEG component (independent of their position in the polymer chain) is approximately 30 %, the polymer forms a highly hydrophobic core. Below this ratio, the dehydrated segments of the polymer are surrounded by still hydrated small segments, leading to the formation of larger aggregates, which possess increased local polarity despite having a larger fraction of less polar monomeric units.

The observed solubility trend from the EPR data is shown as the temperature dependence of the water-rich component obtained through spectral simulation (see **Figure 10 (A)**). We can also represent the polymer behavior in terms of the overall hydrophobicity. Since TEMPO is an amphiphilic spin probe, it can be partitioned in media of different polarity or hydrophilicity/hydrophobicity. We derive a simple measure, the *spectral hydrophobicity*, to quantify change in the polymer samples' hydrophobicities using the high-field line intensity of TEMPO:

$$\text{Spectral hydrophobicity} = 1 - \frac{I_{\text{baseline}} - I_{\text{min},T}}{I_{\text{max},90} - I_{\text{baseline}}} \quad (\text{eq 4})$$

$I_{\text{max},90}$ represents the intensity at the maximum of the high-field peak measured at 90 °C, and $I_{\text{min},T}$ is the intensity of the minimum of the “first component” (the hydrophilic component) in the high-field peak at measured temperature T . The latter is subtracted from the baseline in the numerator, while the baseline intensity was subtracted from the denominator. This type of normalization ensures that the resulting intensity has a positive absolute value. The maximum of this absolute value corresponds to the maximum of the high-field peak at 90 °C and is therefore defined as “100 %”, so that all resulting values range between 0 and 1. The intensity of the more hydrophobic peak in the equation is normalized by dividing it by $I_{\text{max},90}$, since the parameter reflects hydrophobicity (see **Figure S47**). The smaller $I_{\text{min},T}$, the higher is the hydrophobicity value, i.e. the larger the weighted fraction of the hydrophobic spectral component. If the height above or below the baseline of both extremes is the same, a hydrophobicity value of 0 is determined. If the

respective minimum is greater than the maximum at 90 °C, negative *spectral hydrophobicity* values are obtained, which can be translated as less hydrophobic environment.

For rPEGs with a GME content of 35 mol% or less, this measure is not relevant, since they remain water soluble at all measured temperature ranges. In case of medium-content GME polymers (e.g., **rPEG₁₀₅^{0.64}**), there is gradual increase in spectral hydrophobicity with increasing temperature (see **Figure 10 (B)**). This is indicative of TEMPO exchange between both hydrated (aqueous) and dehydrated (collapsed, aggregated segments) nanodomains in the sample. As soon as the first hydrophobic domains form (for example start of polymer collapse at sites with more GME monomers present in the random sequence), TEMPO diffuses to these domains as it prefers them to the aqueous medium.²² When increasing temperature, such dehydrated regions form larger hydrophobic patches (due to physical crosslinking) and finally TEMPO cannot, on the relevant EPR timescale of several nanoseconds, exchange anymore and resides only in the now dominant dehydrated polymer segments. We termed this “dynamic inhomogeneities” that occur during temperature variation of thermoresponsive polymers.²³ It should be noted that the choice of comonomer and its hydrophobicity are crucial in determining whether dynamic or static inhomogeneities are ultimately present.^{21,22} It is also noteworthy that there is an apparent thermal hysteresis, such that the system does not reach the original values before the heating cycle. This is indicative of long-term changes in local hydration induced through the collapse that do not reach the initial level on the timescale of our experiments (several hours).

For the maximum GME content sample, the homopolymer **PGME₁₀₃**, high water-content (reflected in negative hydrophobicity) up to 60 °C and then a sharp increase in the sample hydrophobicity up to 90 °C is observed, indicative of a clear and distinct hydration behavior upon temperature increase above a certain threshold (here 60 °C). The reverse trend is observed during the cooling cycle (see **Figure S48**).

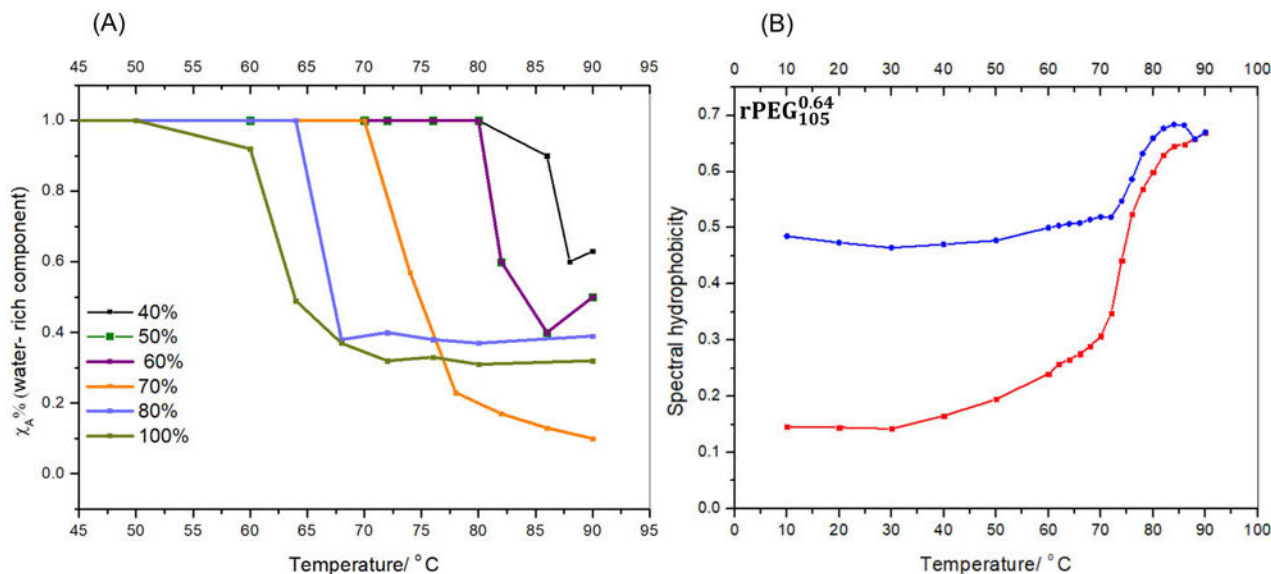


Figure 10. Solubility and spectral hydrophobicity of polymer samples, as detected by EPR spectroscopy. (A) Temperature dependency of the fraction of TEMPO in aqueous phase for samples with medium to high (40-100 mol%) content GME. (B) Spectral hydrophobicity of polymer sample with 64 % GME content that increases by the increase of temperature. Data points during heating and cooling cycles are shown in red and blue.

The cloud point temperatures for all polymer samples, as measured by the two techniques in our study, are illustrated in **Figure 11**. The plot clearly indicates that EPR spectroscopy can be used as an early detector of the transition temperature range, well before it becomes apparent by optical methods at conventional concentrations. This is due to temperature-induced changes on the nanoscopic level that take place with a *local* equilibrium between hydrophilic and hydrophobic parts within the polymer solution. The volume fraction of these initial collapse regions is so small that it is not detectable via optical methods. The numerical values of cloud points obtained by both methods are collected in **Table 5**.

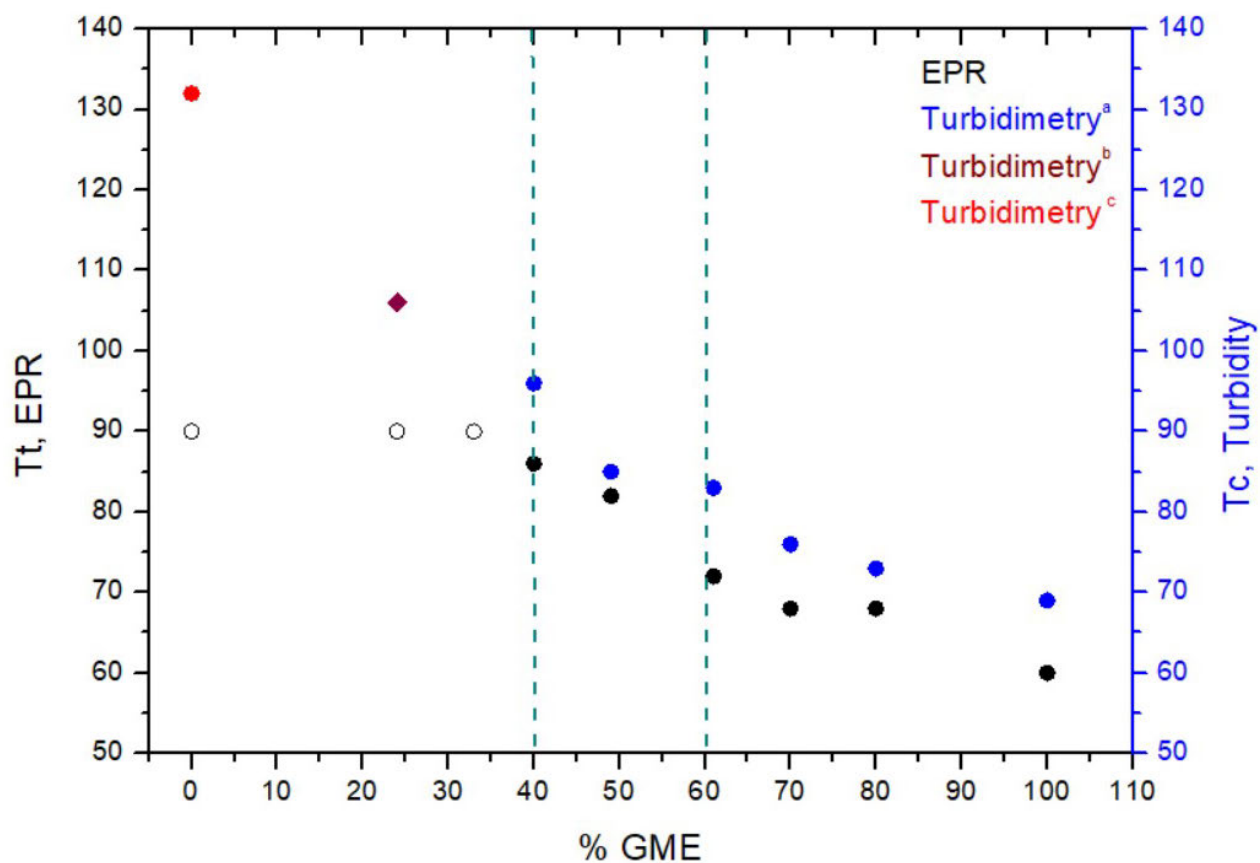


Figure 11. Transition temperatures obtained by EPR spectroscopy (T_t) and cloud points by turbidity (T_c), as a function of GME mole fraction. Turbidity data (a) from this work, (b) predicted by linear fits (c) taken from Prausnitz *et al*³⁵. The polymers with medium content GME (shown as area between two dashed lines), experience a sharp drop of transition temperature of ~ 14 °C. Note that the EPR-based transition temperatures of the low-GME-content samples (0 mol%, 26 mol%, 35 mol%) can only be given as a lower limit of 90°C and are shown as open symbols.

Table 5. Cloud point temperatures of rPEGs measured by optical and EPR spectroscopies.

<i>Polymer</i>	<i>T_{cp}</i>	<i>T_{on}</i>	<i>T_{cp}</i>	<i>T_{cp}</i>	<i>T_{cp}</i>
	(turbidity)	(onset EPR ^b)	(prediction ^c)	(turbidity)	(prediction ^d)
	/ °C	/ °C	/ °C	/ °C	/ °C
	(100 mg mL ⁻¹)	(100 mg mL ⁻¹)	(100 mg mL ⁻¹)	(5 mg mL ⁻¹)	(5 mg mL ⁻¹)
<i>mPEG</i> ₁₀₆	124.6 ^a	> 90	--	132.2 ^a	--
<i>rPEG</i> ₁₁₁ ^{0.26}	--	> 90	96.8	--	106.5
<i>rPEG</i> ₁₁₁ ^{0.35}	--	> 90	89.3	--	99.2
<i>rPEG</i> ₁₀₆ ^{0.41}	85.6	86	--	96.0	94.8
<i>rPEG</i> ₁₁₀ ^{0.51}	--	82	78.7	85.8	--
<i>rPEG</i> ₁₀₅ ^{0.64}	71.6	72	--	83.2	--
<i>rPEG</i> ₁₀₅ ^{0.73}	--	68	69.7	76.6	--
<i>rPEG</i> ₁₁₅ ^{0.82}	68.4	68	--	73.2	--
<i>PGME</i> ₁₀₃	--	60	67.7	69.9	--

^a lit. value taken from Prausnitz *et al*⁴⁷^b temperature at which beginning of chain collapse can be observed^c values predicted by polynomial function (eq 3)^d values predicted by polynomial function (eq 2)

Conclusion

rPEGs are structural isomers of PEG, regardless of their composition based on EO and GME. Since high hydrophilicity is imperative for the application of rPEGs as alternative PEGylation agents for nanomedicine, their behavior in aqueous solution has to be assessed in detail. Therefore, cloud point behavior and nanoscopic processes involving small probe molecules that can be seen as models for, e.g. amphiphilic drug molecules, of rPEGs have been investigated by turbidity and EPR measurements. Turbidity measurements were performed at low concentration (5 mg mL^{-1}) and high concentration (100 mg mL^{-1}) in Millipore water while EPR measurements were performed for high concentrations.

The data shows that T_{cp} strongly depends on the molar fraction of GME in the polymer backbone. As expected, higher contents of GME lower the hydrophilicity of the polymers also on the nanoscale and in interaction with amphiphilic small molecules compared to mPEG and therefore lead to a decrease in T_{cp} . This is due to the rather hydrophobic nature of the methyl groups of GME. Therefore, by increasing the GME content, phase separation between hydrophilic and hydrophobic components becomes more pronounced.

The cloud points measured by both methods are in good agreement. We found that for molar GME contents below 41 mol%, no cloud point temperatures were observed up to 96°C . The biggest drop in transition temperature is observed for samples with medium content GME, where an increase of $\sim 20 \text{ mol\%}$ of GME results in $\sim 14^\circ\text{C}$ lower transition temperatures. For **PGME₁₀₃**, transition temperatures obtained by EPR are $5 - 10^\circ\text{C}$ lower than the values measured by turbidimetry and polynomial prediction (even at 100 mg mL^{-1}). These findings suggest a comparably large drop in hydrophilicity between 82 and 100 mol% GME content. This demonstrates the importance of even small amounts of EO units in the backbone for stabilization of the hydration shell. Predicted values exhibit slight overestimation compared to values found by EPR but still provide a good approximation of cloud point temperatures for rPEGs up to 82 mol% GME.

Finally, all determined cloud points for rPEG and PGME samples, were found to be in a range between $60 - 95^\circ\text{C}$. Even at a concentration of 100 mg mL^{-1} – magnitudes above concentrations used in medical applications – no T_{cp} below 60°C was observed. Additionally, the onset of chain

collapse determined by EPR demonstrates that no partial collapse of the polymer chain, even on the nanometer scale, is to be expected below apparent precipitation of the copolymers which would decrease hydrophilicity and potentially trigger an immune response. Overall, high hydrophilicity of the copolymers in the temperature range below 60 °C could be confirmed for molar GME contents between 26 mol% and 82 mol% as well as for PGME, providing no indication of safety issues for rPEGs in biomedical applications with respect to their water solubility.

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Supporting Information

Nanoscopic characterization of the thermal phase behavior
of random poly(ethylene glycol – glycidyl methyl ether)
copolymers (rPEGs)

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1. Materials and Methods

The calculation of the GME content and total degree of polymerization ($D_p = D_{p,GME} + D_{p,EO}$) in the rPEG samples is not feasible *via* end group analysis from the respective ^1H NMR spectra. Because of the signal overlap of the methoxy group deriving from the initiator and the methoxy methylene side groups, no reference signal can be chosen in the spectrum. Therefore, the GME content and the degree of polymerization were determined by a combination of MALDI TOF MS and ^1H NMR spectroscopy.

For calculation of the molar fraction of GME f_{GME} , the ratio of the methoxy signal between 3.36 – 3.10 ppm (I_{MeO}) and the backbone signal between 4.00 – 3.37 ppm ($I_{backbone}$) has to be determined.

Hereby, I_{MeO} is derived by the signals of the methoxy groups of the GME units and the methoxy signal of the initiator.

$$I_{MeO} = 3 \cdot D_{p,GME} + 3 \quad (\text{eq S1})$$

The integral of the backbone signal is composed of the methylene and methine groups of the GME units as well as the methylene groups of the EO units.

$$I_{backbone} = 5 \cdot D_{p,GME} + 4 \cdot D_{p,EO} \quad (\text{eq S2})$$

$$\left(\frac{I_{backbone}}{I_{MeO}} \right) = \frac{5 \cdot D_{p,GME} + 4 \cdot D_{p,EO}}{3 \cdot D_{p,GME} + 3} \quad (\text{eq S3})$$

Since only the relative ratio of monomers needs to be calculated, a theoretical value for $D_{p,GME}$ can be chosen arbitrarily for equation S3. To minimize errors, the targeted value for a polymer of $D_p^{target} = 113$ with the respective targeted molar fraction of GME f_{GME}^{target} is chosen.

$$D(theo)_{p,GME} = D_p^{target} \cdot f_{GME}^{target} \quad (\text{eq S4})$$

With the value of $\left(\frac{l_{backbone}}{l_{MeO}}\right)$ derived from the NMR spectrum, $D(theo)_{p,EO}$ can be determined by equation S6. The real molar fractions of EO and GME in the backbone can then be calculated by:

$$f_{EO} = \frac{D(theo)_{p,EO}}{D(theo)_{p,EO} + D(theo)_{p,GME}} \quad (\text{eq S5})$$

and

$$f_{GME} = \frac{D(theo)_{p,GME}}{D(theo)_{p,EO} + D(theo)_{p,GME}} \quad (\text{eq S6})$$

To receive the real degree of polymerization D_p , the molar mass determined by MALDI TOF MS $M_{n,MALDI}$ is used.

To receive the molar mass of the monomer units, the mass of the initiator methoxy M_{MeO} group is subtracted from $M_{n,MALDI}$. The degree of polymerization is then calculated by:

$$D_p = D_{p,GME} + D_{p,EO} = \frac{M_{n,MALDI} - M_{MeO}}{M_{EO} \cdot f_{EO} + M_{GME} \cdot f_{GME}} \quad (\text{eq S7})$$

Since

$$M_{GME} = 2 \cdot M_{EO} \quad (\text{eq S8})$$

and

$$f_{GME} = 1 - f_{EO} \quad (\text{eq S9})$$

equation S10 can be simplified to:

$$D_p = \frac{M_{n,MALDI} - M_{MeO}}{M_{EO}} (2 - f_{EO})^{-1} \quad (\text{eq S10})$$

To give an example, the calculations are performed for rPEG₁₀₆^{0.41} (**Figure S10** and **Figure S19**).

$$D(theo)_{p,GME} = 113 \cdot 0.40 \approx 45$$

$$\left(\frac{I_{backbone}}{I_{MeO}} \right) = 3.53$$

Using equation (S3) results in

$$D(theo)_{p,EO} \approx 66$$

and with equations (S5) and (S6): $f_{EO} = 0.59$; $f_{GME} = 0.41$

The real degree of polymerization is then calculated using the values of f_{EO} and $M_{n,MALDI}$ in equation S10:

$$D_p = \frac{6,629 \frac{g}{mol} - 31.03 \frac{g}{mol}}{44.05 \frac{g}{mol}} (2 - 0.59)^{-1} \approx 106$$

All values obtained by these calculations can be found in **Table 1** of the main manuscript. The integrals of the NMR spectra of the copolymers (**Figure S7** to **Figure S14**) are adjusted for the calculated real values of f_{EO} , f_{GME} and D_p . It is important to note that the 2 EO repeating units of the initiator are included in D_p .

1.1. Materials

Ethylene oxide was purchased from *Air Liquide Deutschland*. All solvents were purchased from *Thermo Fisher Scientific*. Other substances were purchased from *TCI Chemicals*, *Sigma-Aldrich*, and *Thermo Fisher Scientific*. All substances were used as received.

1.2. ^1H NMR (nuclear magnetic resonance)

^1H and ^{13}C NMR spectra were recorded on a *Bruker Avance III HD 400* spectrometer with 400 and 100 MHz, respectively, and referenced internally to residual proton signals of the deuterated solvent. All spectra were acquired at 23 °C. Spectra were processed and analyzed utilizing the *MestReNova 14.3.3-33362* software. Deuterated chloroform was purchased from *Deutero GmbH*.

1.3. Matrix-assisted laser desorption ionization using time-of-flight (MALDI TOF) spectrometry

MALDI TOF MS measurements were carried out at a *Bruker autoflex maX MALDI-TOF/TOF* using a Smartbeam-II solid-state laser with a wavelength of 337 nm. Spectra were recorded using the software *Bruker flexControl 3.4* and analyzed using *Bruker flexAnalysis 3.4* and *Bruker polytools 1.31*. The potassium salt of trifluoroacetic acid (KTFA) and trans-2-[3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) were utilized as ionization salt and matrix, respectively. For sample preparation, the polymers were dissolved in chloroform at 10 mg mL⁻¹. 20 µL of this solution was combined with 20 µL of a 10 mg mL⁻¹ solution of the matrix in chloroform. 5 µL of a 0.1 M solution of the salt in methanol were added and 1 µL of the resulting mixture was spotted onto a *MTP 384 ground steel target plate*. The solvents were allowed to evaporate completely before the measurement.

1.4. Size exclusion chromatography (SEC)

Measurements were conducted using an *Agilent 1100 series* HPLC system, which included a degasser, isocratic pump (G1310A), autosampler (G1313A), column oven (G1316A), and detectors for refractive index (RI) (G1310A) and variable wavelength (VWD) (G1314A). Separations were carried out employing a four-column set-up (*MZ-Analysentechnik GmbH*) connected sequentially:

- i) HEMA-40 guard column (40 Å pore size, 10 µm particle size, 50 x 8.0 mm)
- ii) HEMA-40 analytical column (40 Å pore size, 10 µm particle size, 300 x 8.0 mm)
- iii) HEMA-100 analytical column (100 Å pore size, 10 µm particle size, 300 x 8.0 mm)
- iv) HEMA-300 analytical column (300 Å pore size, 10 µm particle size, 300 x 8.0 mm)

The eluent consisted of DMF (*Fisher Chemical*) with 1 mg mL⁻¹ anhydrous LiBr (*Acros Organics*), delivered at a flow rate of 1 mL min⁻¹. Both the column oven and RI detector cell were maintained at 50 °C. Calibration was performed using well-defined poly(ethylene glycol)s from PSS (*PSS Standards Kit*) with molar mass values (M_p) ranging from 106 to 42700 g mol⁻¹. Samples were dissolved in DMF (with 1 mg mL⁻¹ anhydrous LiBr) at a concentration of 1 mg mL⁻¹ with the addition of 1 drop of toluene. The injection of 100 µL of the stock solutions was carried out via the autosampler, with a measurement duration of 45 min. Elution times were referenced using toluene as an internal standard. RI traces were analyzed using the *PSS WinGPC Unichrom V8.31* software.

1.5. Cloud point measurements

Turbidimetric measurements were performed with a *JASCO* UV-Vis Spectrometer (V730) at a light wavelength of 600 nm and a heating rate of 1 K min⁻¹ using the software *JASCO Spectra Manager Ver.2*. Polymers were dissolved in Millipore water at various concentrations. Pure Millipore water was utilized as a reference value of 100% transmittance and was measured prior to each experiment. All measurements were performed in a quartz glass cuvette from *Hellma Analytics* with a light path of 10 mm. Cloud point temperatures (T_{cp}) were determined at a transmittance of 50%. Raw data was normalized to maximum and minimum values of the respective heating curves.

1.6. Differential scanning calorimetry (DSC)

DSC measurements were performed on a *DSC250* device from *TA Instruments* using indium and *n*-octane as calibrations standards. 3 – 5 mg of the polymer samples were weighed in *Tzero* aluminum pans and measured under nitrogen atmosphere against an empty pan as a reference point. The following steps were performed for all samples:

- i) Heating from room temperature to 100 °C at a heating rate of 10 °C min⁻¹
- ii) Cooling from 100 °C to – 90 °C at a cooling rate of 10 °C min⁻¹
- iii) Temperature kept isothermally at – 90 °C for 10 min
- iv) Heating from – 90 °C to 180 °C at a heating rate of 10 °C min⁻¹
- v) Cooling from 180 °C to – 90 °C at a cooling rate of 10 °C min⁻¹
- vi) Heating from – 90 °C to 180 °C at a heating rate of 10 °C min⁻¹

Melting temperatures (T_g) and glass transition temperatures (T_g) were calculated from the last heating cycle to ensure prior removal of solvent residues as well as thermal history of the sample.

1.7. EPR Spectroscopy

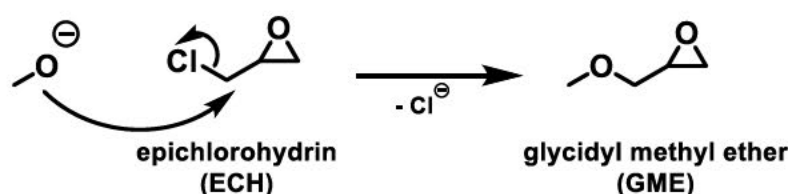
A solution of 10 wt% of each sample was prepared by dissolving the polymer in a freshly prepared 0.2 mM aqueous solution of TEMPO (2,2,6,6-Tetramethylpiperidine-1-oxyl) if not mentioned otherwise.

The effect of the heating rate on the phase transition was minimized by employing a constant heating rate of 1 K min⁻¹.^{1–3} According to previous studies⁴, the cloud point temperature is not significantly influenced when a consistent heating rate is applied. Temperature-dependent CW EPR spectra at X-band frequencies ($\nu \approx 9.4$ GHz) were recorded using a *MiniScope MS400* (Magnetech/Freiberg Instruments, now Bruker BioSpin) equipped with the *MS400 temperature control unit, TC H02*. EPR spectra were measured at 10 K steps, ranging from 10 °C to 90 °C. To see if there is any change between warmed up polymer with the cooled down ones, the procedure was repeated by measurements from 90 °C to 10 °C. Then they finely screened at temperature intervals of 2.0 ± 0.2 °C, around transition point temperature. Reference sample measurements on TEMPO solutions were performed accordingly. Each spectrum was acquired with a sweep width of 15 mT, a modulation amplitude of 500 mG (0.05 mT), and a microwave attenuation of 20 dB (0.3 mW). Each spectrum was the result of three accumulated scans. EPR spectral simulations were performed using home written *Matlab* scripts (version 2022b) using the *EasySpin* software package⁵ (version 6.0.5) and “chili” function. This function reflects slow, non-isotropic tumbling of nitroxide radicals based on the Schneider-Freed theory.⁶

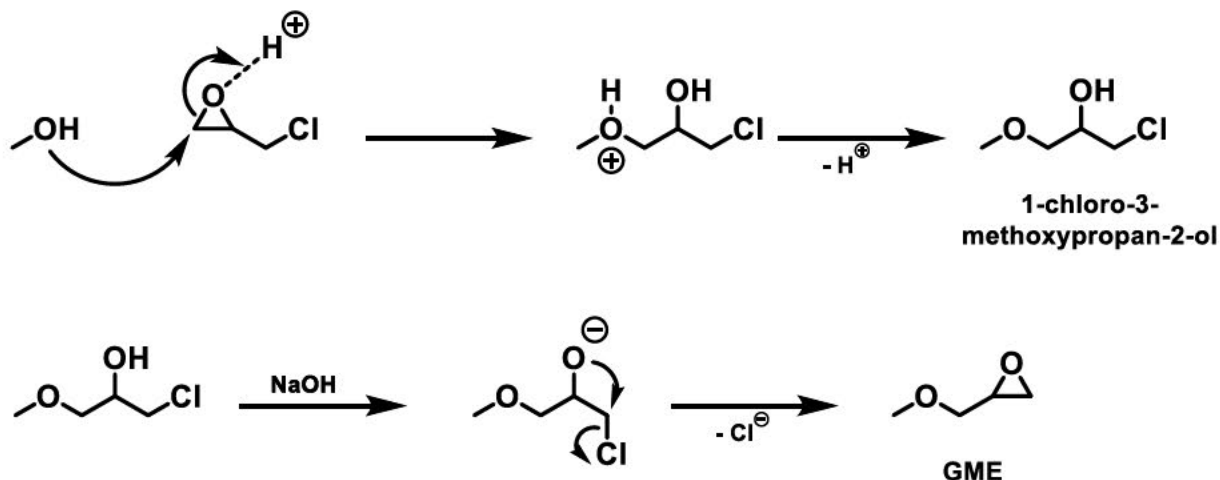
2. Preparative procedures

We recently utilized an alternative method for the synthesis of GME which does not rely on a base-catalyzed one step substitution but instead a reaction of epichlorohydrin under acid-catalyzed hydrolysis of the epoxide ring followed by ring closure via an intramolecular nucleophilic substitution to reform the epoxide ring. The reaction of 1-chloro-3-methoxypropan-2-ol to GME was originally published by Nakatsuji *et al.*⁷ The mechanism is given in **Scheme S1**.

industrial route:



alternative route:



Scheme S1. Mechanism of commercial production of GME and the alternative route via intramolecular nucleophilic substitution.

The 2-step synthesis ensures complete hydrolysis of epichlorohydrin. Additionally, purification by distillation of 1-chloro-3-methoxypropan-2-ol is much more effective since the boiling points of the product (170 – 172 °C at normal pressure⁸) and ECH (115 °C at normal pressure⁹) are much more distinct than ECH and GME (105 – 107 °C at normal pressure⁷). According to our analytics, fractional distillation over CaH₂ and purification over *t*-BuOK as described in the literature¹⁰ were insufficient to remove all residuals of ECH. A comparison of the NMR spectra excerpted from our previous work can be seen in **Figure S1**.

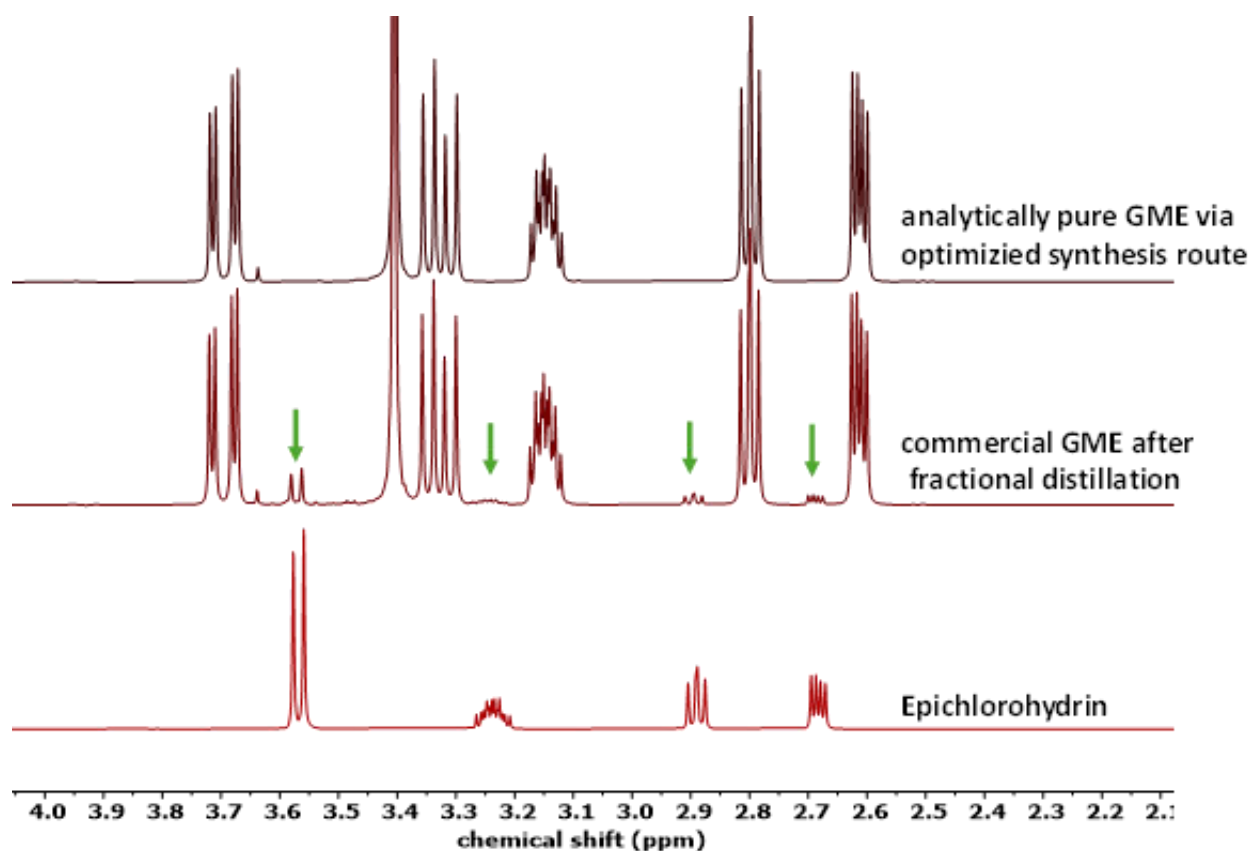


Figure S1. Comparison of ¹H-NMR spectra of GME synthesized by the optimized 2-step synthesis route (top), commercially available GME (middle) and epichlorohydrin (bottom). Residuals of ECH in commercial GME are highlighted (green arrows). Taken from our previous work (Published by American Chemical Society, licensed under CC-BY 4.0).¹¹

2.1. Monomer synthesis

1-Chloro-3-methoxypropan-2-ol In a flame-dried 1000 mL three neck round bottom flask equipped with a magnetic stirring bar, a dropping funnel and a Dimroth condenser, 233 mL (183.9 g, 5.74 mol, 3.00 eq) of methanol were combined with 4 mL (7.5 g, 0.08 mol, 0.04 eq) conc. sulfuric acid (98 %). Under rigorous stirring and argon atmosphere 150 mL (177.0 g, 1.91 mol, 1.00 eq) of epichlorohydrin were added dropwise to the mixture. After the addition was finished the mixture was stirred under reflux overnight. After cooling to room temperature, 22.65 g (0.11 mol, 0.06 eq) of barium carbonate were added and the suspension was stirred for 1 h. Finally, methanol is removed at normal pressure before the product is purified at reduced pressure (Bp.: 100 – 102 °C, 60 mbar) in a fractional distillation. 191 g (80 %) of product were received as a clear, colorless liquid.

^1H NMR (CDCl_3 , 400 MHz) δ : 3.99 – 3.95 (dd; J = 5.32 Hz, 5.26 Hz; 1H), 3.65 – 3.55 (qd; J = 11.13 Hz, 5.56 Hz; 2H), 3.50 – 3.48 (d; J = 5.01 Hz; 2H), 3.39 (s; 3H), 2.65 – 2.64 (d; J = 5.50 Hz; 1H).

Glycidyl methyl ether In a 500 mL three neck round bottom flask equipped with an electric overhead stirrer, a Dimroth condenser and a thermometer, 172 mL (200.0 g, 1.61 mol, 1.00 eq) of 1-chloro-3-methoxypropan-2-ol were combined with 400 mL of diethyl ether. The mixture was cooled to 0 °C in an ice bath and 77.07 g (1.93 mol, 1.20 eq) of sodium hydroxide were added in small portions (over a timespan of appr. 2 h) while the temperature was kept under 15 °C. Under constant stirring, the mixture was allowed to reach room temperature after 5 h. The residue was filtered off and flask and filter were washed 4 times with diethyl ether. The combined organic phases were dried over magnesium sulfate and diethyl ether was removed by distillation at normal pressure. Afterwards, the product was purified by fractional distillation at reduced pressure (Bp.: 70 – 72 °C, 150 mbar) to receive 95.6 g (68 %) of a clear, colorless liquid.

^1H NMR (CDCl_3 , 400 MHz) δ : 3.70 – 3.66 (dd; J = 11.37 Hz, 5.26 Hz; 1H), 3.39 (s; 3H), 3.34 – 3.28 (dd; J = 11.32 Hz, 5.82 Hz; 1H), 3.15 – 3.10 (m; 2H), 2.80 – 2.77 (dd; J = 4.62 Hz, 4.57 Hz; 1H), 2.61 – 2.58 (dd; J = 5.00 Hz, 2.71 Hz; 1H).

^{13}C NMR (CDCl_3 , 101 MHz) δ [ppm]: 73.09, 59.10, 50.63, 44.05.

2.2. Polymerization procedure

The ideally random copolymer composition of rPEGs is generally described with $\text{rPEG}_{D_p}^{f_{\text{GME}}}$, where f_{GME} is the relative molar content of GME in the polymer backbone and D_p is the total degree of polymerization. For mPEG and poly (glycidyl methyl ether) (PGME), only the D_p is given. All samples carry a methoxy and hydroxy group at the α - and ω -group, respectively.

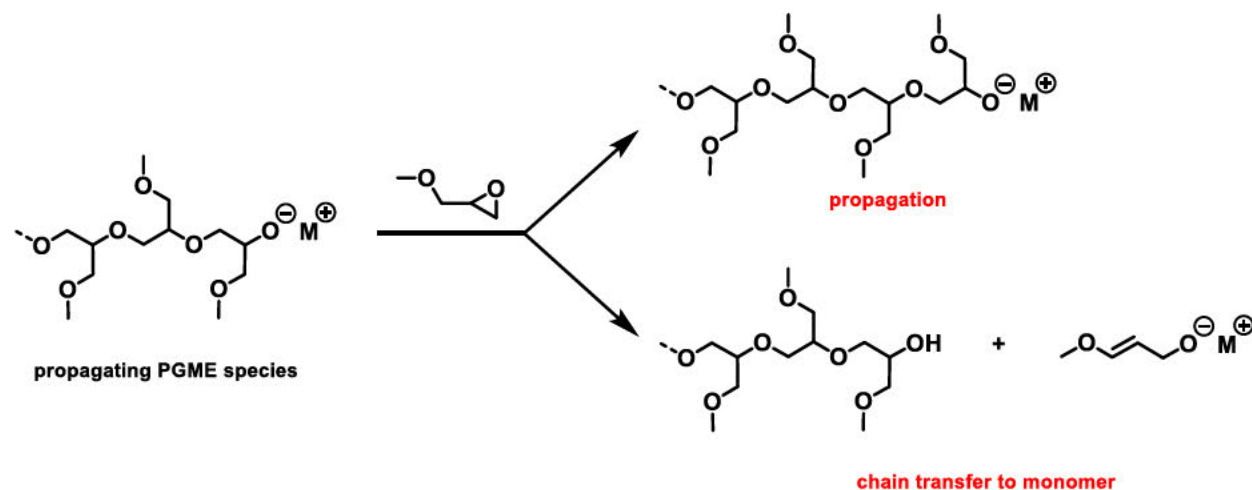
Polymer synthesis was performed with identical procedures for most copolymers, varying only the ratio of monomers in the reaction mixture. It is exemplary described for the synthesis of $\text{rPEG}_{110}^{0.51}$ in the following paragraph.

GME was freshly dried before every polymerization by stirring over calcium hydride overnight in a flame-dried flask and subsequently cryo-transferred immediately before use.

Diethylene glycol monomethyl ether (38.7 mg, 322 μmol , 1.0 eq) was dissolved in benzene (5 mL) and transferred via syringe into a flame-dried flask equipped with a stirrer bar, stop cock, and septum. Potassium *tert*-butoxide (32.5 mg, 290 μmol , 0.9 eq) was dissolved in freshly distilled THF (3 mL) and added to the solution in the flask via syringe. The mixture was stirred for 20 min before the solvents were slowly removed under vacuum. The resulting initiator salt was dried at 60 °C under high vacuum overnight. The flask was sealed and the initiator salt was dissolved in dry DMSO (6 mL). The flask was subsequently cooled to – 90 °C with an ethanol/nitrogen cooling bath. GME (1589 mL, 18.0 mmol, 56 eq) was added via syringe into the cooled flask. Ethylene oxide (750 μL , 17.7 mmol, 55 eq) was condensed into a graduated ampule at – 90 °C and then transferred to the flask. After sealing the flask, the cooling bath was removed and the mixture was allowed to reach room temperature. After stirring for 48 h at 30 °C the polymerization was terminated by flushing the flask with argon and adding a mixture of 1M HCl and methanol (1:1, 1 mL). The reaction mixture was taken up in chloroform (20 mL) and extracted twice with deionized water and subsequently with brine. The organic phase was dried with magnesium sulfate, filtered, and the solvent was removed under reduced pressure. The polymer was dialyzed against methanol (regenerated cellulose, MWCO = 2000 g mol⁻¹) for 24 h, affording a pale-yellow viscous liquid (2,073 mg, 87%).

For mPEG₁₀₆ and PGME₁₀₃, only 1 type of monomer was used. Additionally, rPEG₁₁₅^{0.82} and PGME₁₀₃ were synthesized using 2 consecutive monomer additions of 50 % of the required monomers each. The second addition in these cases was made by cooling the reaction mixture 24 h after initiation again to – 90 °C and adding the second portion of the monomer(s). Afterwards, the mixture was thawed at room temperature and polymerization was continued at 30 °C.

Synthesis of PGME homopolymers proved challenging. Many polymerizations only resulted in PGME with a degree of polymerization of $D_p \approx 80 - 85$, even after prolonged reaction times of several days. The reaction time was expanded from 48 h – the usual reaction time for the synthesis of rPEGs – to more than one week. Still, no increase in chain length was observed and the degree of polymerization remained at about 80 – 85. Possibly the solubility of the propagating species of PGME in DMSO decreases and the reaction solution separates into a polymer-rich and a monomer-rich phase so that chain growth is interrupted. Further attempts at increasing the monomer consumption by altering the degree of deprotonation between 90 – 98 % and increasing the reaction temperature from room temperature to up to 50 °C also proved unsuccessful. At the same time, the transfer-to-monomer side reaction (**Scheme S2**) was observed to increase when the reaction temperature was elevated. Deprotonation was then performed with cesium hydroxide instead of potassium *t*-butoxide, but only a D_p of 70 was obtained and increased transfer-to-monomer was observed as well. Similar results were found when potassium *t*-butoxide was used in combination with two equivalents of 18-crown-6 to bind the potassium ions and increase the reactivity of the active chain end. In this case, a mixture of DMSO and toluene (3:1) was employed as solvent for the polymerization to increase the solubility of the crown ether.



Scheme S2. Chain propagation of the active PGME species vs transfer-to-monomer side reaction.

Another attempt was made by using a previously synthesized **PGME**₅₅ as a macroinitiator to add more monomer to the chain, but no polymerization was achieved – presumably because potassium *t*-butoxide was not able to deprotonate the macroinitiator.

3. ^1H NMR spectra

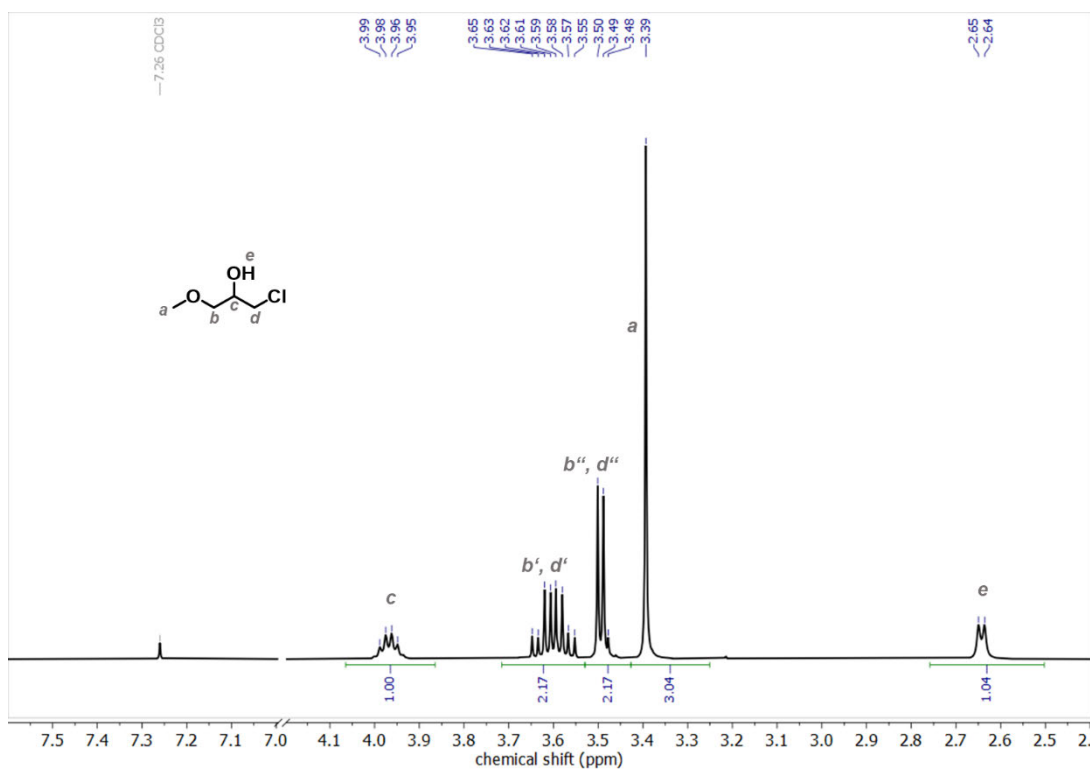


Figure S2. ^1H NMR spectrum (CDCl₃, 400 MHz) of 1-chloro-3-methoxypropan-2-ol.

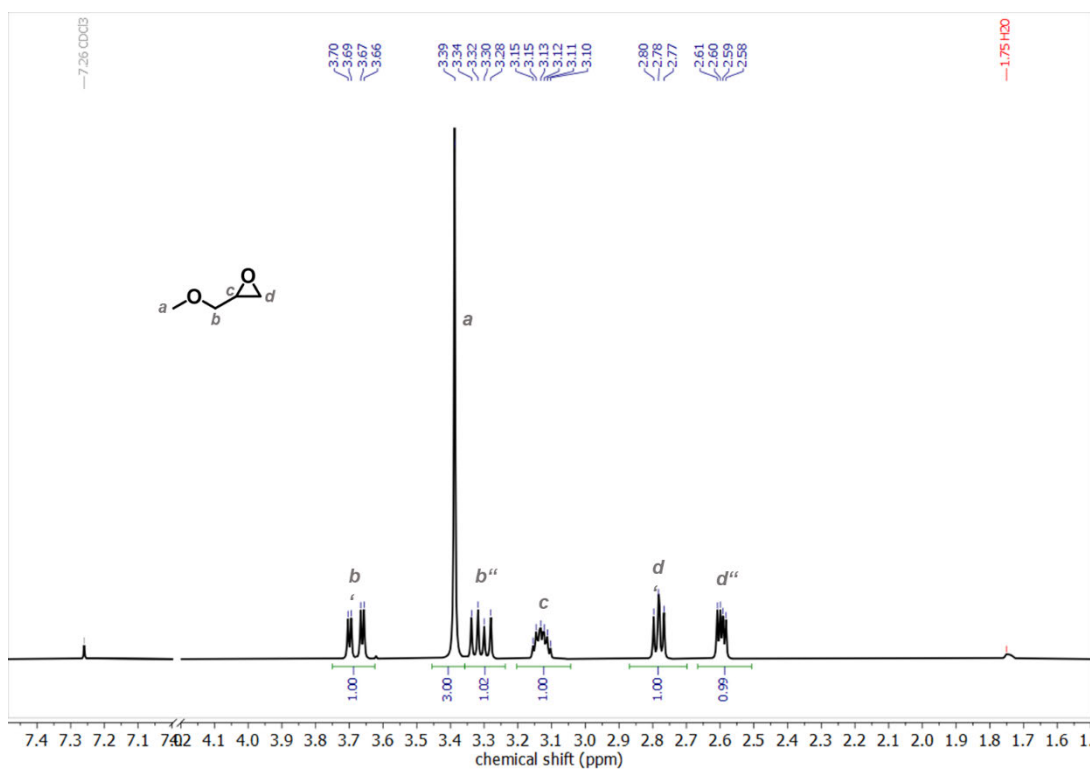


Figure S3. ^1H NMR spectrum (CDCl₃, 400 MHz) of glycidyl methyl ether.

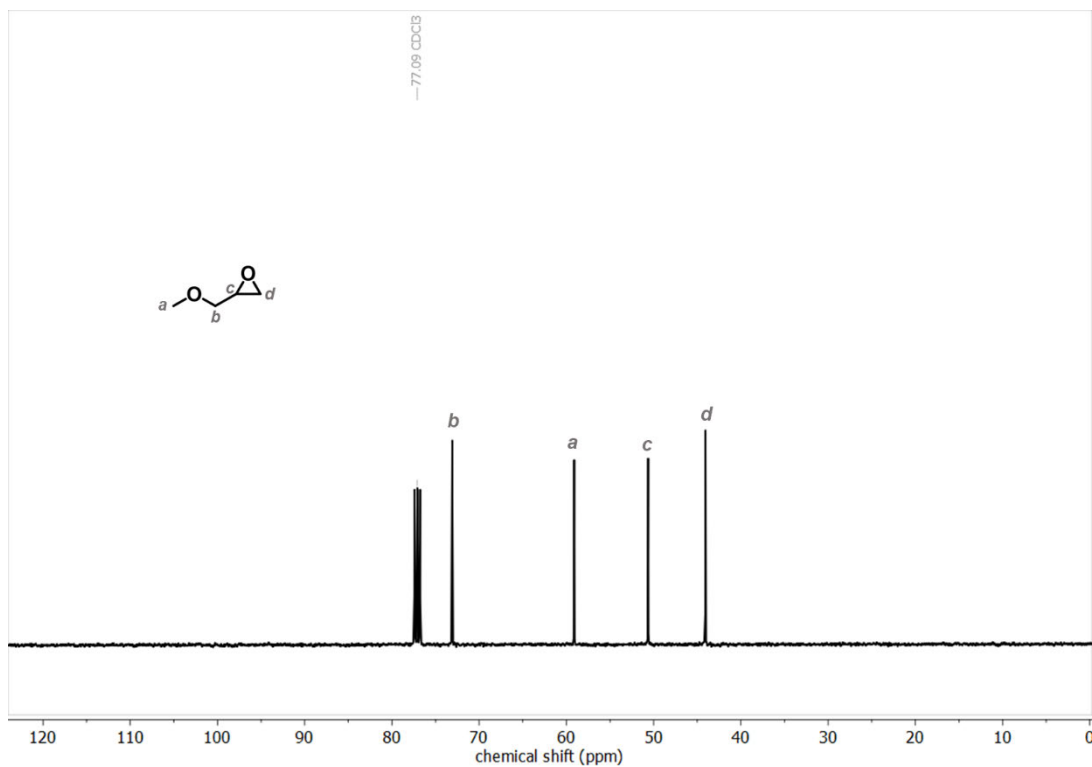


Figure S4. ${}^{13}\text{H}$ NMR spectrum (CDCl_3 , 101 MHz) of glycidyl methyl ether.

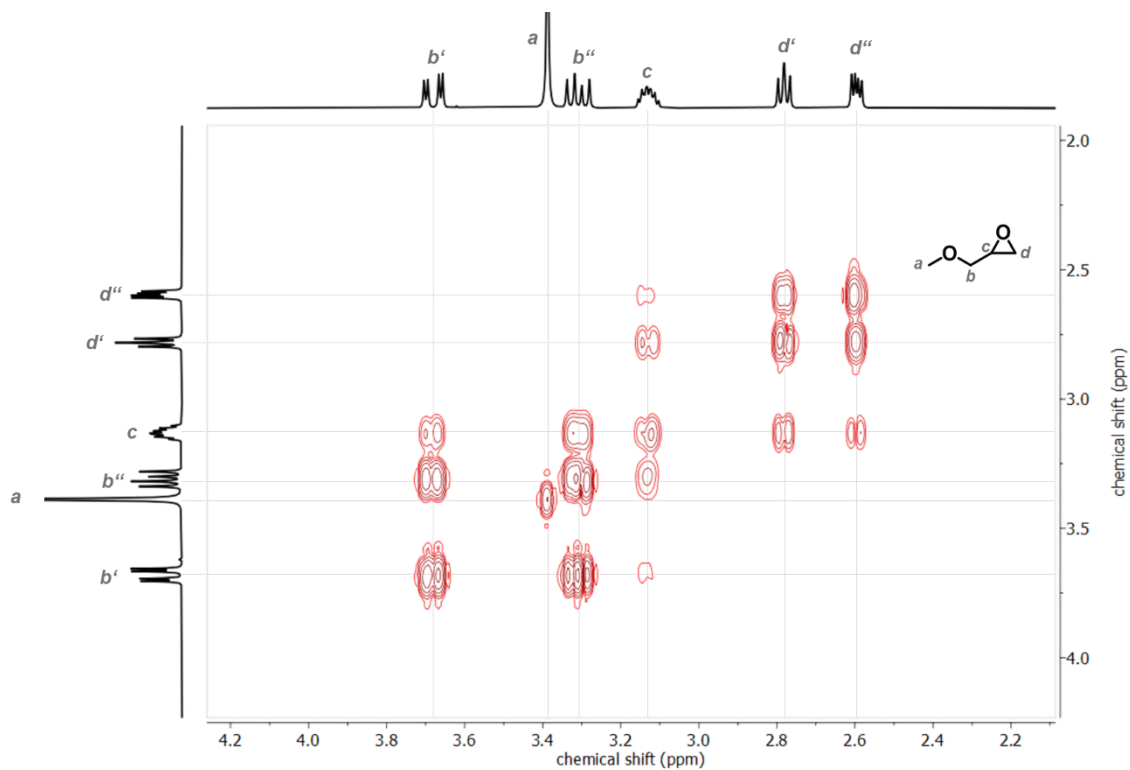


Figure S5. 2D $[{}^1\text{H}, {}^1\text{H}]$ COSY NMR spectrum of glycidyl methyl ether.

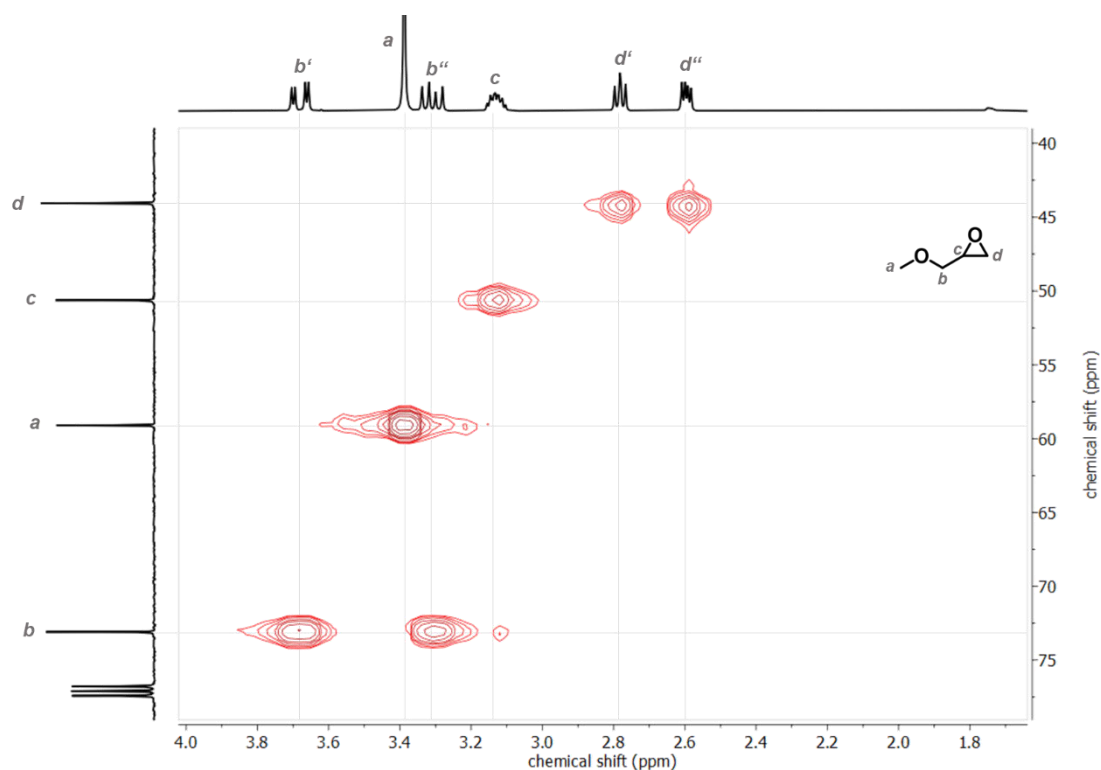


Figure S6. 2D ^1H , ^{13}C HSQC NMR spectrum of glycidyl methyl ether.

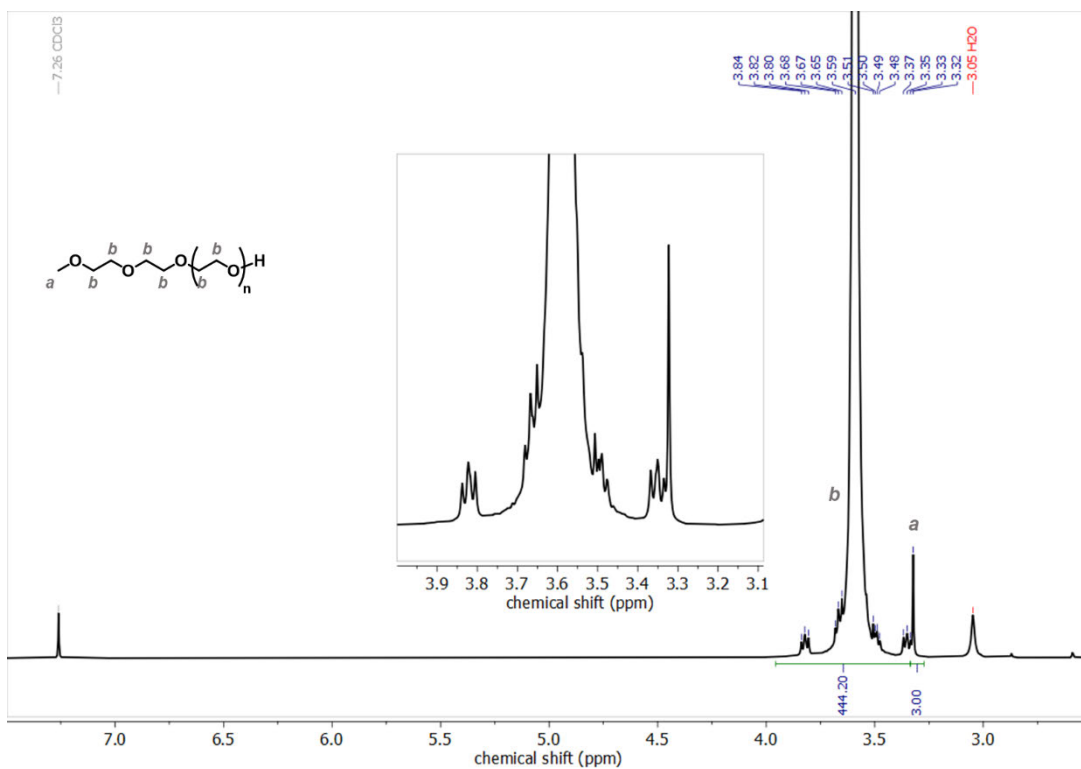


Figure S7. ^1H NMR spectrum (CDCl₃, 400 MHz) of mPEG₁₀₆.

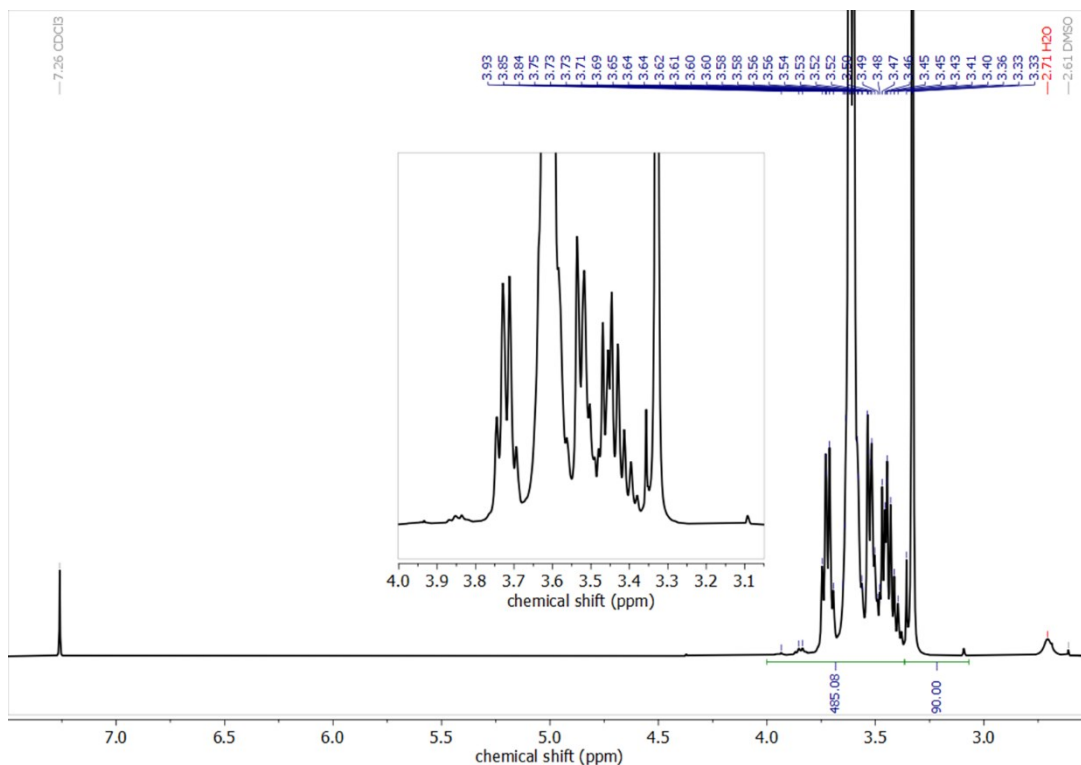


Figure S8. ¹H NMR spectrum (CDCl₃, 400 MHz) of rPEG₁₁₁^{0.26}.

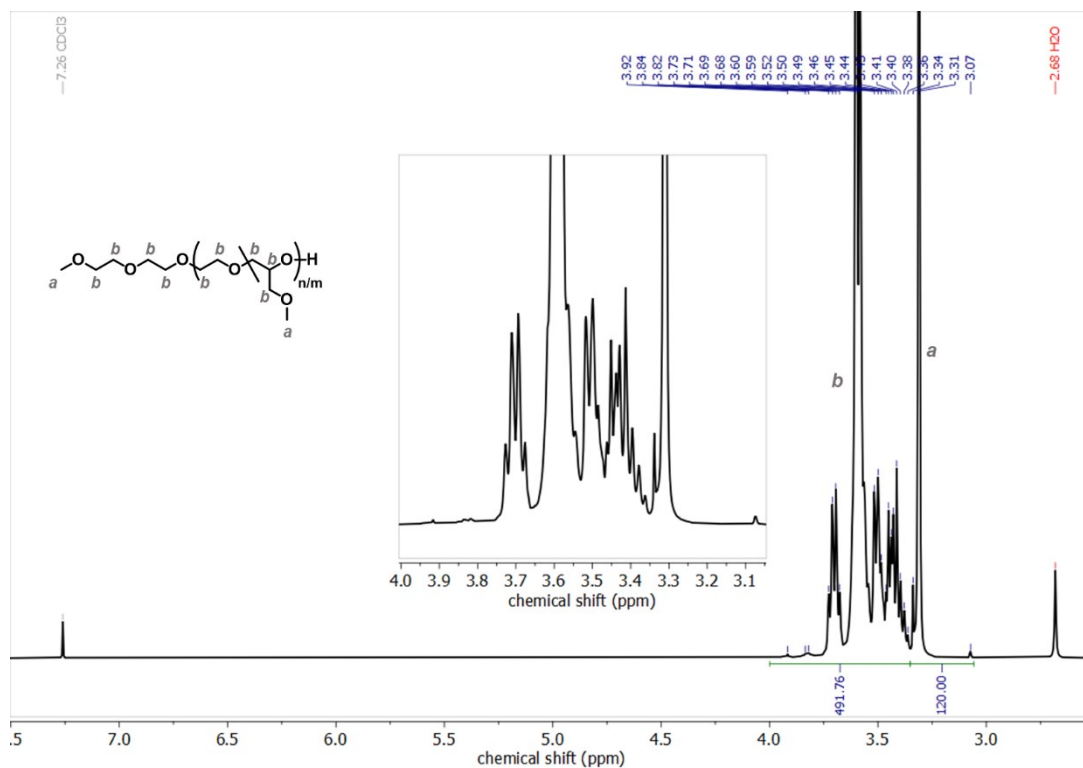


Figure S9. ¹H NMR spectrum (CDCl₃, 400 MHz) of rPEG₁₁₁^{0.35}.

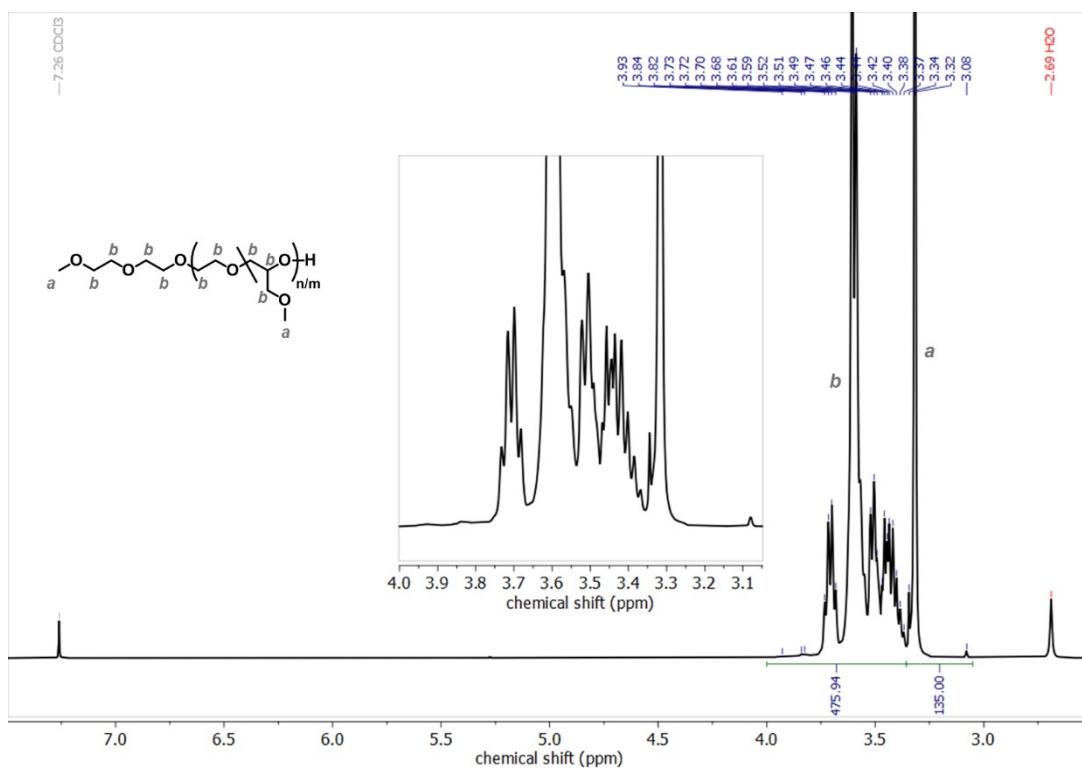


Figure S10. ^1H NMR spectrum (CDCl_3 , 400 MHz) of $\text{rPEG}^{0.41}_{106}$.

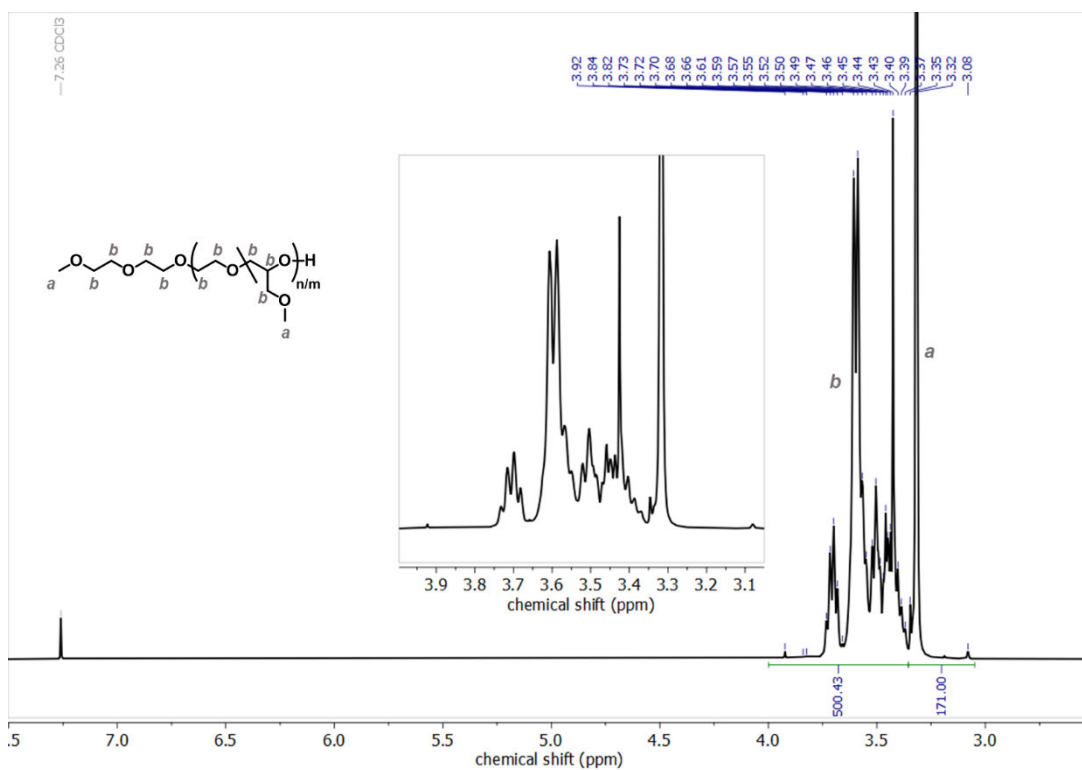


Figure S11. ^1H NMR spectrum (CDCl_3 , 400 MHz) of $\text{rPEG}^{0.51}_{110}$.

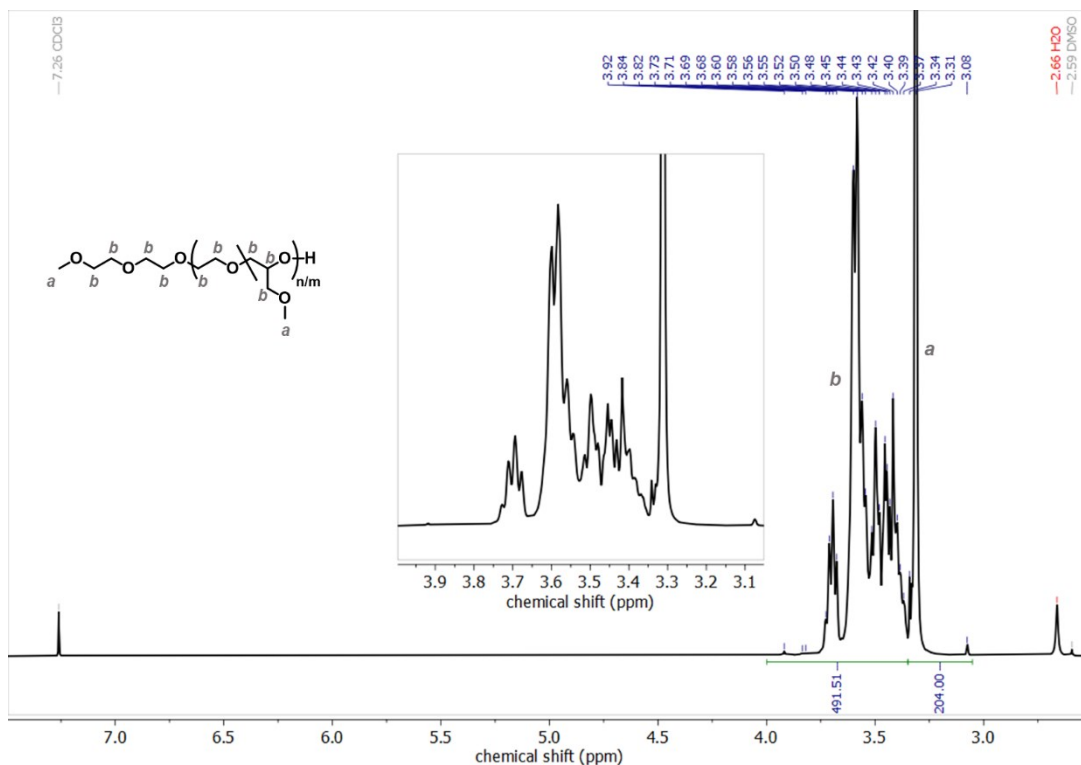


Figure S12. ^1H NMR spectrum (CDCl_3 , 400 MHz) of $\text{rPEG}_{105}^{0.64}$.

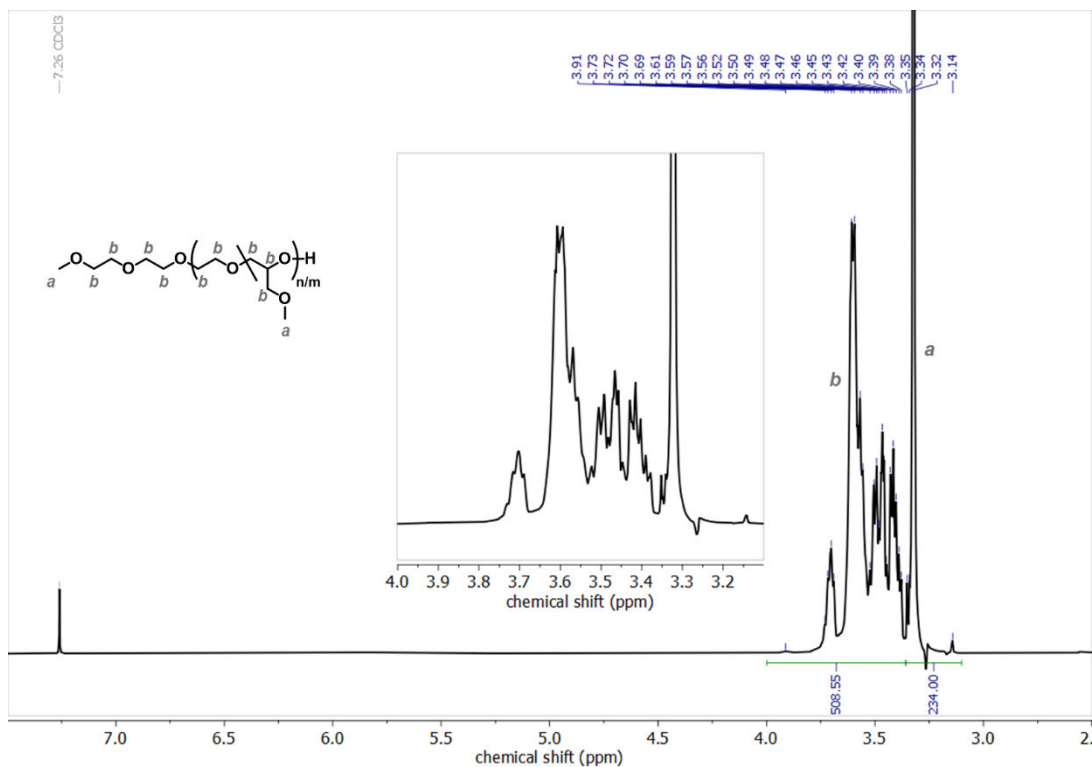


Figure S13. ^1H NMR spectrum (CDCl_3 , 400 MHz) of $\text{rPEG}_{105}^{0.73}$.

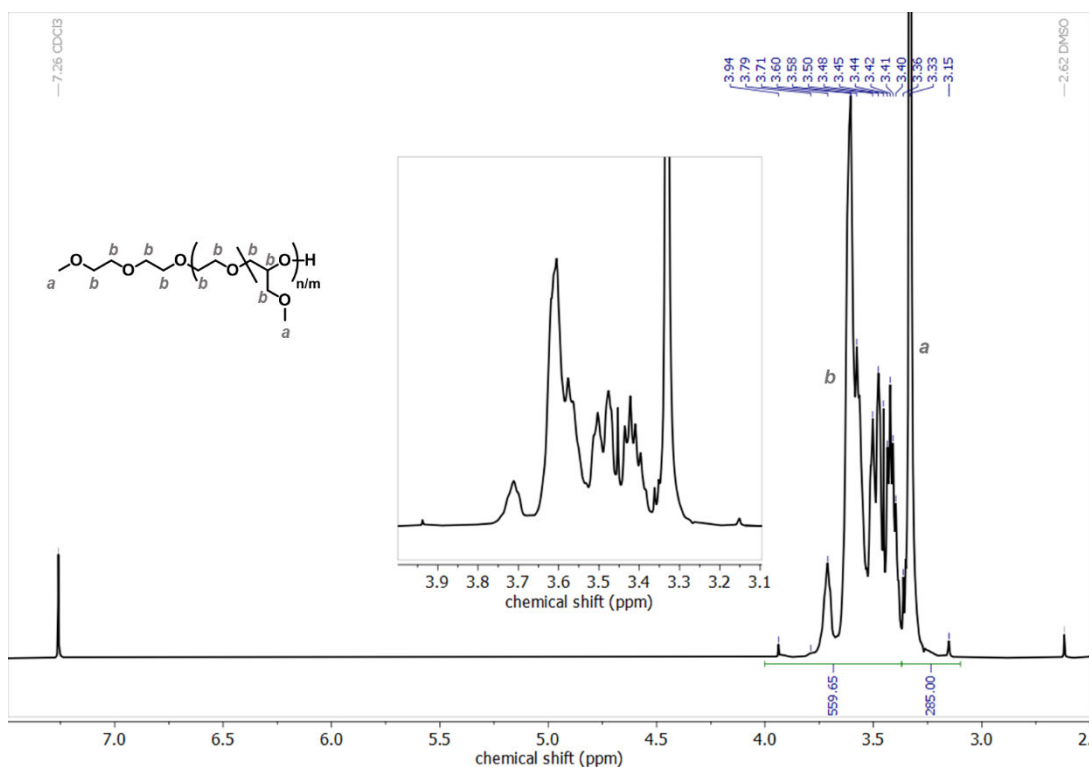


Figure S14. ^1H NMR spectrum (CDCl_3 , 400 MHz) of rPEG $^{0.82}_{115}$.

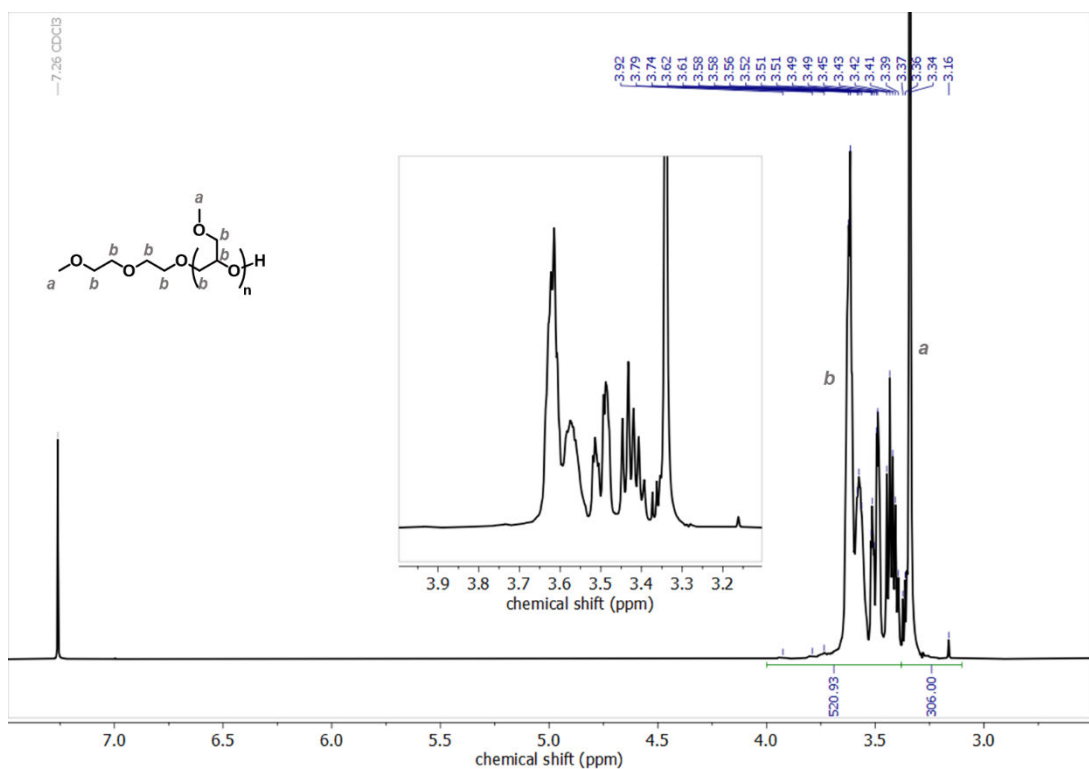


Figure S15. ^1H NMR spectrum (CDCl_3 , 400 MHz) of PGME $_{103}$.

4. MALDI TOF mass spectra

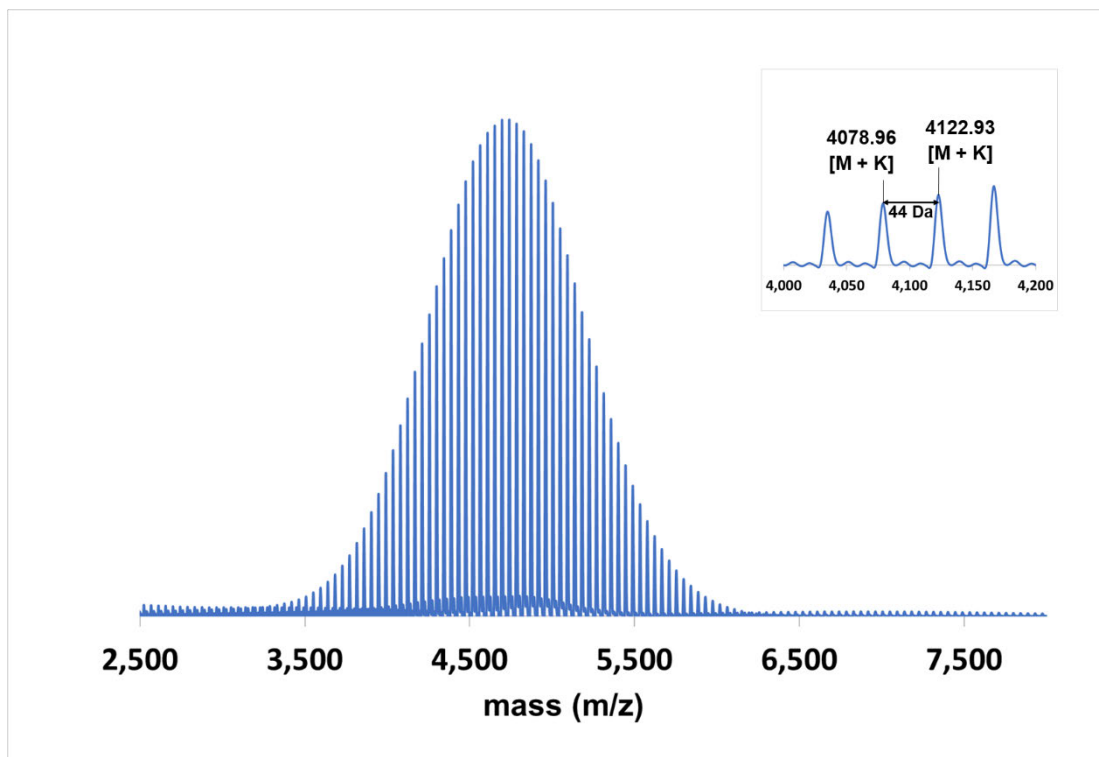


Figure S16. MALDI TOF mass spectrum of mPEG₁₀₆.

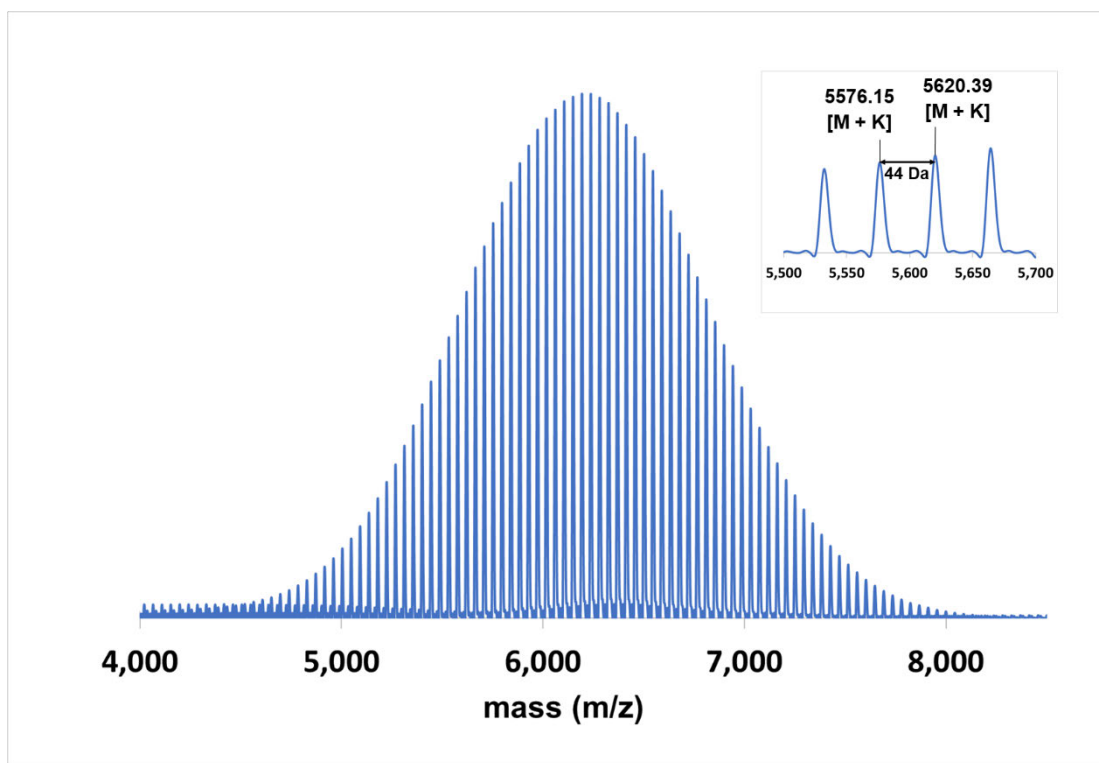


Figure S17. MALDI TOF mass spectrum of rPEG₁₁₁^{0.26}.

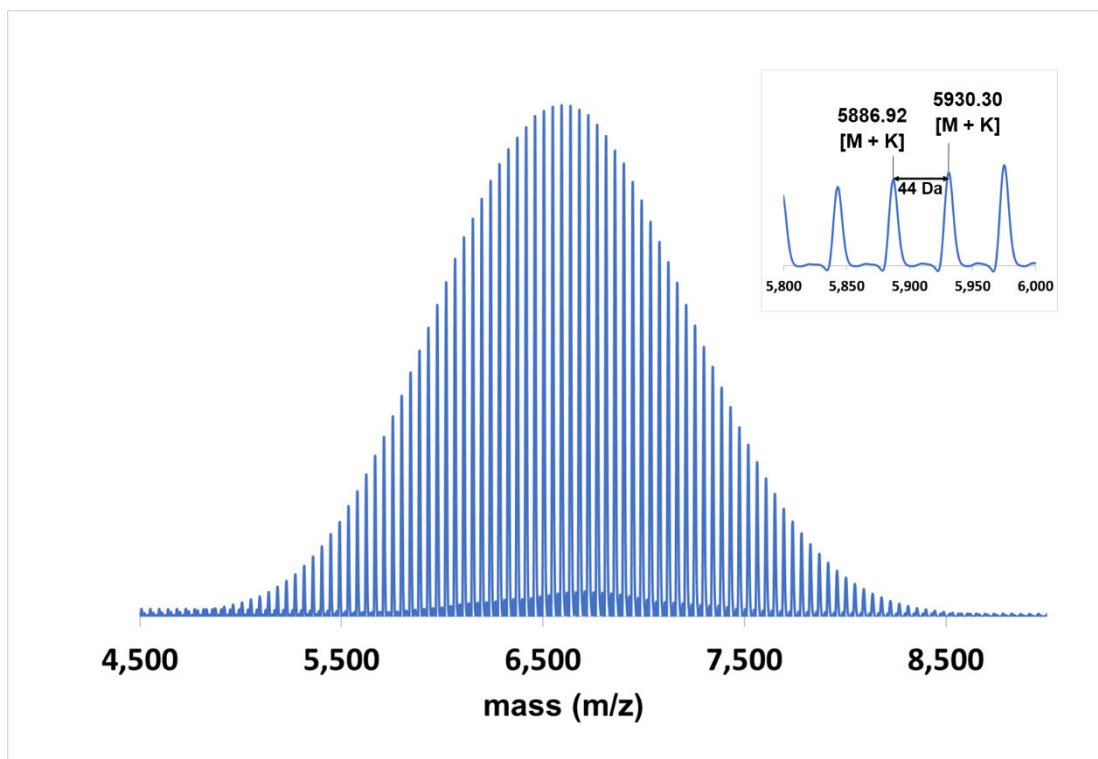


Figure S18. MALDI TOF mass spectrum of rPEG₁₁₁^{0.35}.

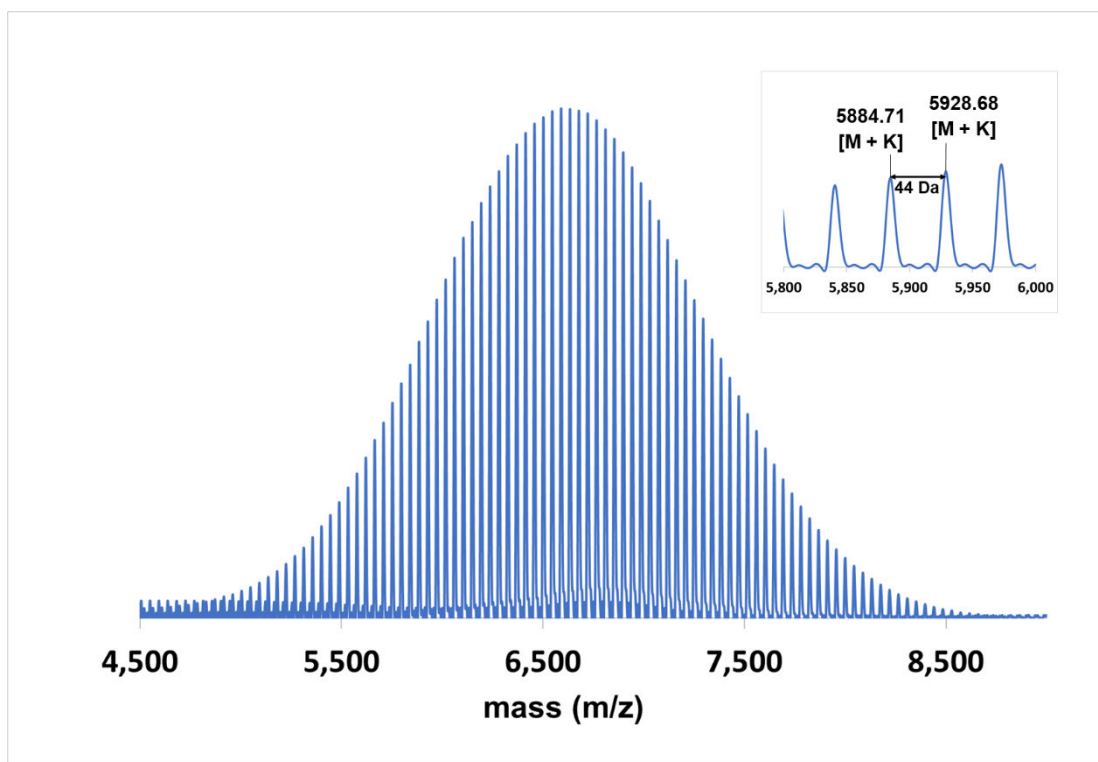


Figure S19. MALDI TOF mass spectrum of rPEG₁₀₆^{0.41}.

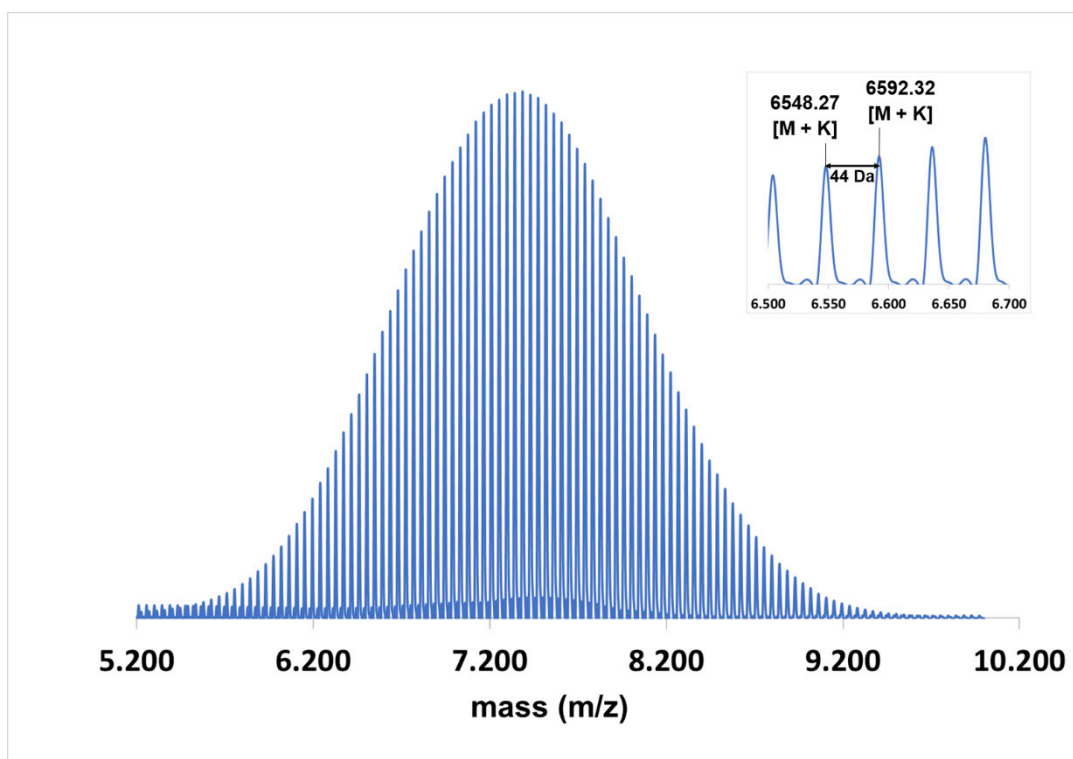


Figure S20. MALDI TOF mass spectrum of rPEG₁₁₀^{0.51}.

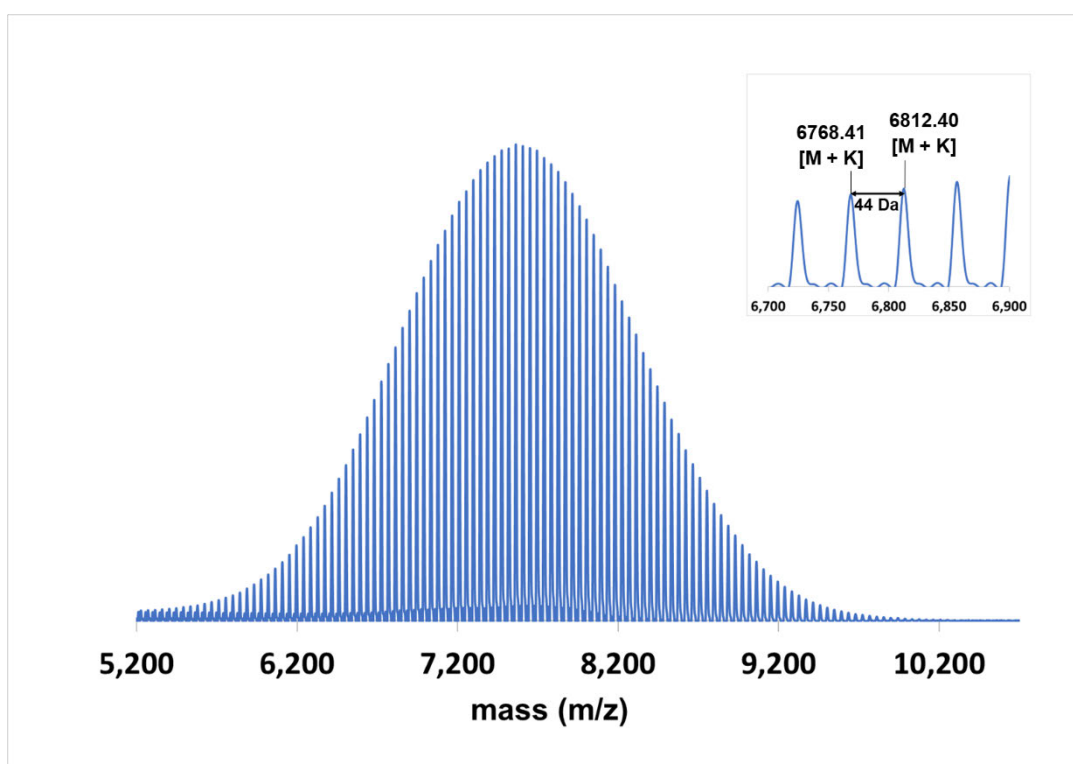


Figure S21. MALDI TOF mass spectrum of rPEG₁₀₅^{0.64}.

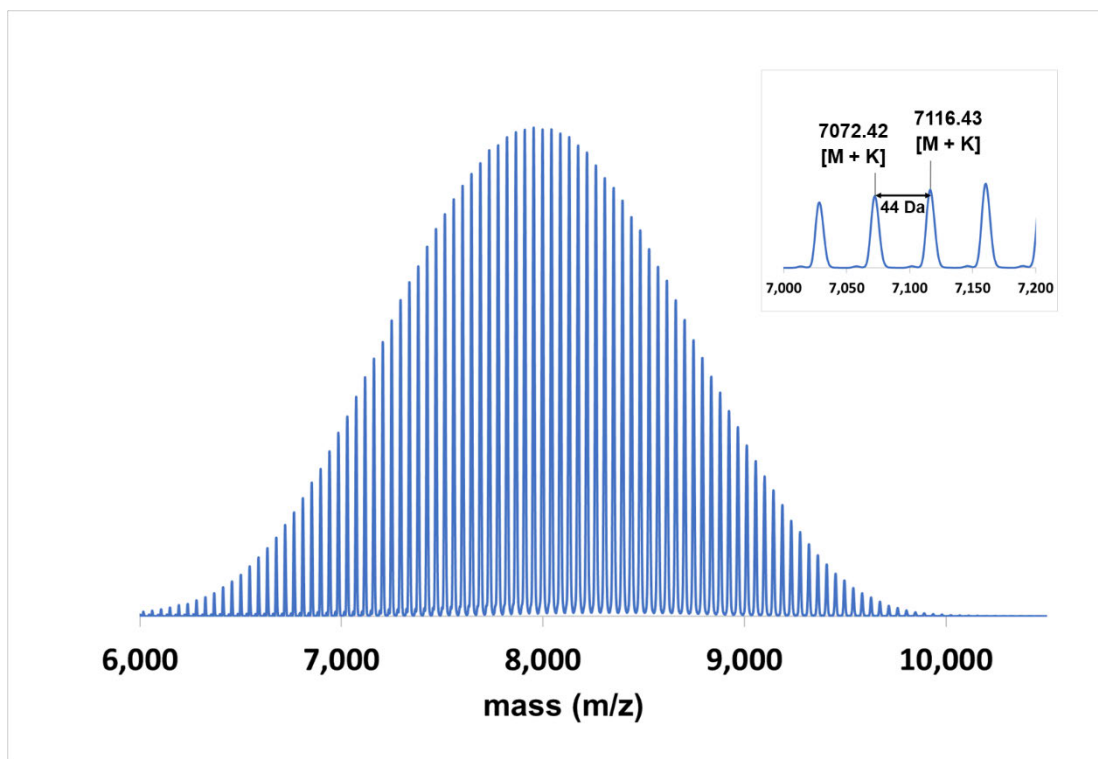


Figure S22. MALDI TOF mass spectrum of rPEG₁₀₅^{0.73}.

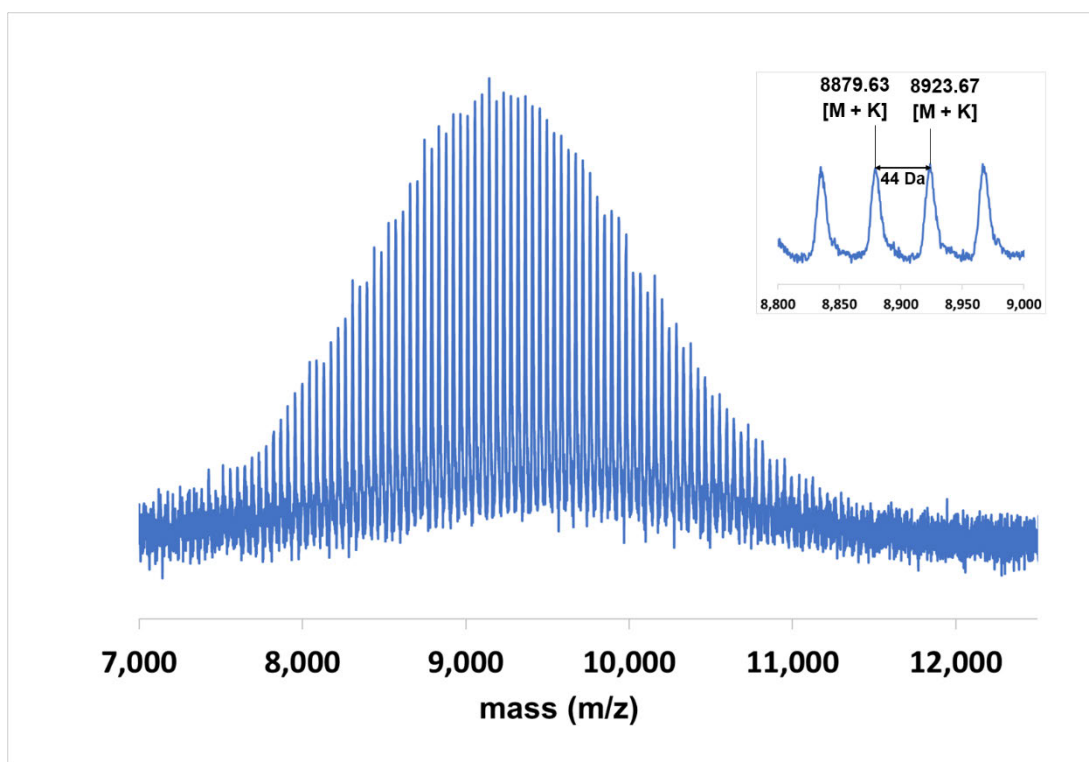


Figure S23. MALDI TOF mass spectrum of rPEG₁₁₅^{0.82}.

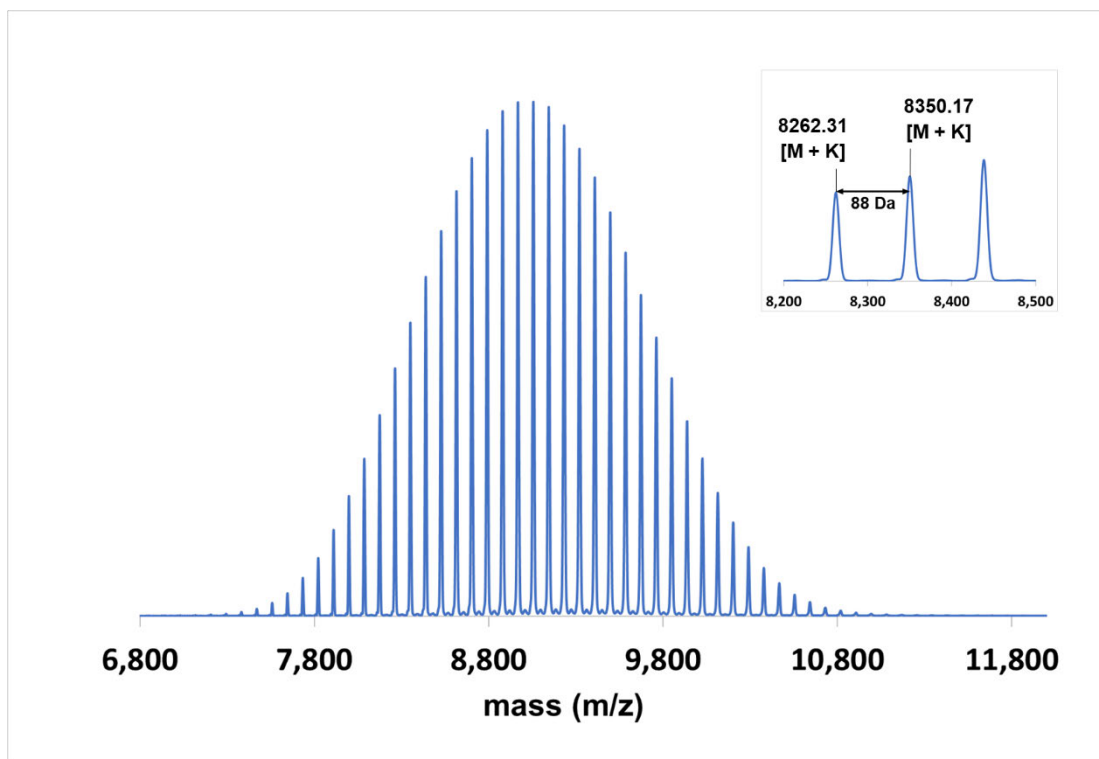


Figure S24. MALDI TOF mass spectrum of PGME₁₀₃.

5. SEC curves

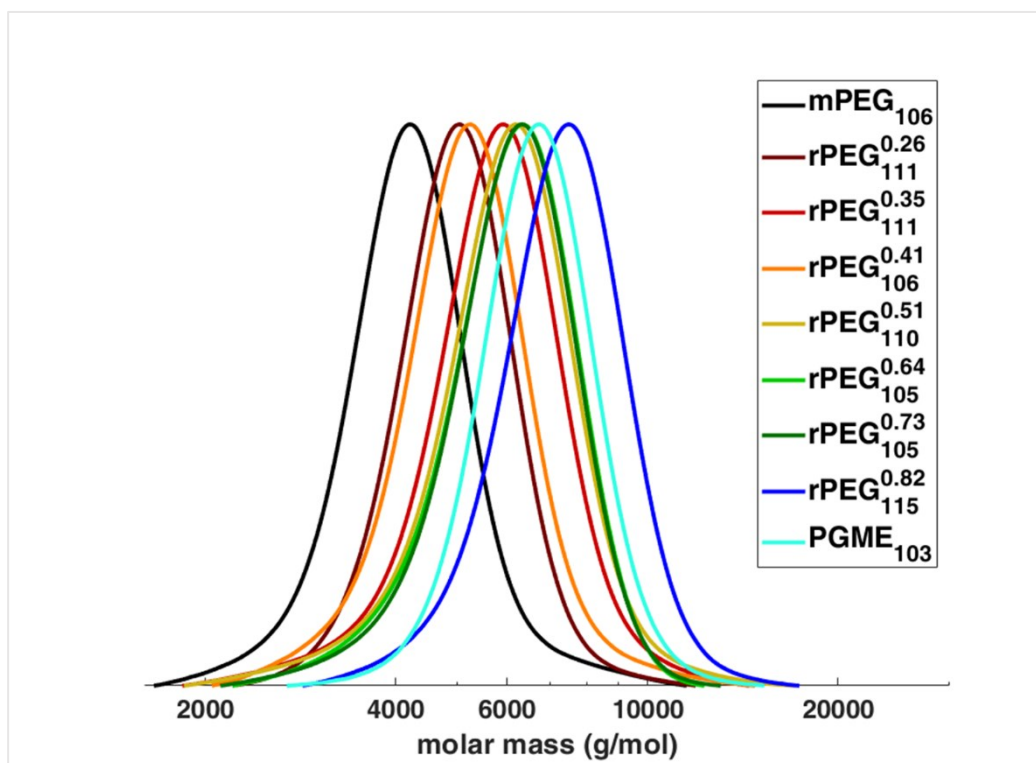


Figure S25. SEC curves (solvent: DMF; calibration: PEG) of the synthesized copolymers.

6. Cloud point measurements (turbidity curves)

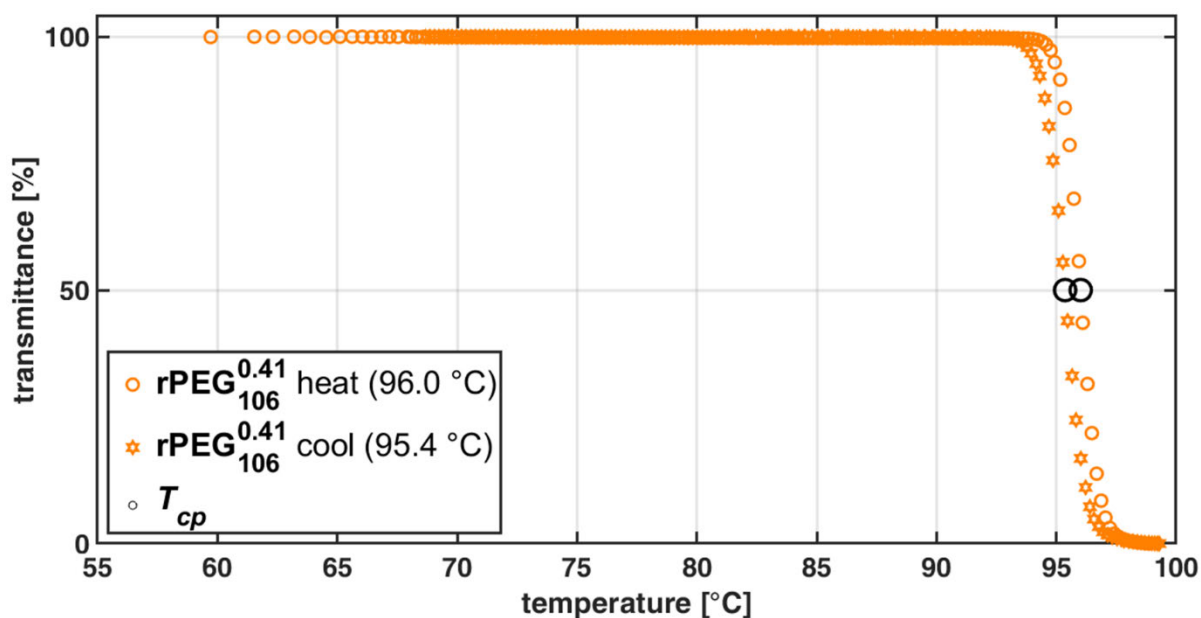


Figure S26. Turbidity curves (heating and cooling) of $\text{rPEG}_{106}^{0.41}$ measured at 5 mg mL^{-1} in Millipore water. Cloud points at 50 % transmittance are marked in the graph.

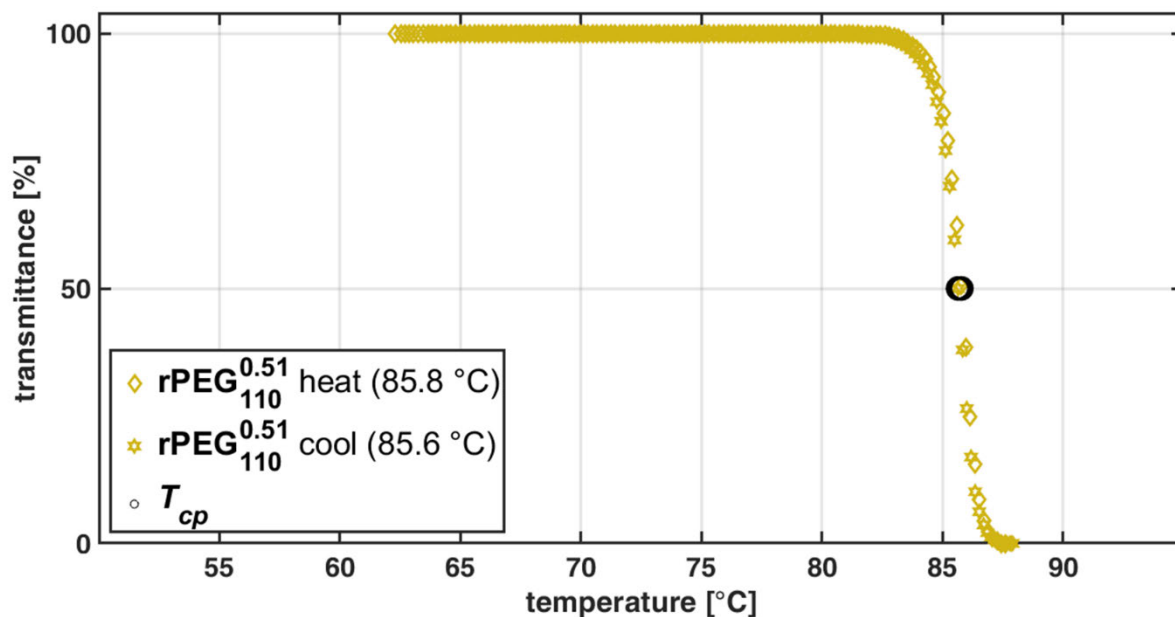


Figure S27. Turbidity curves (heating and cooling) of $\text{rPEG}_{110}^{0.51}$ measured at 5 mg mL^{-1} in Millipore water. Cloud points at 50 % transmittance are marked in the graph.

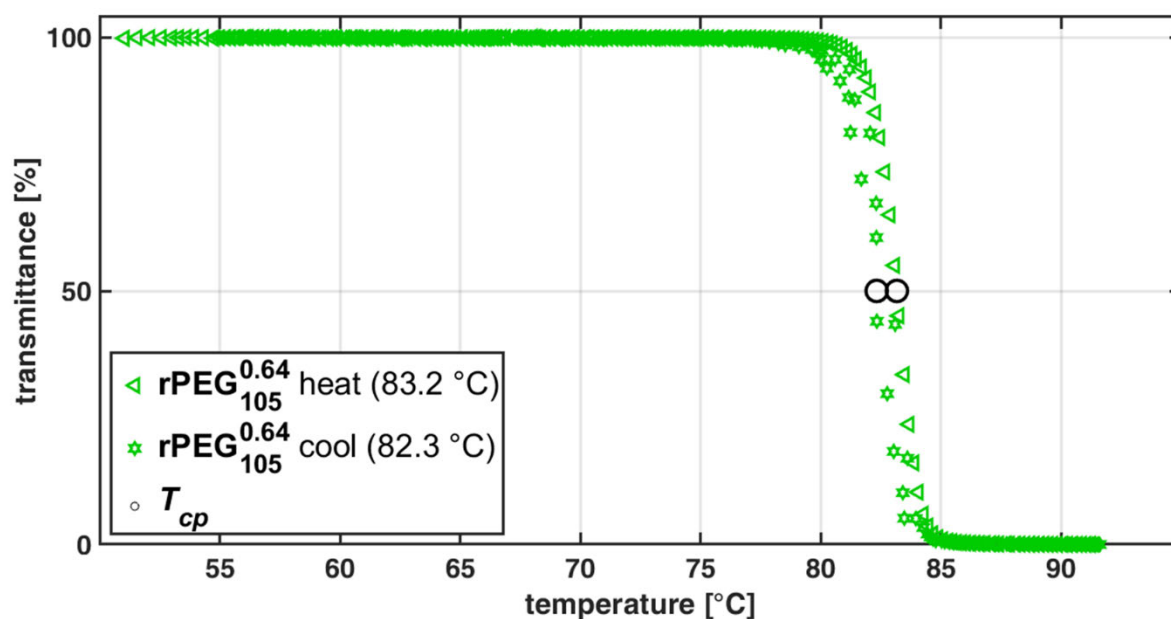


Figure S28. Turbidity curves (heating and cooling) of rPEG₁₀₅^{0.64} measured at 5 mg mL⁻¹ in Millipore water. Cloud points at 50 % transmittance are marked in the graph.

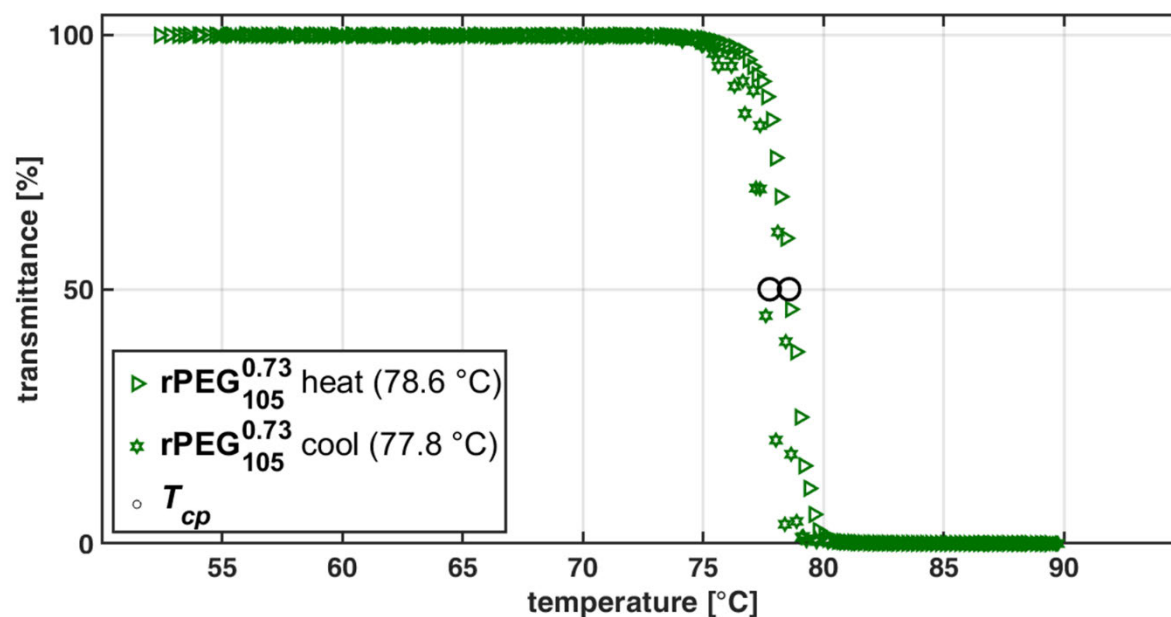


Figure S29. Turbidity curves (heating and cooling) of rPEG₁₀₅^{0.73} measured at 5 mg mL⁻¹ in Millipore water. Cloud points at 50 % transmittance are marked in the graph.

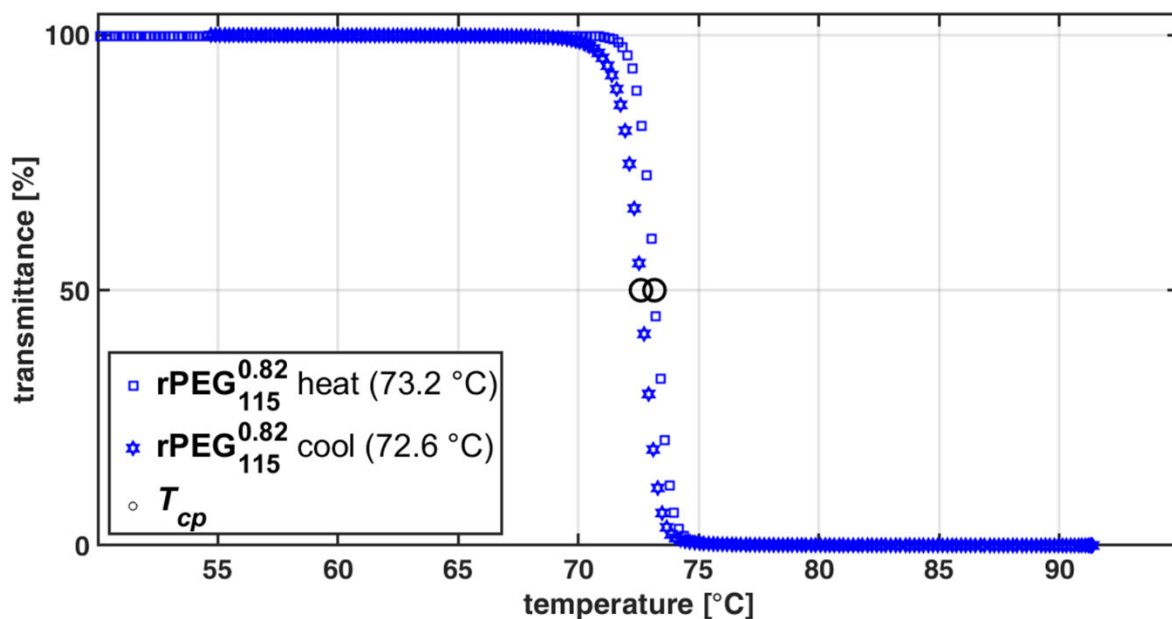


Figure S30. Turbidity curves (heating and cooling) of rPEG₁₁₅^{0.82} measured at 5 mg mL⁻¹ in Millipore water. Cloud points at 50 % transmittance are marked in the graph.

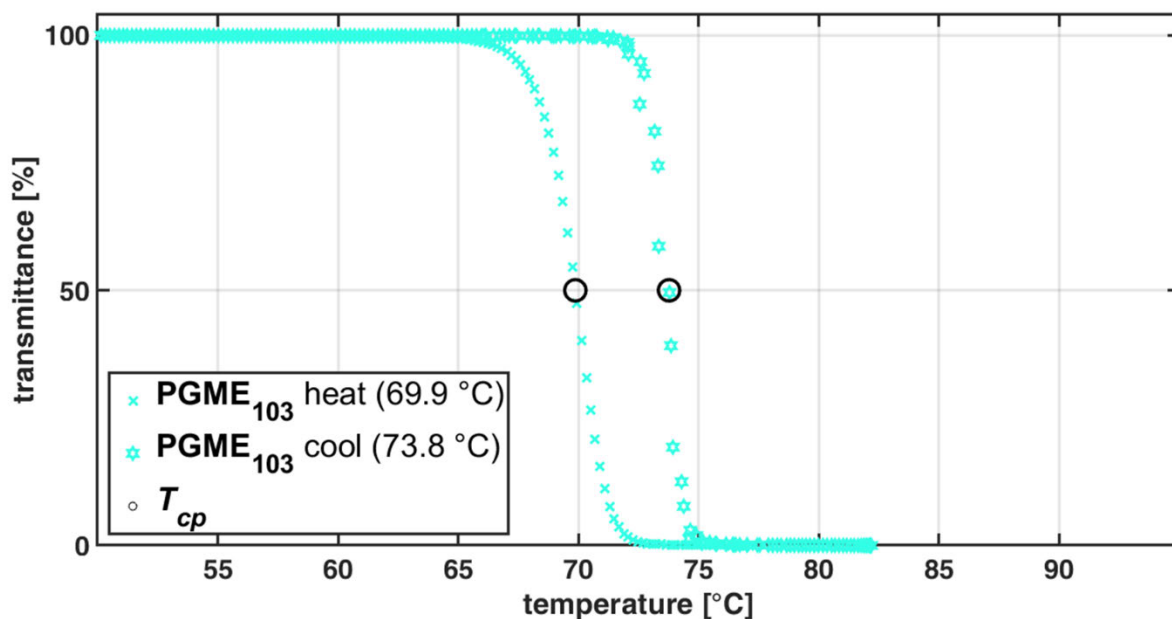


Figure S31. Turbidity curves (heating and cooling) of PGME₁₀₃ measured at 5 mg mL⁻¹ in Millipore water. Cloud points at 50 % transmittance are marked in the graph.

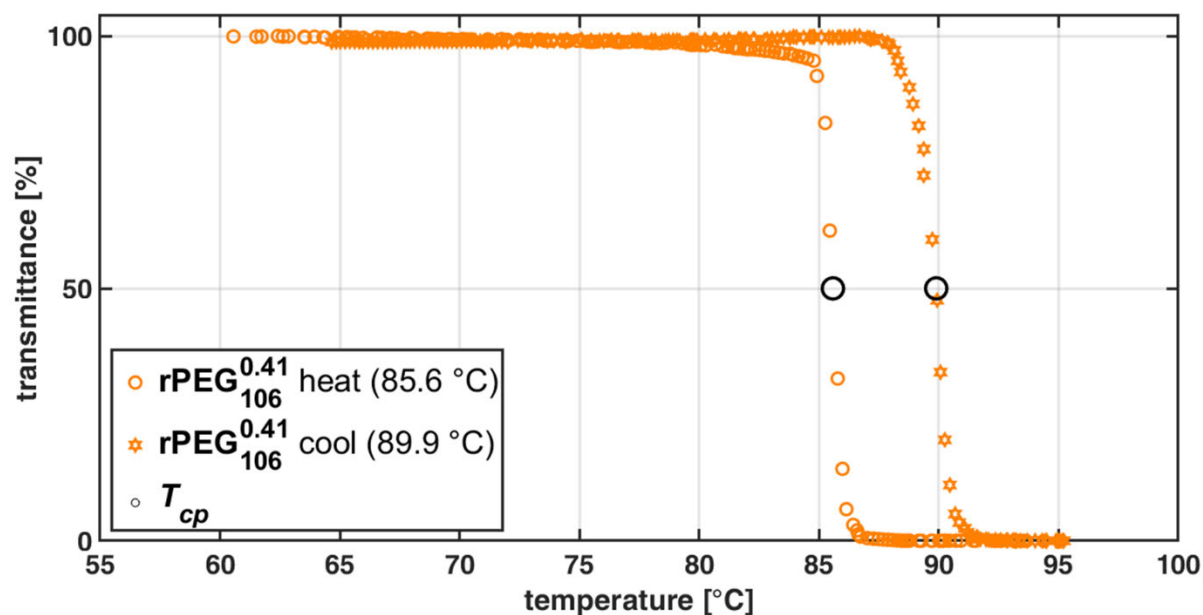


Figure S32. Turbidity curves (heating and cooling) of $\text{rPEG}_{106}^{0.41}$ measured at 100 mg mL^{-1} in Millipore water. Cloud points at 50 % transmittance are marked in the graph.

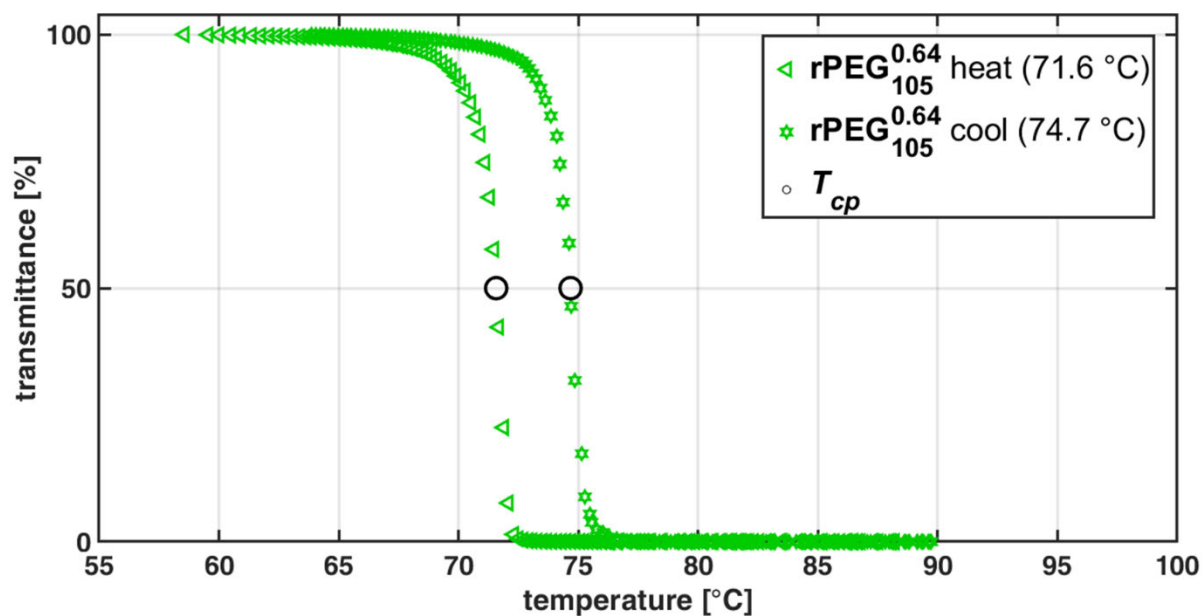


Figure S33. Turbidity curves (heating and cooling) of $\text{rPEG}_{105}^{0.64}$ measured at 100 mg mL^{-1} in Millipore water. Cloud points at 50 % transmittance are marked in the graph.

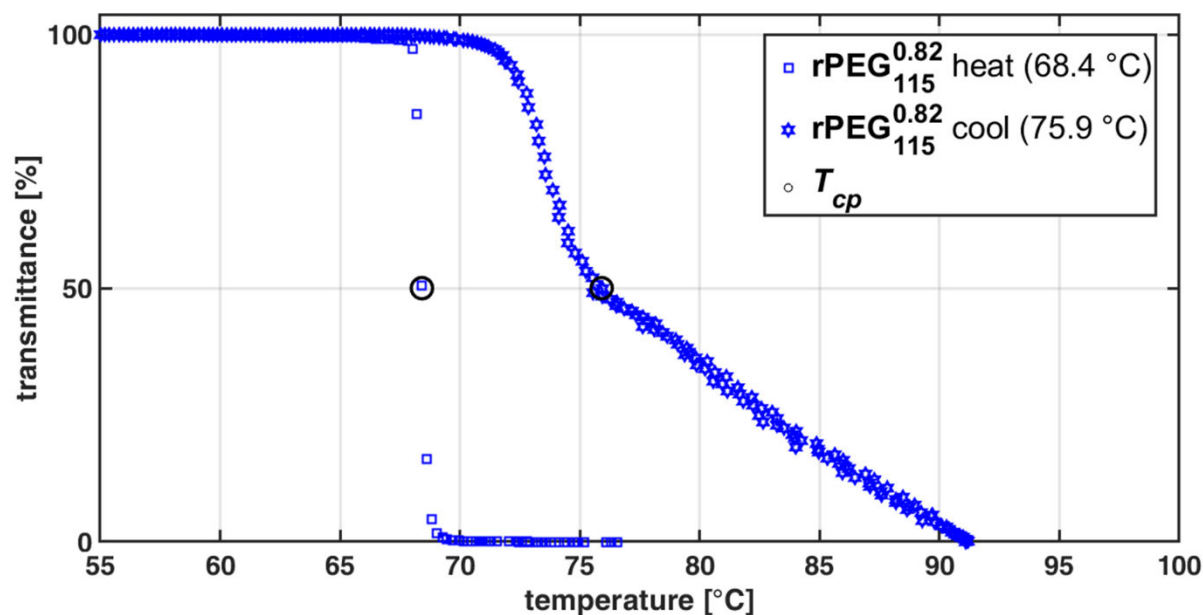


Figure S34. Turbidity curves (heating and cooling) of $\text{rPEG}_{115}^{0.82}$ measured at 100 mg mL^{-1} in Millipore water. Cloud points at 50 % transmittance are marked in the graph.

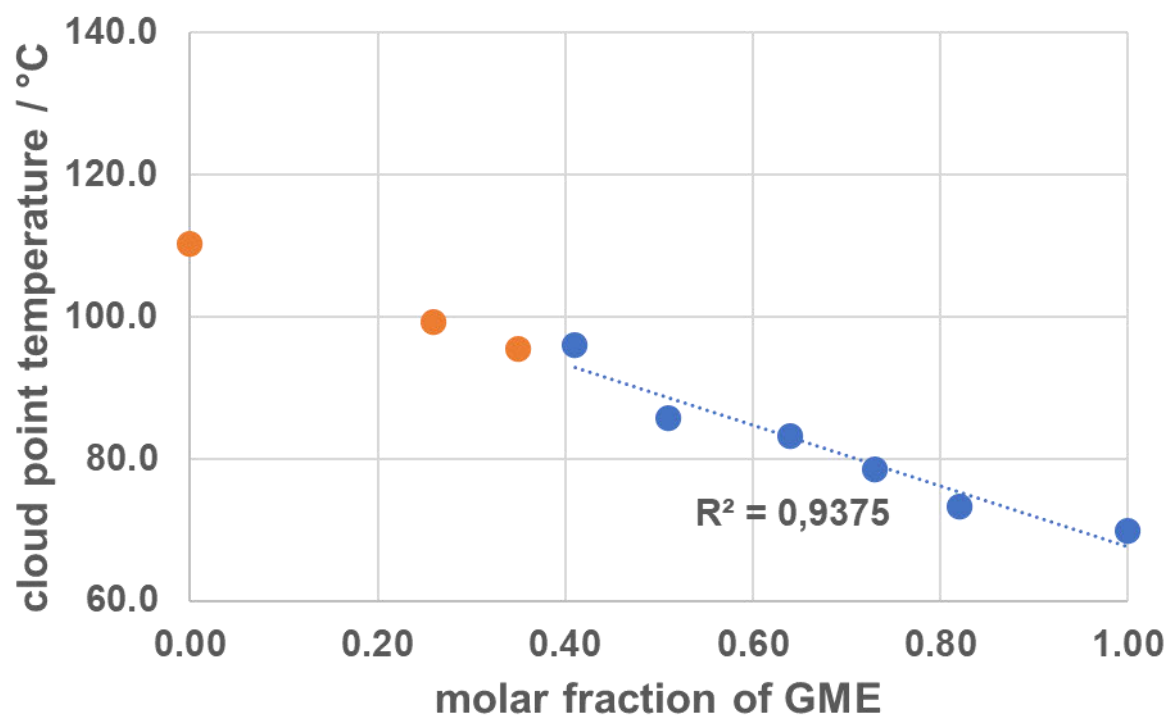


Figure S35. Molar fraction of GME in rPEGs plotted against cloud point temperatures for conc. = 5 mg mL^{-1} (blue = measured values; orange = predicted values based on fit); linear fit of measured cloud points and predicted values based on this fit. Linear fit equation: $T_{cp}(f_{GME}) = -42.69 \cdot f_{GME} + 110.36$.

Table S1. Predicted cloud point temperatures (orange) based on a linear fit of measured values (blue) at conc. = 5 mg mL⁻¹.

Polymer	T_{cp} (for conc. = 5 mg mL⁻¹) / °C
mPEG₁₀₆	110.4
rPEG₁₁₁^{0.26}	99.3
rPEG₁₁₁^{0.35}	95.4
rPEG₁₀₆^{0.41}	96.0
rPEG₁₁₀^{0.51}	85.8
rPEG₁₀₅^{0.64}	83.2
rPEG₁₀₅^{0.73}	78.6
rPEG₁₁₅^{0.82}	73.2
PGME₁₀₃	69.9

7. DSC curves

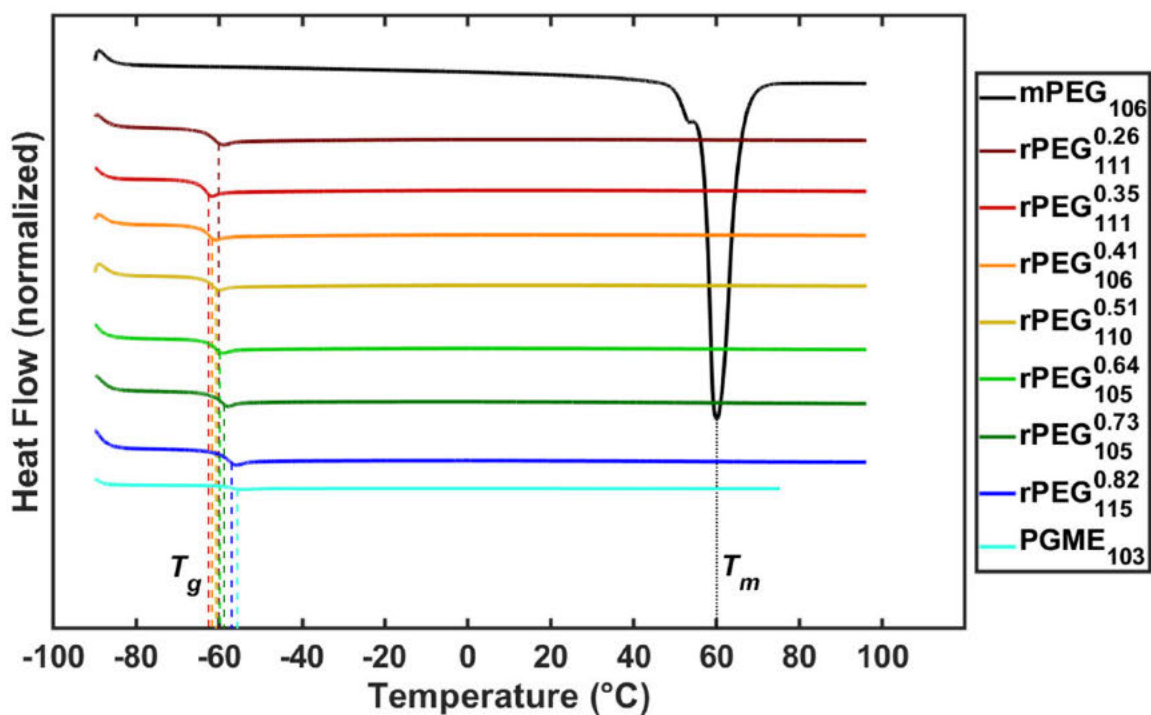


Figure S36. DSC curves of the synthesized copolymers. Glass transition temperatures T_g and melting temperatures T_m are marked in the graph.

Table S2. Glass transition temperatures T_g and melting temperatures T_m of the synthesized copolymers.

<i>Polymer</i>	M_n (MALDI TOF) / g mol^{-1}	T_g / $^{\circ}\text{C}$	T_m / $^{\circ}\text{C}$	ΔH_m / J g^{-1}
mPEG ₁₀₆	4,724	--	60	169.1
rPEG ₁₁₁ ^{0.26}	6,217	– 60	--	--
rPEG ₁₁₁ ^{0.35}	6,617	– 63	--	--
rPEG ₁₀₆ ^{0.41}	6,629	– 62	--	--
rPEG ₁₁₀ ^{0.51}	7,384	– 61	--	--
rPEG ₁₀₅ ^{0.64}	7,593	– 60	--	--
rPEG ₁₀₅ ^{0.73}	8,007	– 59	--	--
rPEG ₁₁₅ ^{0.82}	9,292	– 57	--	--
PGME ₁₀₃	9,085	– 56	--	--

8. EPR Spectroscopy

8.1. Experimental and simulated spectra

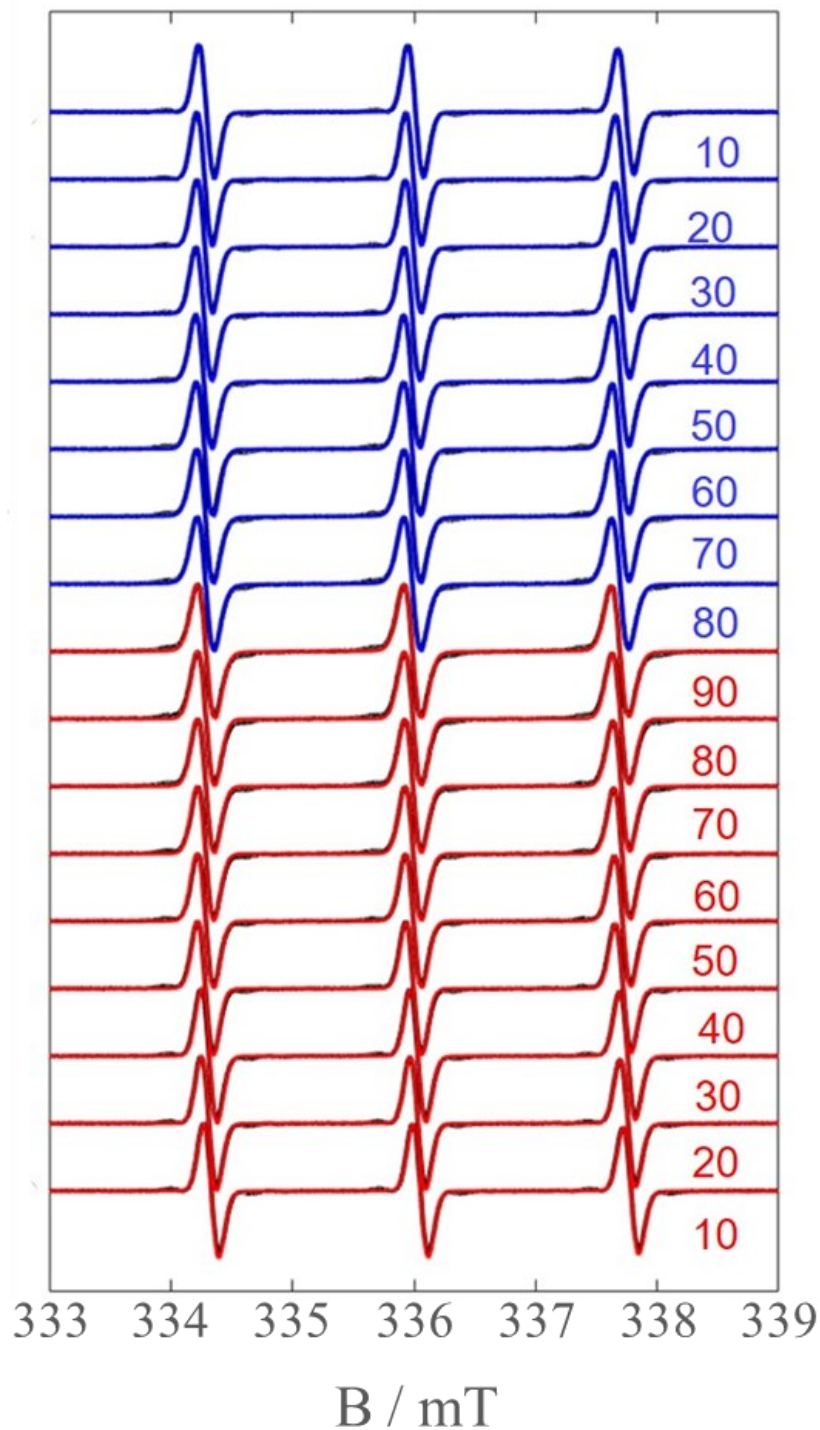


Figure S37. Experimental data (black) and corresponding simulation of EPR spectra of spin probe TEMPO (0.02 mM) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

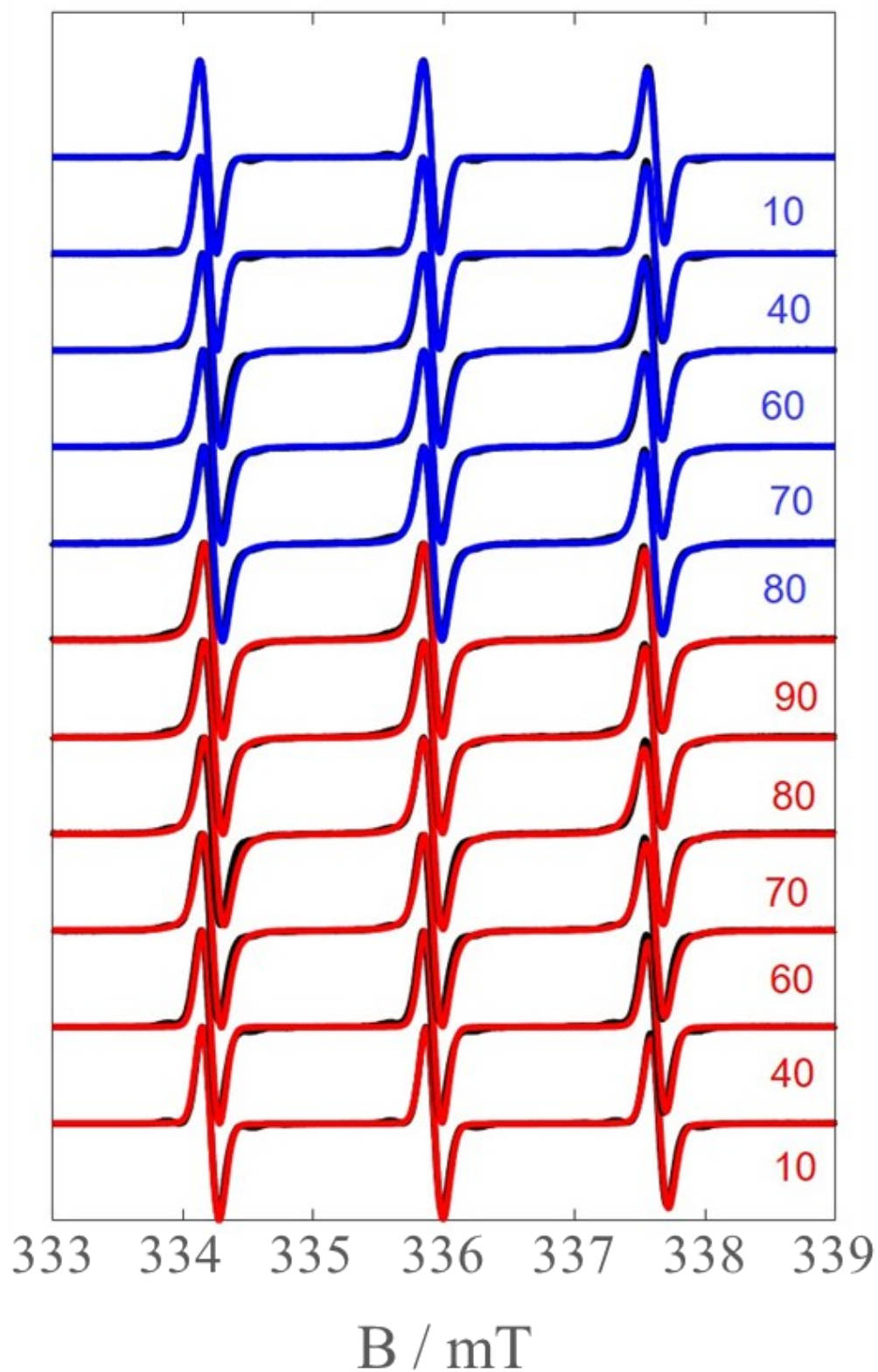


Figure S38. Experimental data (black) and corresponding simulation of EPR spectra of a 10 w% aqueous solution of mPEG₁₀₆ (1.00 mM TEMPO) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

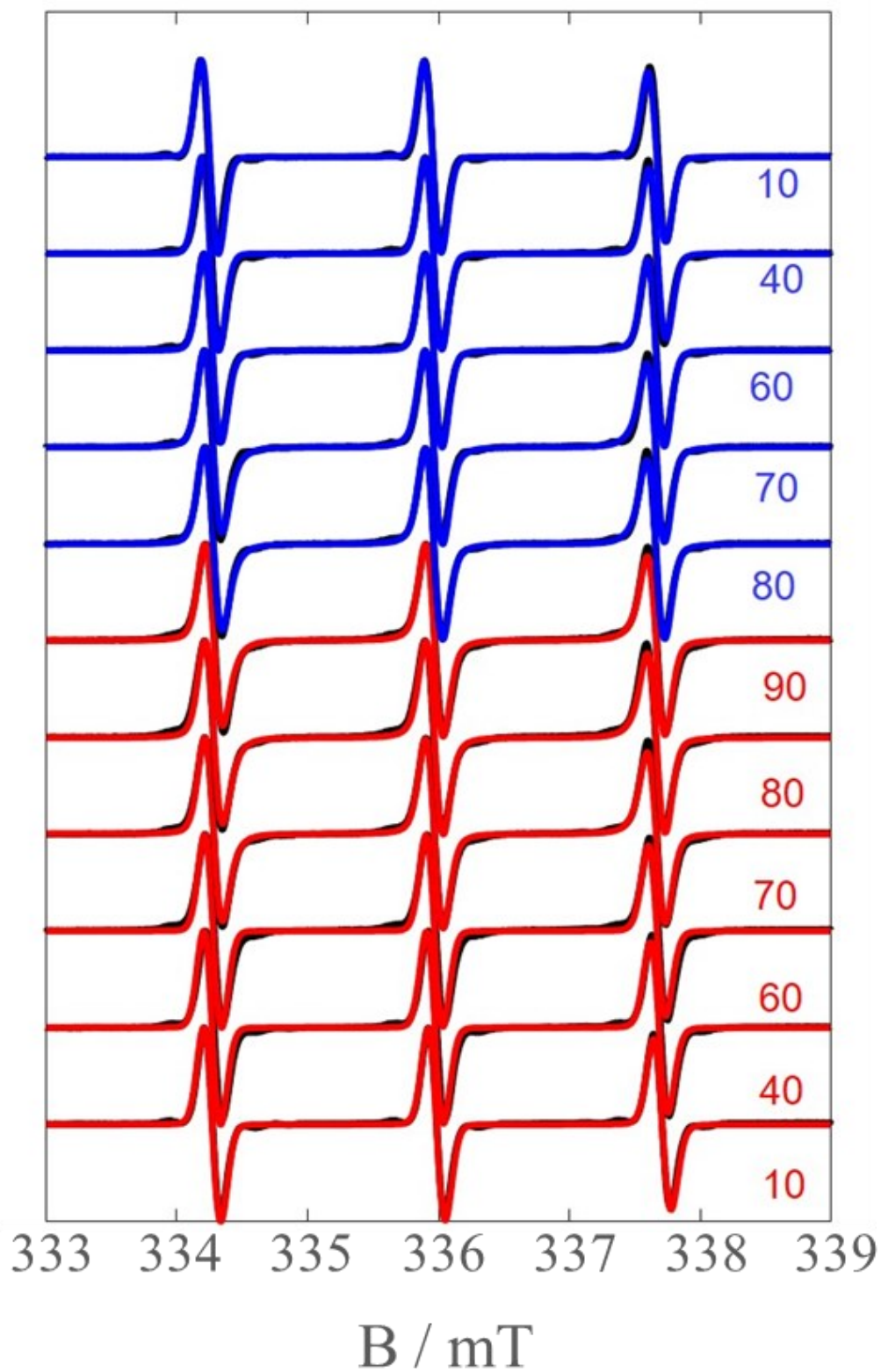


Figure S39. Experimental data (black) and corresponding simulation of EPR spectra of a 10 w% aqueous solution of rPEG₁₁₁^{0.26} (1.00 mM TEMPO) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

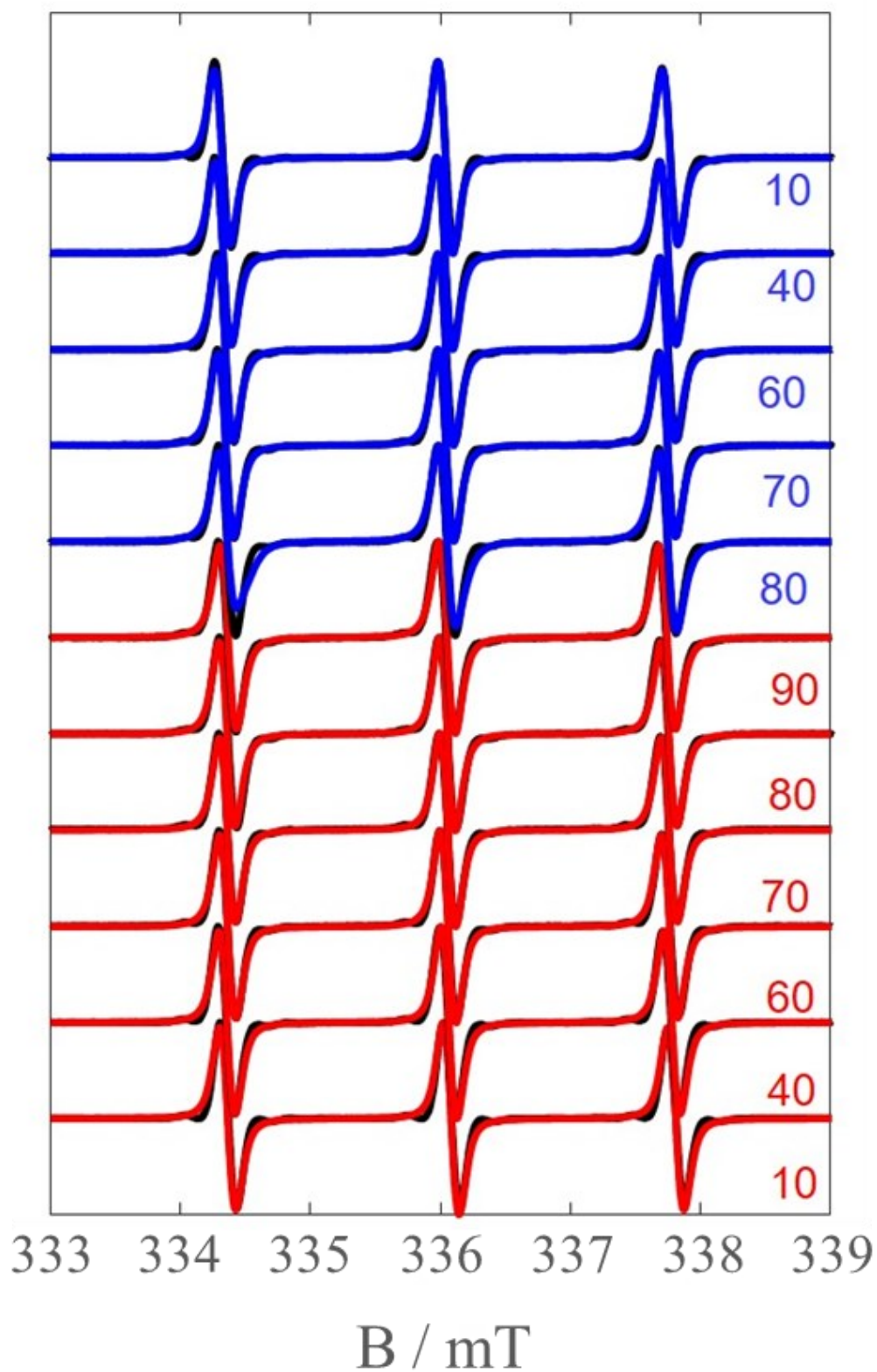


Figure S40. Experimental data (black) and corresponding simulation of EPR spectra of a 10 w% aqueous solution of rPEG₁₁₁^{0.35} (1.00 mM TEMPO) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

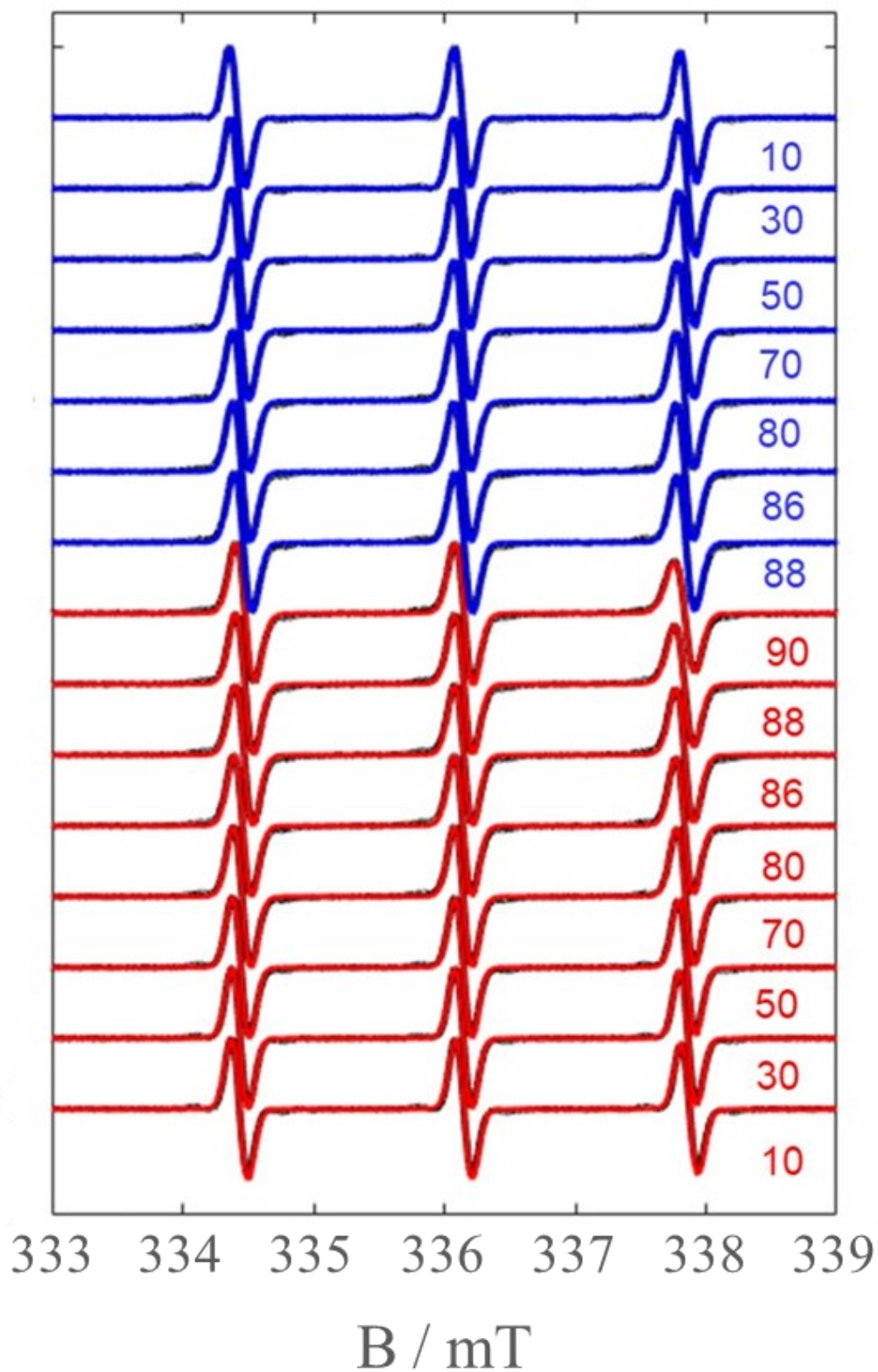


Figure S41. Experimental data (black) and corresponding simulation of EPR spectra of a 10 w% aqueous solution of rPEG₁₀₆^{0.41} (1.00 mM TEMPO) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

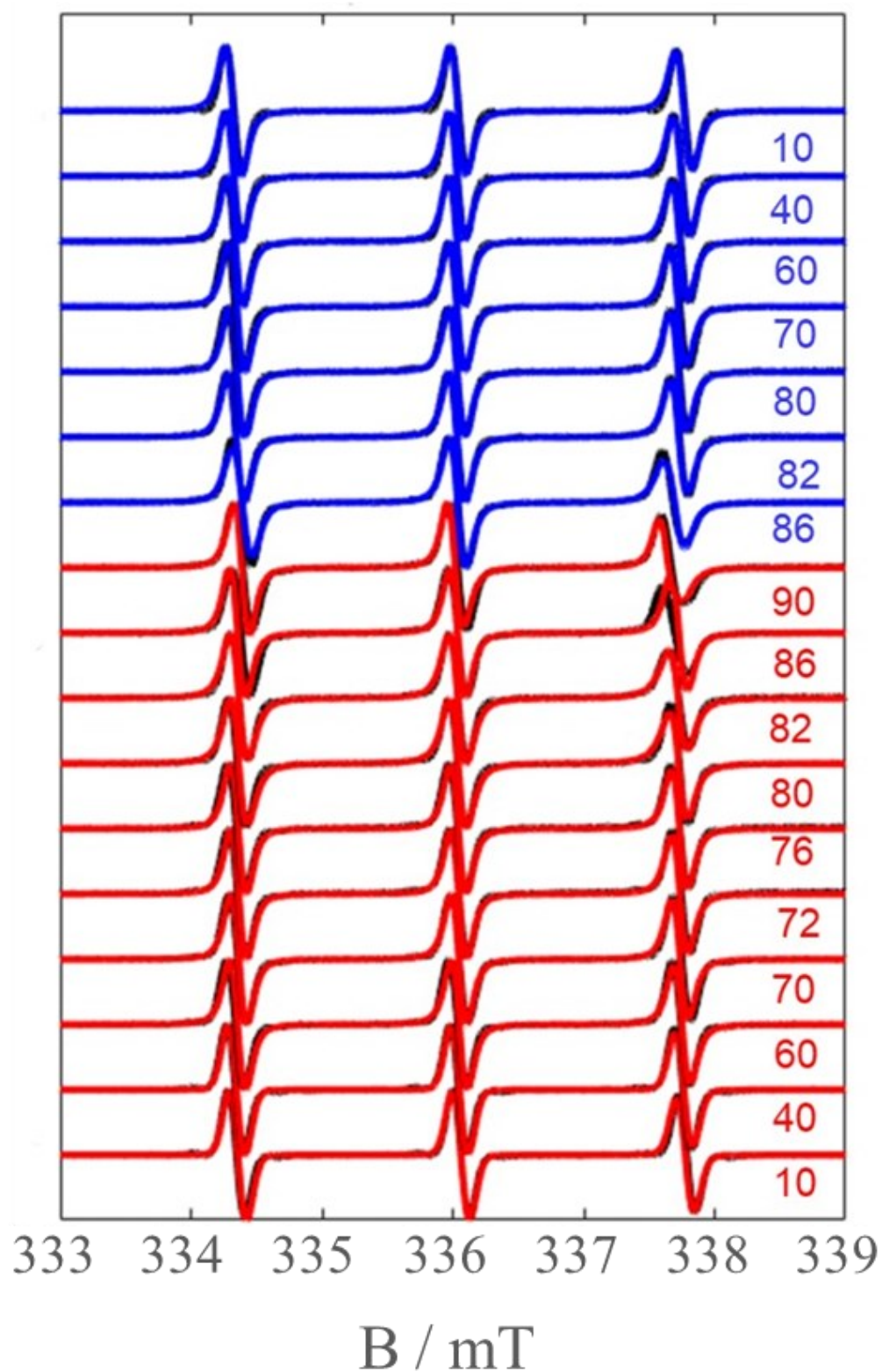


Figure S42. Experimental data (black) and corresponding simulation of EPR spectra of a 10 w% aqueous solution of $\text{rPEG}_{110}^{0.51}$ (1.00 mM TEMPO) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

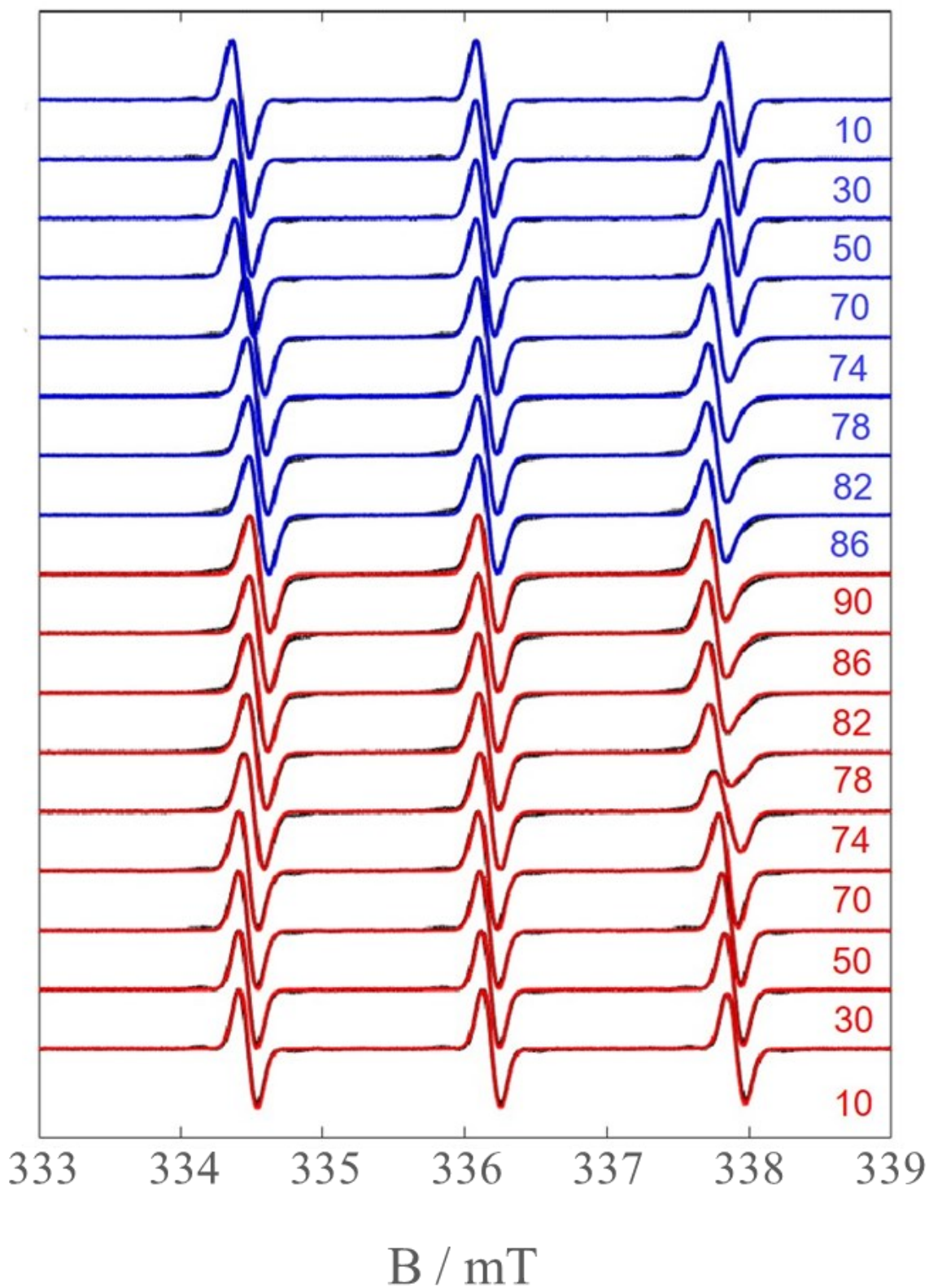


Figure S43. Experimental data (black) and corresponding simulation of EPR spectra of a 10 w% aqueous solution of rPEG^{0.64}₁₀₅ (1.00 mM TEMPO) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

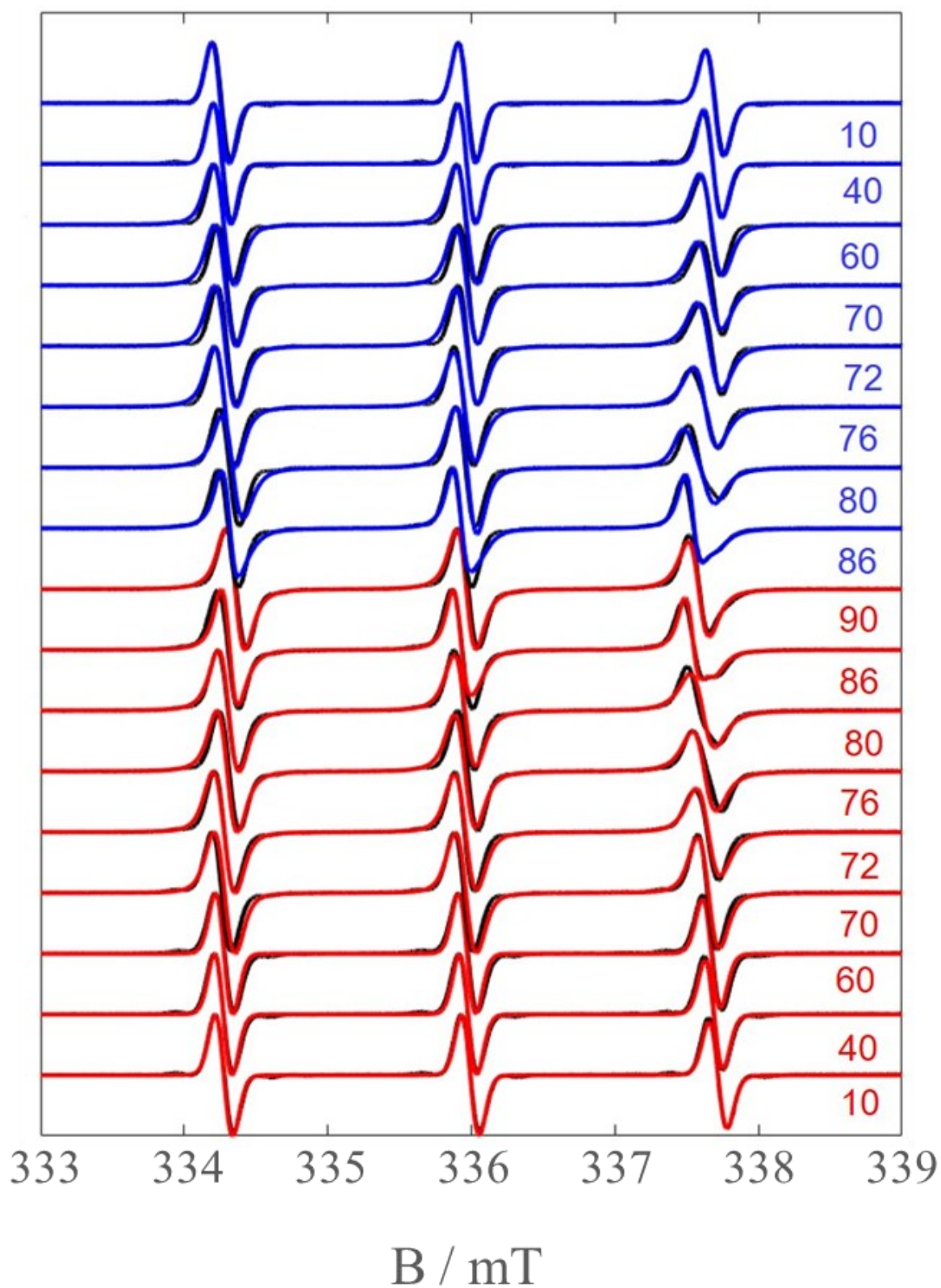


Figure S44. Experimental data (black) and corresponding simulation of EPR spectra of a 10 w% aqueous solution of $\text{rPEG}_{105}^{0.73}$ (1.00 mM TEMPO) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

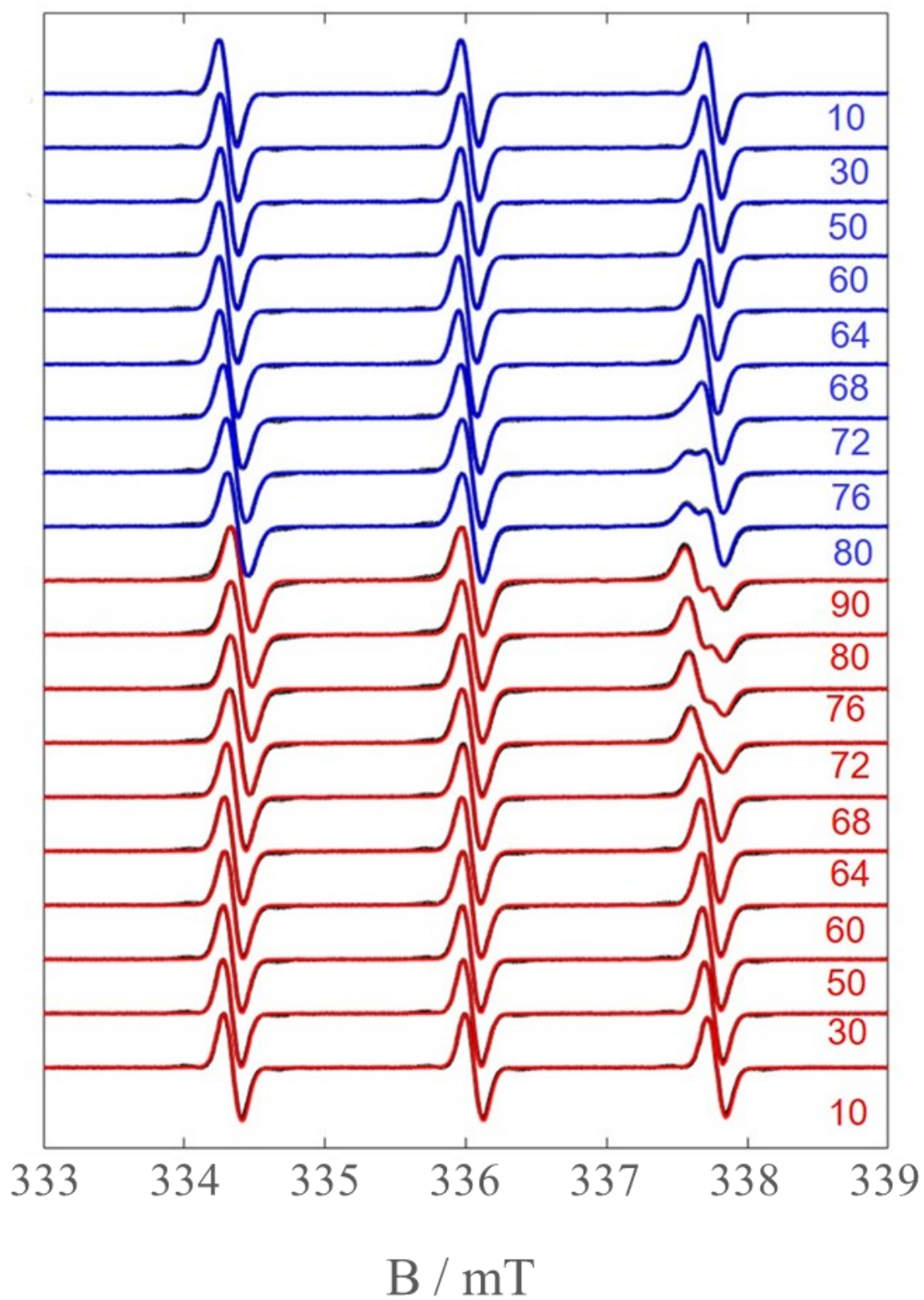


Figure S45. Experimental data (black) and corresponding simulation of EPR spectra of a 10 w% aqueous solution of rPEG^{0.82}₁₁₅ (1.00 mM TEMPO) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

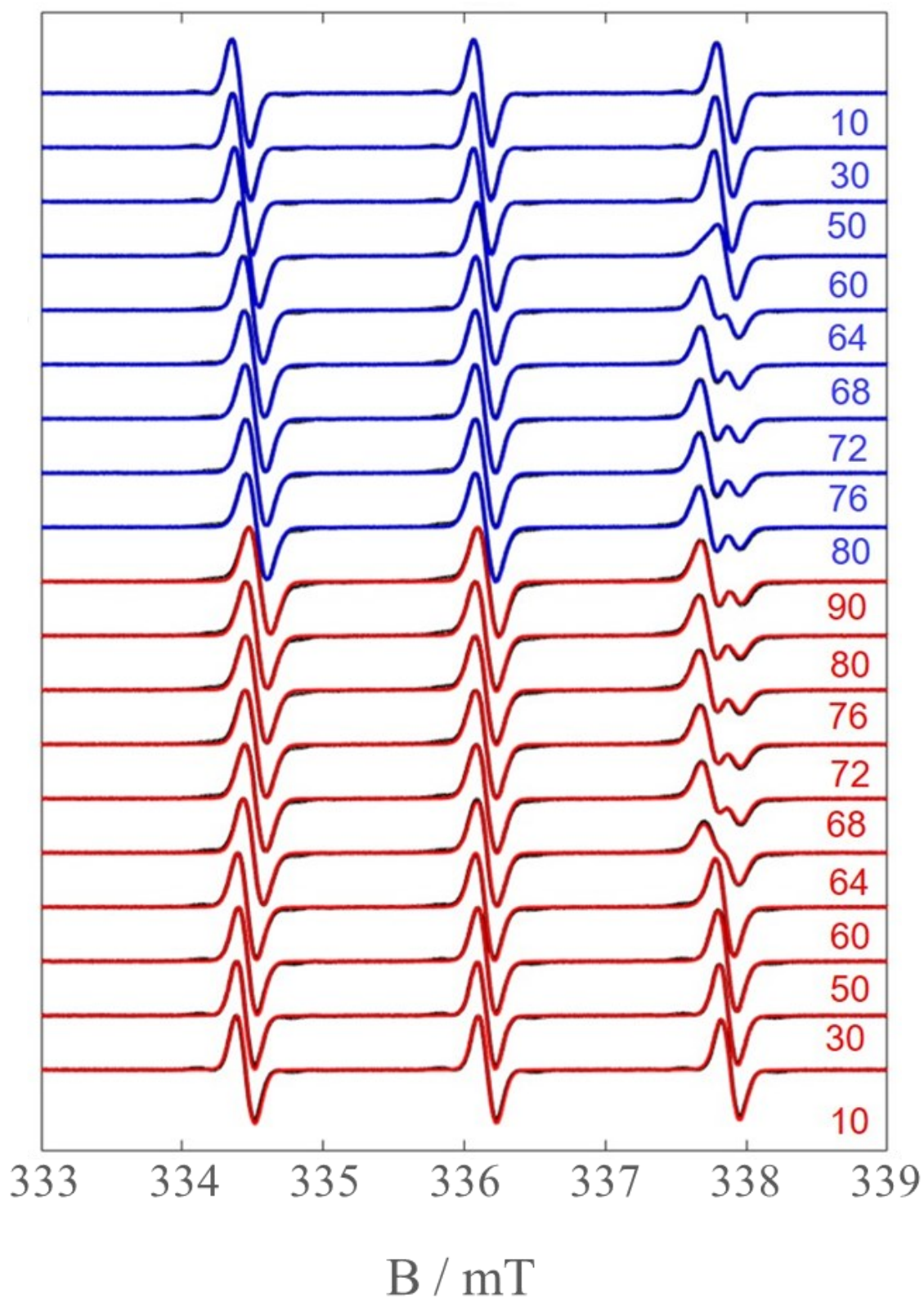


Figure S46. Experimental data (black) and corresponding simulation of EPR spectra of a 10 w% aqueous solution of PGME₁₀₃ (1.00 mM TEMPO) for varying temperatures. Simulated spectra in heating and cooling cycles are shown in red and blue. For the purpose of clarity, only a selection of spectra is shown.

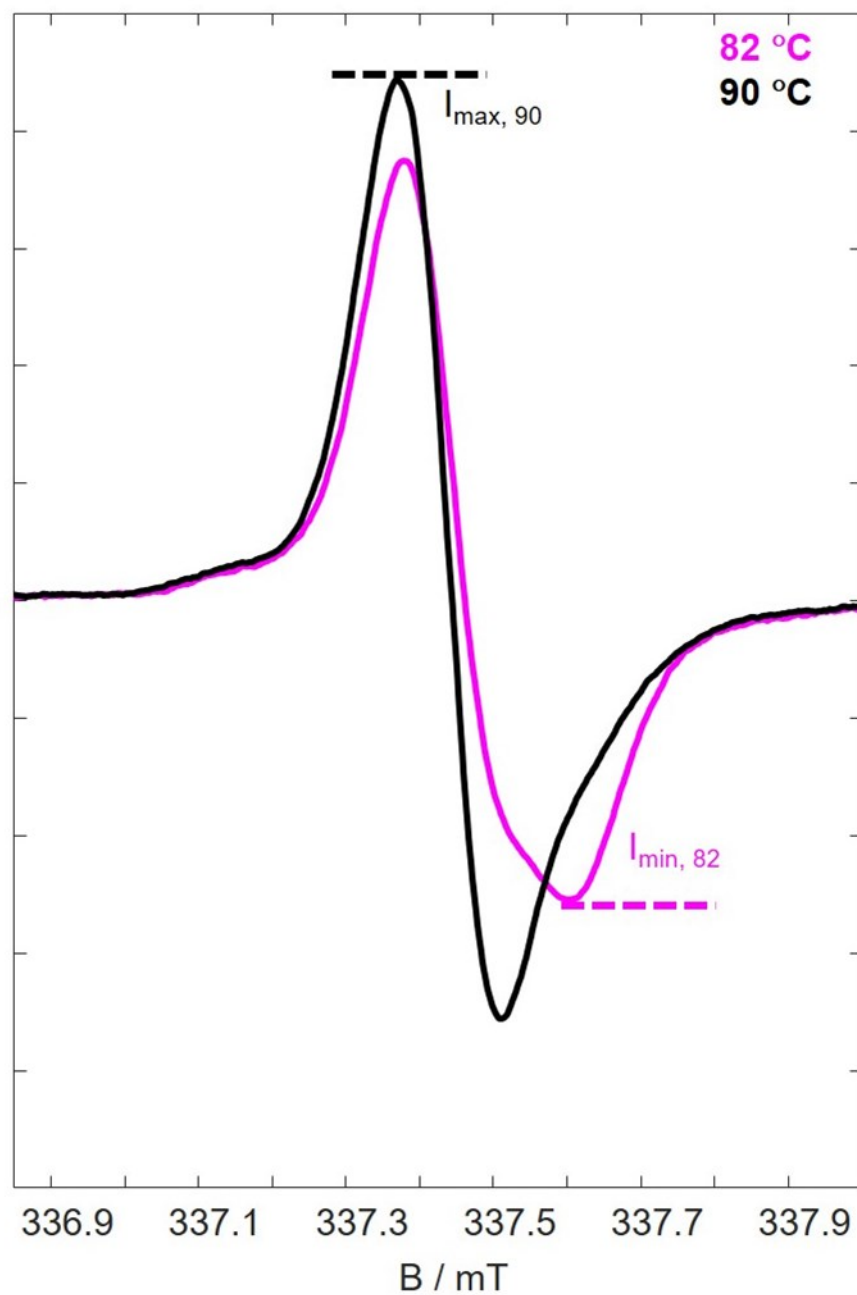


Figure S47. Reading spectral data to calculate overall hydrophobicity. $I_{\max, 90}$ represents the intensity at the maximum of the high-field peak measured at 90 °C, and $I_{\min, T}$ is the intensity of the minimum of the “first component” (the hydrophilic component) in the high-field peak at measured temperature T .

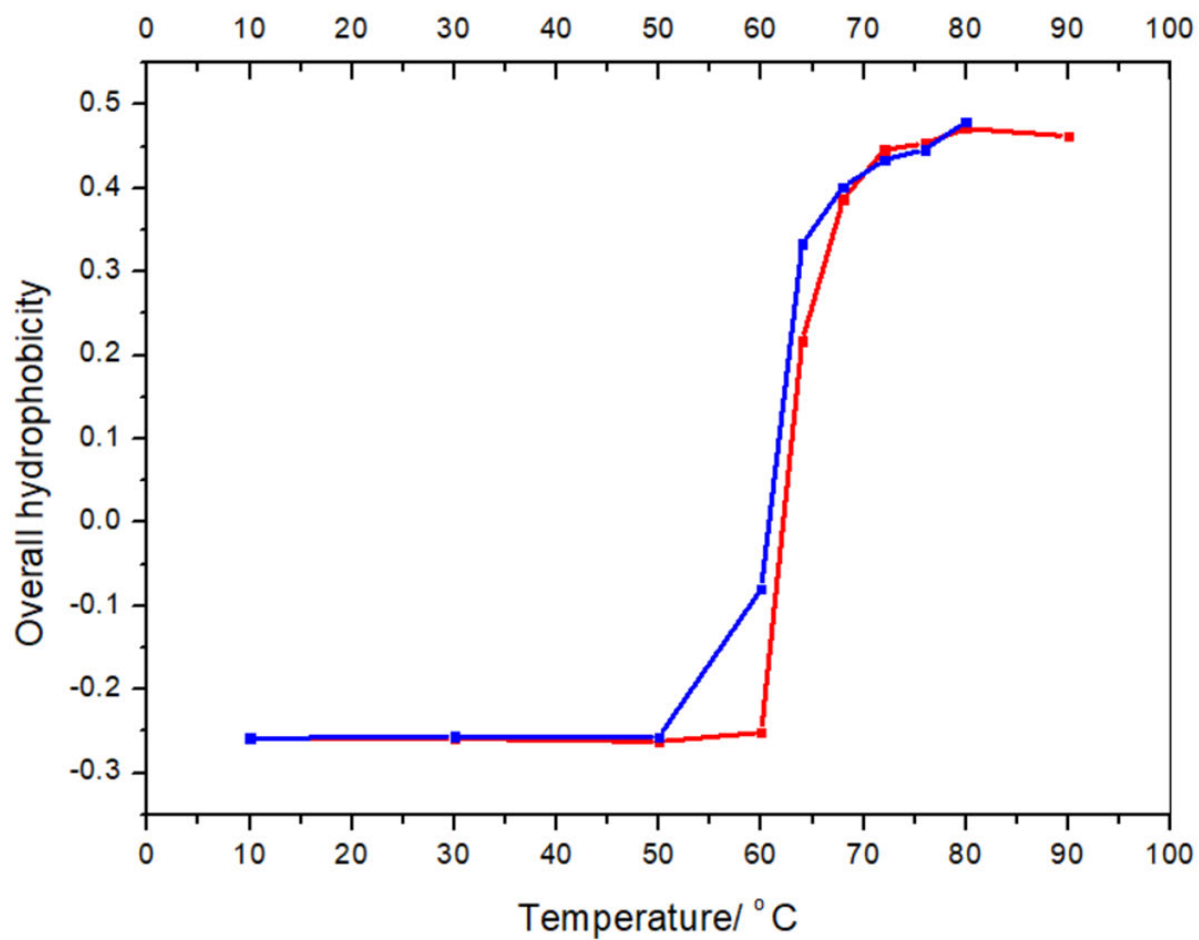


Figure S48. Spectral hydrophobicity of polymer sample with PGME₁₀₃. Data points during heating and cooling cycles are shown in red and blue.

8.2. Spin Hamiltonian parameters derived from simulations

A_{iso} and g_{iso} are the isotropic hyperfine coupling (in MHz) and g -value. The rotational correlation time (τ_c) is given in nanoseconds and spectral contribution of each component in spectrum is given as % *contribution*. Simulation data regarding cooling cycle are shown with index “C”.

$$\tau_c = \frac{1}{6 \sqrt[3]{D_{xx}D_{yy}D_{zz}}} \quad (\text{eq S14})$$

A_{iso} and g_{iso} are the trace of the A and g -tensors, calculated as the following (eq S15, S16):

$$A_{iso} = \frac{A_{xx} + A_{yy} + A_{zz}}{3} \quad (\text{eq S15})$$

$$g_{iso} = \frac{g_{xx} + g_{yy} + g_{zz}}{3} \quad (\text{eq S16})$$

Table S3. Spin Hamiltonian parameters derived from spectral simulations of TEMPO spin probe-reference.

$T/$ °C	$A_{iso}/$ MHz	$2A'_{zz}/$ MHz	$\tau_c /$ ns	g_{iso}	% contribution
10	48.4666	109.4	0.0056	2.0059	100
20	48.366	109.1	0.0081	2.0059	100
30	48.266	108.8	0.0081	2.0059	100
40	48.233	108.7	0.0056	2.0059	100
50	48.1666	108.5	0.0046	2.0059	100
60	48.1	108.3	0.0044	2.0059	100
70	47.966	107.9	0.0043	2.0059	100
80	47.86	107.6	0.0048	2.0059	100
90	47.766	107.3	0.0048	2.0059	100
80-C	47.86	107.6	0.0036	2.0059	100
70-C	47.95	107.85	0.0046	2.0059	100
60-C	48	108	0.0045	2.0059	100
50-C	48.1	108.3	0.0046	2.0059	100
40-C	48.2	108.6	0.0065	2.0059	100
30-C	48.3	108.9	0.0064	2.0059	100
20-C	48.366	109.1	0.0089	2.0059	100
10-C	48.433	109.3	0.0107	2.0059	100

Table S4. Spin Hamiltonian parameters derived from spectral simulation for mPEG₁₀₆.

$T/$ °C	$A_{iso}/$ MHz	$2A'_{zz}/$ MHz	$\tau_c /$ ns	g_{iso}	% contribution
10	48.33	108	0.0385	2.0059	100
40	48	107	0.0385	2.0059	100
60	47.66	106	0.0300	2.0059	100
70	47.33	105	0.0226	2.0059	100
80	47.33	105	0.0226	2.0059	100
90	47.33	105	0.0227	2.0059	100
80-C	47.33	105	0.0385	2.0059	100
70-C	47.33	105	0.0385	2.0059	100
60-C	47.33	105	0.0385	2.0059	100
40-C	48	107	0.0300	2.0059	100
10-C	48	107	0.0300	2.0059	100

Table S5. Spin Hamiltonian parameters derived from spectral simulation for rPEG₁₁₁^{0.26}.

$T /$ °C	$A_{iso} /$ MHz	$2A'_{zz} /$ MHz	$\tau_c /$ ns	g_{iso}	% contribution
10	48.33	108	0.030	2.0059	100
40	48	107	0.030	2.0059	100
60	47.66	106	0.030	2.0059	100
70	46.56	105	0.050	2.0059	45
	47.66	106	0.0260		54
80	46.56	105	0.050	2.0059	54
	47.66	106	0.0057		46
90	46.56	105	0.050	2.0059	54
	47.66	106	0.0057		46
80-C	46.56	105	0.050	2.0059	54
	47.66	106	0.0057		46
70-C	46.56	105	0.050	2.0059	54
	47.66	106	0.0057		46
60-C	46.56	105	0.050	2.0059	28
	47.66	106	0.0057		72
40-C	48	107	0.0350	2.0059	100
10-C	48	107	0.0350	2.0059	100

Table S6. Spin Hamiltonian parameters derived from spectral simulation for rPEG₁₁₁^{0.35}.

$T /$ °C	$A_{iso} /$ MHz	$2A'_{zz} /$ MHz	$\tau_c /$ ns	g_{iso}	% contribution
10	48.33	106	0.0300	2.0061	100
40	48	105	0.0300	2.0061	100
60	47.66	104	0.0300	2.0061	100
70	47.66	104	0.0300	2.0061	100
80	47.66	104	0.0300	2.0061	100
90	47.33	103	0.0300	2.0061	100
80-C	46.56	105	0.0500	2.0059	25
	47.66	104	0.0300	2.0061	75
70-C	47.666	104	0.0300	2.0061	100
60-C	47.66	104	0.0300	2.0061	100
40-C	48	105	0.0300	2.0061	100
10-C	48.33	106	0.0300	2.0061	100

Table S7. Spin Hamiltonian parameters derived from spectral simulation for rPEG₁₀₆^{0.41}.

<i>T</i> / °C	<i>A</i> _{iso} / MHz	2 <i>A</i> ' _{zz} / MHz	τ_c / ns	<i>g</i> _{iso}	% contribution
10	48.26	108.8	0.0138	2.0059	100
30	48.066	108.2	0.0079	2.0059	100
50	47.86	107.6	0.0063	2.0059	100
70	47.566	106.7	0.0054	2.0059	100
80	47.333	106.0	0.0053	2.0059	100
86	46.733	104.2	0.0630	2.0059	10
	47.333	106.0	0.0040	2.00589	90
88	46.733	104.2	0.0631	2.0059	40
	47.34	106.1	0.0040	2.00586	60
90	46.166	102.5	0.050	2.0059	37
	47.566	106.7	0.0030	2.00584	63
88-C	46.266	102.8	0.0501	2.0059	5
	47.533	106.6	0.0031	2.00588	95
86-C	47.533	106.6	0.0050	2.0059	100
80-C	47.6	106.8	0.0063	2.0059	100
70-C	47.766	107.3	0.0063	2.0059	100
50-C	47.966	107.9	0.0050	2.0059	100
30-C	48.2	108.6	0.0050	2.0059	100
10-C	48.366	109.1	0.0138	2.0059	100

Table S8. Spin Hamiltonian parameters derived from spectral simulation for rPEG₁₁₀^{0.51}.

$T /$ °C	$A_{iso} /$ MHz	$2A'_{zz} /$ MHz	$\tau_c /$ ns	g_{iso}	% contribution
10	48	107	0.0300	2.0059	100
40	48	107	0.0300	2.0059	100
60	47.66	107	0.0227	2.0059	100
70	47.333	106	0.0227	2.0059	100
72	47.333	106	0.0227	2.0059	100
76	47.333	106	0.0227	2.0059	100
80	47.333	106	0.0227	2.0059	100
82	46.66	103	0.0894	2.0059	40
	47.333	106	0.0300	2.0059	60
86	46	101	0.0959	2.0059	60
	47.333	106	0.0300	2.0059	40
90	45.333	101	0.2491	2.0059	50
	46.66	104	0.1053	2.0059	50
86-C	45.66	101	0.0430	2.0059	60
	47.333	106	0.0300	2.0059	40
82-C	47.333	107	0.0531	2.0059	40
	47.33	106	0.0300	2.0059	60
80-C	47.33	106	0.0488	2.0059	100
70-C	47.333	105	0.0488	2.0059	100
60-C	47.333	106	0.0488	2.0059	100
40-C	48	106	0.0488	2.0059	100
10-C	48.33	106	0.0488	2.0059	100

Table S9. Spin Hamiltonian parameters derived from spectral simulation for rPEG₁₀₅^{0.64}.

<i>T</i> / °C	<i>A</i> _{iso} / MHz	2 <i>A</i> ' _{zz} / MHz	<i>τ</i> _c / ns	<i>g</i> _{iso}	% contribution
10	48.33	107	0.0300	2.0059	100
40	48	106	0.0260	2.0059	100
60	47.66	105	0.0260	2.0059	100
70	47.33	104	0.0260	2.0059	100
72	47.33	104	0.1042	2.0059	100
76	46.66	104	0.1263	2.0059	100
80	46.33	103	0.1491	2.0059	100
86	45.33	101	0.0571	2.0061	59
	46.33	103	0.1133	2.0057	41
90	45.33	98	0.0770	2.0059	100 ^a
86-C	45.33	101	0.0494	2.0061	52
	46.00	103	0.2230	2.0057	48
80-C	45.00	100	0.0426	2.0061	27
	46.00	103	0.4217	2.0057	73
76-C	47.00	104	0.1263	2.0059	100
72-C	47.33	104	0.1042	2.0059	100
70-C	47.33	104	0.0260	2.0059	100
60-C	47.66	105	0.0260	2.0059	100
40-C	48.00	106	0.0260	2.0059	100
10-C	48.33	107	0.0300	2.0059	100

^a entirely hydrophobic contribution

Table S10. Spin Hamiltonian parameters derived from spectral simulation for rPEG₁₀₅^{0.73}.

$T /$ °C	$A_{iso} /$ MHz	$2A'_{zz} /$ MHz	$\tau_c /$ ns	g_{iso}	% contribution
10	48.23	108.7	0.0126	2.0059	100
30	48.03	108.1	0.0079	2.0059	100
50	47.766	107.3	0.0069	2.0059	100
70	47.433	106.3	0.0054	2.0059	100
74	45.73	101.2	0.0100	2.0059	43
	47.53	106.6	0.0050	2.00576	57
78	45.56	100.7	0.0100	2.0059	77.5
	47.96	107.9	0.0050	2.0057166	22.5
82	45.266	99.8	0.0100	2.0059	83
	47.666	107.0	0.002511	2.0057	17
86	45.133	99.4	0.0100	2.0059	87
	47.46	106.4	0.0025	2.0057	13
90	45.033	99.1	0.0100	2.0059	90
	47.366	106.1	0.0025	2.0057	10
86-C	45.1	99.3	0.0100	2.0059	90
	47.43	106.3	0.0025	2.0057	10
82-C	45.3	99.9	0.0100	2.0059	90
	47.633	106.9	0.0025	2.0057	10
78-C	45.533	100.6	0.0100	2.0059	89
	47.833	107.5	0.0025	2.0057	11
74-C	45.7	101.1	0.0100	2.0059	87
	48	108.0	0.0031	2.0057	13
70-C	47.8	107.4	0.0044	2.0059	100
50-C	48.033	108.1	0.0044	2.0059	100
30-C	48.26	108.8	0.0081	2.0059	100
10-C	48.4	109.2	0.0091	2.0059	100

Table S11. Spin Hamiltonian parameters derived from spectral simulation for rPEG₁₁₅^{0.82}.

$T /$ °C	$A_{iso} /$ MHz	$2A'_{zz} /$ MHz	$\tau_c /$ ns	g_{iso}	% contribution
10	48.23	108	0.0134	2.0059	100
30	48.23	108	0.0089	2.0059	100
50	47.766	107.3	0.0075	2.0059	100
60	47.6	106.8	0.0061	2.0059	100
64	47.5	106.5	0.0061	2.0059	100
68	47.06	105.2	0.025	2.0059	62
	47.566	106.7	0.0061	2.0057	38
72	45.7	101.1	0.025	2.0059	60
	48	108	0.0061	2.0057	40
76	45.3	99.9	0.025	2.0059	62
	47.8	107.4	0.0061	2.0057	38
80	45.1	99.3	0.025	2.0059	63
	47.8	107.4	0.0061	2.005683	37
90	44.9	98.7	0.0501	2.0059	61
	47.73	107.2	0.0061	2.0056	39
80-C	45.2	99.6	0.0501	2.0059	39
	47.9	107.7	0.0057	2.0056	61
76-C	45.36	100.1	0.0501	2.0059	34
	47.9	107.7	0.0057	2.00571	66
72-C	45.86	101.6	0.0501	2.0059	23
	48	108	0.0057	2.0058	77
68-C	47.8	107.4	0.0234	2.0059	100
64-C	47.86	107.6	0.0144	2.0059	100
60-C	47.93	107.8	0.0107	2.0059	100
50-C	48.03	108.1	0.0079	2.0059	100
30-C	48.2	108.6	0.0072	2.0059	100
10-C	48.4	109.2	0.0123	2.0059	100

Table S12. Spin Hamiltonian parameters derived from spectral simulation for PGME₁₀₃.

$T /$ °C	$A_{iso} /$ MHz	$2A'_{zz} /$ MHz	$\tau_c /$ ns	g_{iso}	% contribution
10	48.233	108.7	0.0135	2.0059	100
30	48.066	108.2	0.0155	2.0059	100
50	47.733	107.2	0.0158	2.0059	100
60	46.5	103.5	0.0316	2.0059	8
	47.63	106.9	0.0158	2.0059	92
64	45.466	100.4	0.0355	2.0059	51
	47.8	107.4	0.0141	2.005683	49
68	45.166	99.5	0.0316	2.0059	62.9
	47.966	107.9	0.0126	2.00565	37.1
72	44.96	98.9	0.0316	2.0059	66.5
	47.9	107.7	0.0126	2.0056	32.5
76	44.83	98.5	0.0282	2.0059	67
	47.9	107.7	0.0112	2.0056	33
80	44.8	98.4	0.0199	2.0059	68.8
	47.9	107.7	0.0112	2.0056	31.2
90	44.63	97.9	0.0199	2.0059	68
	47.66	107	0.0112	2.0056	32
80-C	44.733	98.2	0.0199	2.0059	68
	47.83	107.5	0.0126	2.0056	32
76-C	44.84	98.5	0.0199	2.0059	66
	47.9	107.7	0.0126	2.0056	34
72-C	44.96	98.9	0.0199	2.0059	65
	47.93	107.8	0.0126	2.0056	35
68-C	45.033	99.1	0.0251	2.0059	63
	47.93	107.8	0.0126	2.0056	37
64-C	45.266	99.8	0.0251	2.0059	58
	47.9	107.7	0.0126	2.0056	42
60-C	45.4	100.2	0.0251	2.0059	22
	47.666	107.0	0.0158	2.0057	78
50-C	47.733	107.2	0.0158	2.0059	100
30-C	48	108	0.0158	2.0059	100
10-C	48.23	108.7	0.0151	2.0059	100

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Chapter 3

Overcoming PEG antigenicity: Statistical PEG isomers reduce antibody binding

Overcoming PEG antigenicity: Statistical PEG isomers reduce antibody binding

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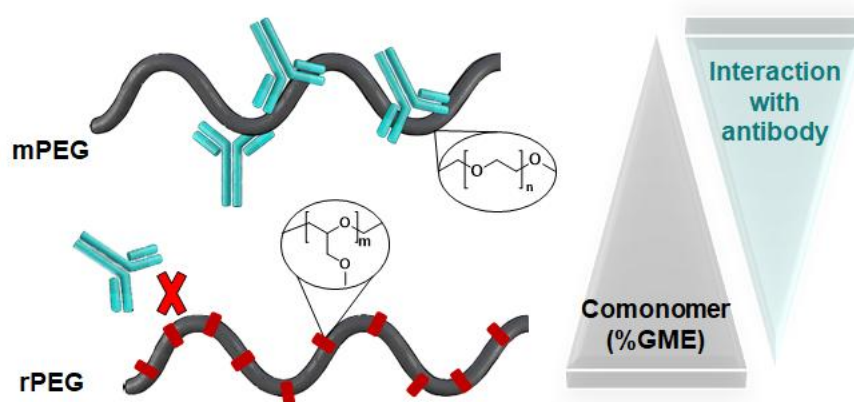
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TOC graphic



ABSTRACT

Poly(ethylene glycol) (PEG) is widely used in bioconjugation and nanomedicine due to its advantageous properties including solubility, biocompatibility, and pharmacokinetic benefits. However, PEG exposure can elicit anti-PEG antibodies (APAs), resulting in accelerated blood clearance and immune responses. To address this, we synthesized “randomized PEG” (rPEG) by incorporating the comonomer glycidyl methyl ether into PEG via anionic ring-opening polymerization. This structural isomer introduces "synthetic point mutations", impeding APA recognition.

We systematically investigated the interaction between rPEGs and APAs in buffer solution using microscale thermophoresis and fluorescence correlation spectroscopy. Increasing comonomer content drastically reduced antibody binding, with rPEGs containing 52 mol% comonomer effectively preventing APA interaction. Monte Carlo simulations confirmed a reduction in consecutive ethylene glycol sequences crucial for APA recognition.

These results establish rPEG as a promising PEG alternative, mitigating immune recognition while retaining key properties. This is of key relevance for next-generation stealth polymers with improved biocompatibility for biomedical applications.

Introduction

Poly(ethylene glycol) (PEG) has been the gold-standard polymer in the fields of bioconjugation and nanomedicine for several decades, as it enhances the pharmacokinetic properties of a wide range of drugs. PEG is the most widely used polyether in pharmaceutical applications and cosmetics, comprising repeating ethylene glycol units (-CH₂-CH₂-O-). Pharma-grade PEG is produced on an industrial scale via anionic ring-opening polymerization (AROP) of ethylene oxide (EO).^{1,2} Its excellent water solubility at physiologically relevant temperatures² and excellent biocompatibility³⁻⁵ have made it widely applicable for pharmaceutical, medical and cosmetic applications. In the human body, PEG is primarily excreted via renal clearance up to a molecular weight of 30 kDa, while for larger PEGs, hepatic clearance or pinocytosis/phagocytosis is favored.^{6,7} However, due to the high chemical stability of ether bonds, degradation of PEG has not been observed to any medically significant extent.⁸ Additionally, no pattern of adverse events indicative of potential PEG toxicity was detected in the past, even at exposure to ultrahigh doses of PEG in preclinical toxicology studies.^{8,9} Therefore, PEG is classified as “non-toxic” by the US Food and Drug Administration (FDA).¹⁰ The so-called “PEGylation” strategy involves the covalent attachment of PEG to proteins, peptides, nanoparticles, and other molecules.¹¹ It is widely employed to improve solubility, stability, and supposedly reduce the immunogenicity of the conjugated molecules. This so-called “stealth effect” of PEG considerably prolongs the retention time of active pharmaceutical ingredients and nanocarriers in the bloodstream and has been fundamental in the development of numerous FDA-approved therapies.¹²⁻¹⁴

However, recent literature has indicated that exposure to PEGylated pharmaceutical ingredients induces the formation of anti-PEG antibodies (APAs), which have also been detected in healthy individuals with no previous medication including PEGylated drugs.¹⁵⁻¹⁸ The presence of these antibodies can lead to accelerated blood clearance of PEGylated drugs, resulting in reduced drug efficacy, which is contrary to the intended purpose of PEGylation.^{19,20} Furthermore, immune responses can trigger hypersensitivity reactions and severe side effects in a fraction of patients.²¹⁻²⁴ To counteract the potentially increasing issue of immunogenic reactions and other unclear side effects of PEG, it is crucial to develop alternatives for PEGylation.²⁵ Several polymers have been developed as a non-immunogenic alternative to PEG. These include poly(oxazolines) (POx)²⁶,

poly(vinylpyrrolidone) (PVP)^{26,27} and polysarcosine (PSar)²⁸, which have been investigated in recent years.

Other variations of PEG architectures like branched PEGs have also been investigated and shown to reduce accelerated blood clearance in mice.²⁹ Reduced immune response in mice has also been shown for bottlebrush polymers like poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA).³⁰ However, these polymers possess regular PEG segments like the aforementioned PEG alternatives and can therefore be subjected to immune adaptation. Statistical copolymers of ethylene oxide and propylene oxide generally show a significantly lower hydrophilicity than PEG.³¹

We have recently developed an approach for the synthesis of well-defined, isomerized PEG structures via AROP, consisting of the building blocks EO and glycidyl methyl ether (GME). The GME units are randomly distributed along the polyether chains resulting in PEGs with "synthetic point mutations" – so-called randomized PEG (rPEG).³² Contrary to the homopolymers investigated as PEG alternatives, the random sequence of comonomers in rPEGs could make adaptation of the immune system more difficult and potentially provide a long-term solution to PEG immunogenicity. Our polymers show excellent aqueous solubility and – like conventional PEG – exhibited no apparent cytotoxicity, as demonstrated with a variety of murine and human cell lines. As recent work by Lai *et al.*³³ shows, binding between PEG and the Fab-fragment of the backbone-specific PEG-antibody is driven by multiple van der Waals interactions and requires a regular chain of 16 consecutive ethylene glycol units. In our previously published work, we found indications in competitive ELISA assays that the randomly distributed side chains impede the polymer-antibody interaction. However, the correlation between GME fraction – i.e. the frequency of "point mutations" in a single rPEG chain – and antibody binding in solution remains to be elucidated.

Accordingly, we investigated the interaction between rPEGs and backbone-specific APAs (immunoglobulin G). For this purpose, the copolymers were functionalized with a fluorescent dye, and their antibody interaction was examined by microscale thermophoresis (MST) and fluorescence correlation spectroscopy (FCS). Relying on these methods, correlation of the polymer structure with antibody binding affinities can be achieved. Furthermore, we aim to establish a quantification of the minimal fraction of GME required to sufficiently suppress antibody

recognition. Additionally, the significance of the presence of 16 EO unit segments for antibody binding can be tested.

Results and Discussion

Polymer synthesis

The polymers utilized in the MST and FCS investigations were synthesized via AROP under conditions like those employed for the production of pharma-grade PEG. The synthesis of high purity GME can be found in our previous work.³² A well-established route for the functionalization of polyethers was used to attach the fluorescent dye to the rPEGs. To this end, a mesylate moiety was attached to the hydroxyl group of the polymer chains. Subsequently, the mesylate group was replaced by an azide moiety, which was eventually reduced to the amine.³⁴ Finally, reaction with fluorescein-5-isothiocyanate (5-FITC) was employed to produce the dye-functionalized polymer from the amine. Characterization data of the functionalized polymers are given in **Table 1**. Throughout this manuscript and the Supporting Information, the FITC functionalized samples' composition is described with $\text{rPEG}_{D_p}^f$ where f is the molar fraction of GME and D_p is the degree of polymerization of the sample.

Progress of the functionalization was monitored by ^1H -NMR-spectroscopy, size exclusion chromatography (SEC) and matrix-assisted laser desorption ionization using time-of-flight (MALDI TOF) mass spectrometry. The general structure of the functionalized copolymers is illustrated in **Figure 1a**. The molar amount of GME units in the copolymers can be determined via ^1H -NMR by calculating the relation between the signal of the methyl group of the GME side chain (3.30 – 3.35 ppm in CDCl_3) and the signal of the methylene and methine groups of the backbone (3.37 – 4.00 ppm in CDCl_3). To determine the average molar mass M_n , MALDI TOF measurements were performed. Additionally, the mass of the polymer end group was determined using mass spectrometry. This proved important to control the completion of the end group functionalization reactions, since in the ^1H -NMR spectrum characteristic signals of the end group partially overlapped with the backbone signal and therefore could not be integrated. The azide functionalization could only be monitored via ^1H -NMR by the disappearance of the mesylate

signals of the precursor, since the signal of the azide-attached methylene group overlaps completely with the polyether backbone signals. This observation aligns with reports in literature, which report the resonance at the same chemical shift as the signal of the GME methyl groups (3.30 – 3.35 ppm).^{35,36} Finally, SEC was measured to determine accurate values for the dispersities $\bar{D}$ of the polymer samples. All NMR and MALDI TOF spectra as well as the SEC curves are available in the **Supporting Information**. As illustrated in **Figure 1c**, the results of the Monte Carlo simulation of copolymers with varying compositions reveal the reduced abundance of the epitope consisting of 16 consecutive EO units within the chains. This is evident in the simulation, which illustrates five random copolymer chains for molar GME contents of 15 mol% and 50 mol%. The data demonstrates a substantial decrease in the epitopal 16 EO units with increasing GME content, reaching a value below 0.1 % at 50 mol% GME content (**Figure 1b** and **1c**).

Table 1. Analytical data of the FITC functionalized polymers.

Polymer	mol% ^{GME} (target)	mol% ^{GME} _[a]	D_p (target)	D_p ^[b]	M_n (target) / g mol ⁻¹	M_n ^[c] / g mol ⁻¹	M_n ^[b] / g mol ⁻¹	$\bar{D}$ ^[c]
mPEG ₁₁₄	0	0	114	114	5,405	4,860	5,300	1.06
rPEG ₉₈ ^{0.17}	15	17	98	98	5,372	4,170	5,300	1.05
rPEG ₈₉ ^{0.27}	25	27	91	89	5,378	4,110	5,200	1.12
rPEG ₉₁ ^{0.42}	40	42	81	91	5,391	4,440	6,000	1.04
rPEG ₇₆ ^{0.52}	50	52	75	76	5,374	3,520	5,400	1.04

^a calculated by ¹H-NMR

^b calculated by MALDI TOF

^c calculated by SEC

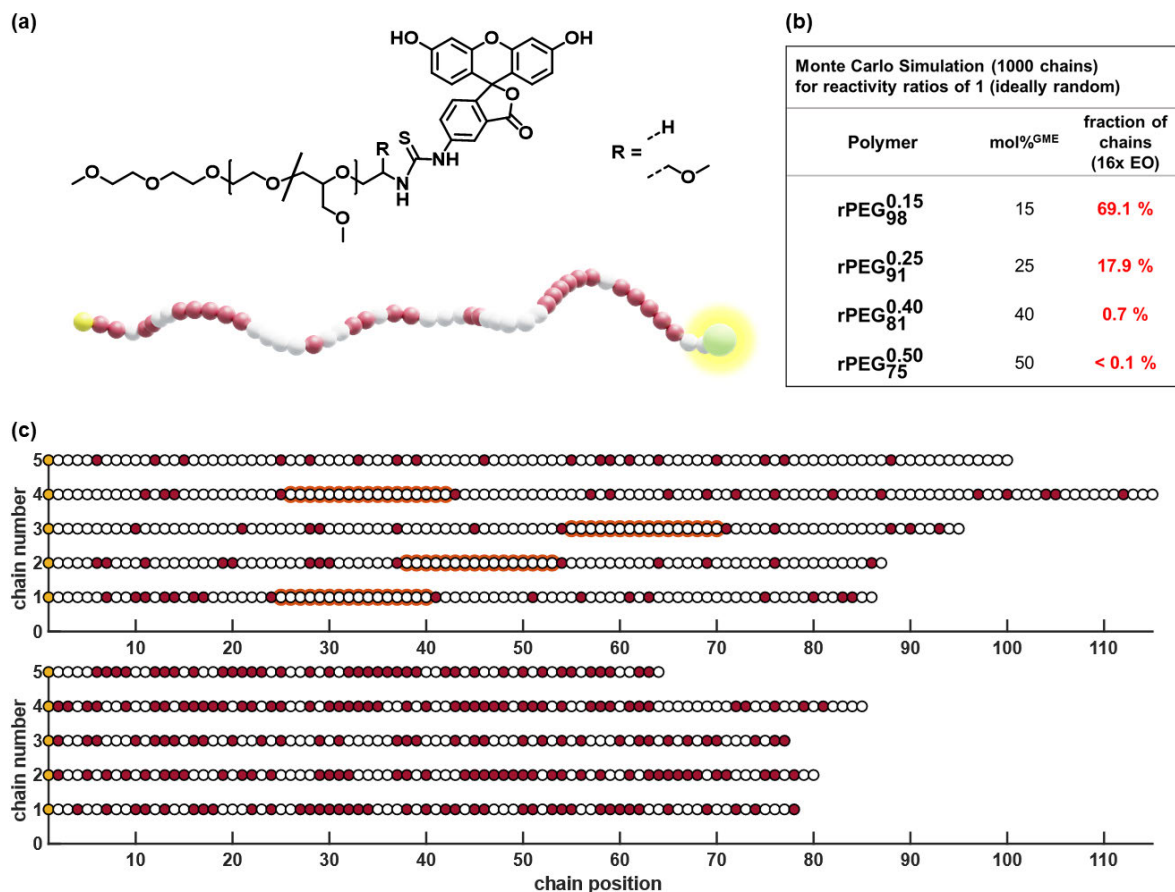


Figure 1. (a) Structure and schematic depiction of the statistical EO/GME copolymers functionalized with FITC at the ω -terminus. (b) Evaluation (Monte-Carlo Simulation) of the occurrence of the epitope consisting of 16 consecutive EO units in statistical copolymers of different compositions. (c) Simulation of monomer sequences of copolymer chains containing 15 mol% GME (top) and 50 mol% GME (bottom), white = EO unit, red = GME unit, orange marking = sequences of ≥ 16 consecutive EO units.

Microscale thermophoresis

Microscale thermophoresis (MST) measurements have been conducted to assess the interaction strength between APAs and free polymers. This technique relies on the temperature-induced fluorescence change of a labeled target polymer to the concentration of a non-fluorescent ligand. In this study, FITC-conjugated rPEGs served as the target molecules. By systematically varying the GME amount in the PEG chain, it was possible to ascertain how the chain composition influences the binding affinity of the APAs, as reflected by the dissociation constant (K_d). To ensure the validity of the experimental results, we utilized a FITC-conjugated PEG with a respective molecular weight of 5,000 g mol⁻¹. The obtained results are displayed in **Figure 2**.

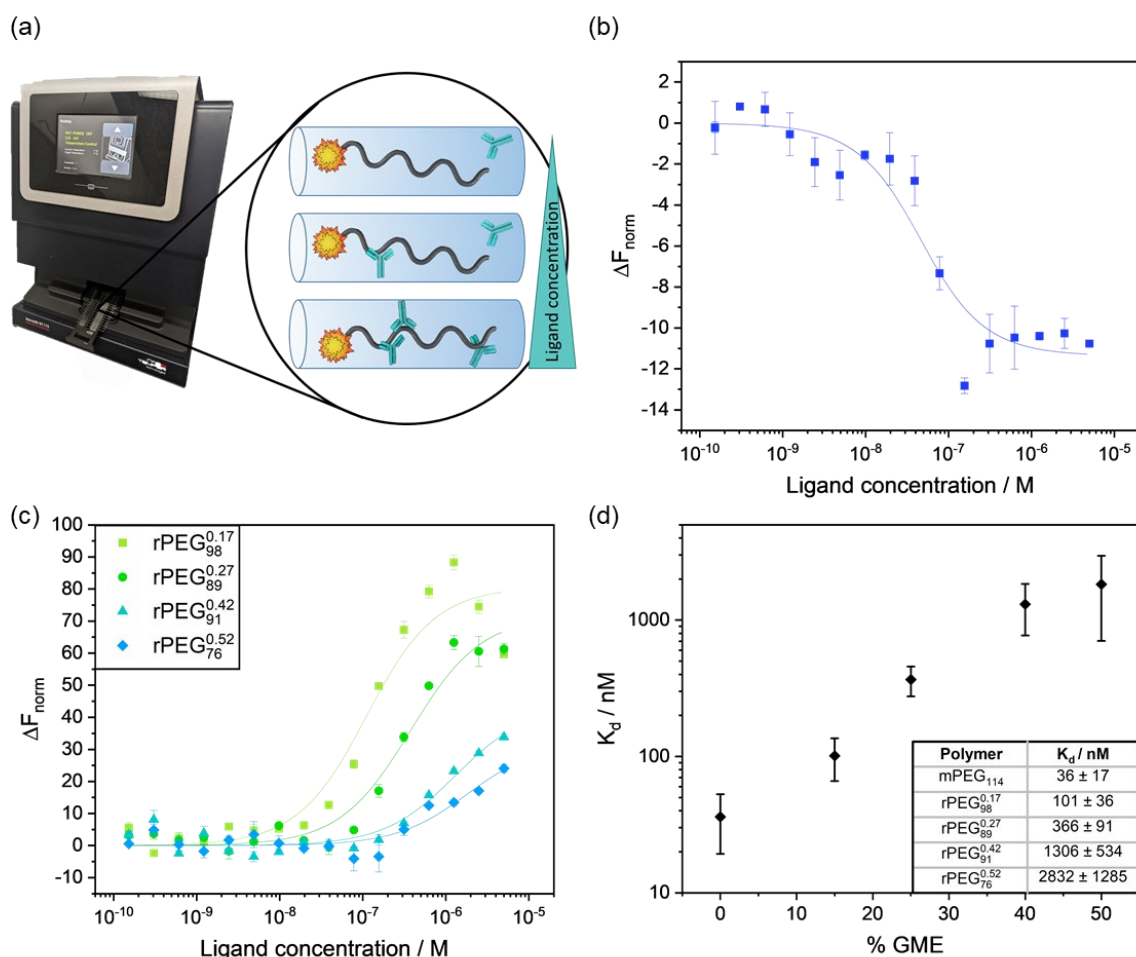


Figure 2. Microscale thermophoresis (MST) measurements of anti-PEG IgG and PEG/rPEG. (a) MST instrument and schematic representation of antibody binding in capillaries. (b) Normalized fluorescence signal (ΔF_{norm}) of anti-PEG IgG binding to PEG together with the corresponding fit. (c) The normalized fluorescence signal of anti-PEG IgG binding to rPEGs together with the corresponding fit. (d) Dissociation constant (K_d) values for PEG/rPEGs plotted against increasing GME content in the PEG chain. Measurements were performed in triplicates.

The MST instrument's setup is illustrated in **Figure 2a**, and a schematic of the interaction of antibodies with the target polymer within the capillary is provided. The normalized change in fluorescence signal of APA binding to PEG (shown in **Figure 2b**) serves as a reference for comparison with modified rPEG derivatives displayed in **Figure 2c** showing the normalized change in fluorescence signal of APA binding to rPEGs containing varying concentrations of GME. As measurements were performed in triplicates, the average of $n = 3$ and corresponding standard deviation is shown (for individual measurements see Figure S62).

The data demonstrate a decrease in the change in fluorescence with increasing GME content, along with a shift of the inflection points towards higher ligand concentrations, showing the correlation between higher GME content and reduced binding affinity. It is important to note that different target-ligand complexes can produce thermophoretic responses in opposite directions, which is the reason for $\Delta F_{\text{norm}} < 0$ in case of PEG and $\Delta F_{\text{norm}} > 0$ in case of rPEGs. The amplitude and direction of the response depend on the specific molecular changes occurring upon binding, including modifications to size, charge, and hydration. This directional variability in thermophoretic response does not affect validity of binding strength comparisons between different target-ligand pairs.

The graph in **Figure 2d** displays the obtained K_d values for PEG and rPEGs with different GME contents, revealing a strong increase in K_d values with higher GME content, which translates to a decrease in binding strength. For rPEG₇₆^{0.52} the substantial error bars and the nearly undetectable binding suggest minimal or no effective, specific binding interaction. For an effective binding detection, the signal-to-noise ratio needs to be above a threshold of 5 and ideally over 12, which was not achieved for all measurements with rPEG₇₆^{0.52}. This underscores the impact of elevated GME content on the elimination of APA binding efficiency.

Fluorescence correlation spectroscopy

The interaction between APAs and rPEG copolymers was further studied with fluorescence correlation spectroscopy (FCS). In a typical FCS experiment, the fluorescence intensity fluctuations caused by the diffusion of fluorescent species, such as small molecules, macromolecules, or nanoparticles through a minute observation volume (e.g. the “focus” of a confocal microscope) are monitored and recorded. The subsequent correlation analysis of these fluctuations yields information on the species’ diffusion coefficient, hydrodynamic radius, and concentration. This information is used to assess their size, aggregation behavior, and interaction with other species.³⁷

According to the results of the MST measurements, three concentrations of APAs were selected to represent unbound (0.05 μM), medium bound (0.5 μM), and fully bound (5 μM) conditions, as depicted in **Figure 3a**. Typical FCS autocorrelation curves (ACFs) measured for fluorescently labeled PEG and rPEG copolymers in solutions with anti-PEG antibodies with increasing concentrations are shown in **Figure 3b**.

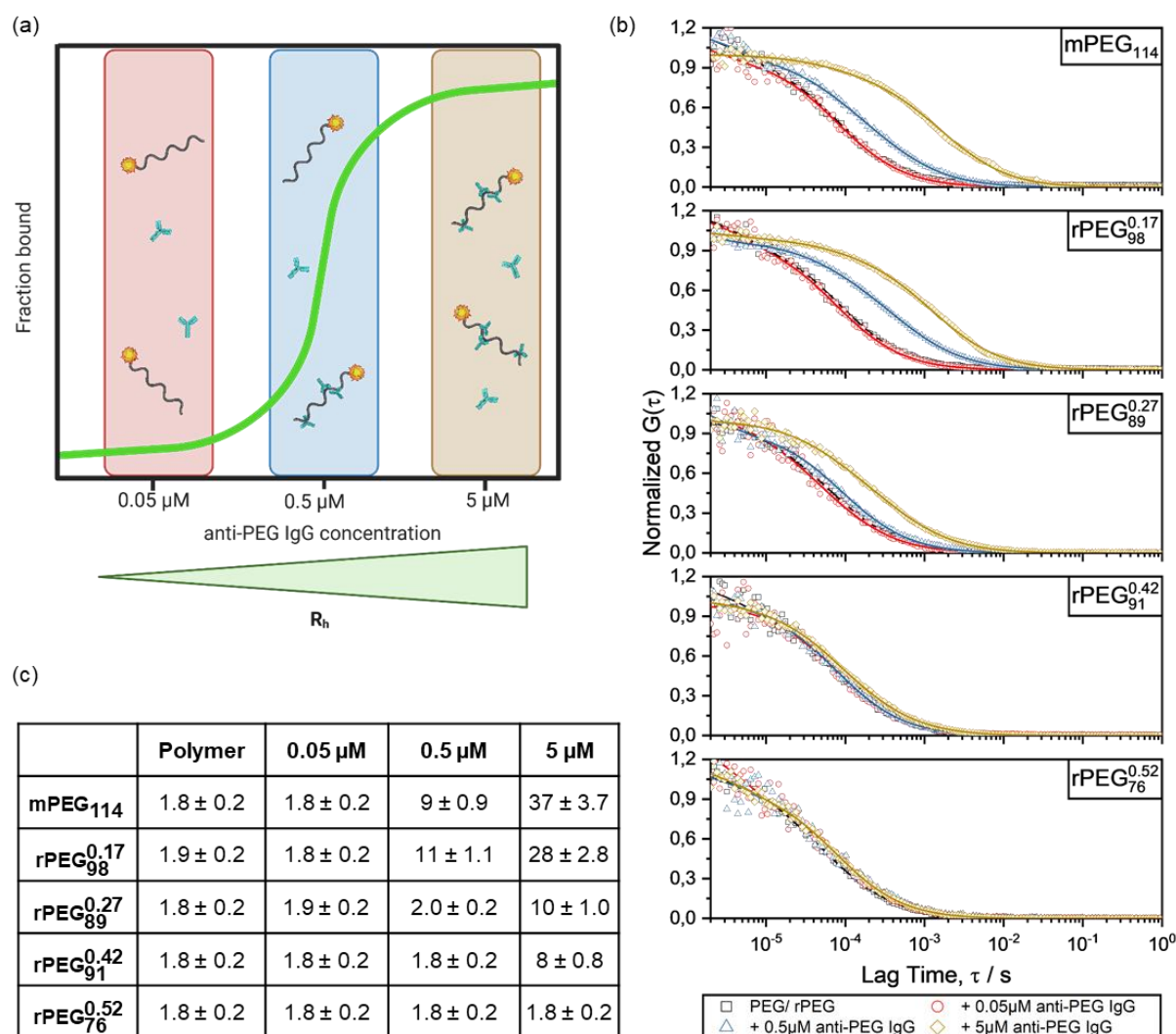


Figure 3. Fluorescence correlation spectroscopy (FCS) measurements of anti-PEG IgG and PEG/rPEG. (a) Scheme of concentrations selected for the experiment and corresponding binding conditions according to the results obtained from the MST measurement. (b) Autocorrelation curves (ACFs) derived from FCS measurements together with the corresponding fits (mono- or biexponential decays). Black = individual PEG/rPEG polymers without anti-PEG IgG addition, red = PEG/rPEG + 0.05 μM anti-PEG IgG, blue = PEG/rPEG + 0.5 μM anti-PEG IgG, yellow = PEG/rPEG + 5 μM anti-PEG IgG. (c) Table of hydrodynamic radii of detected diffusing species obtained from the fits shown in (b).

ACFs acquired in the absence of antibodies are also included for reference. A shift of the ACF towards elevated lag times translates to an increase in the hydrodynamic radius (R_h) of the fluorescent species, thereby reflecting enhanced interactions between APAs and polymers. The table in **Figure 3c** presents the hydrodynamic radii of the diffusing fluorescence species observed at different APA concentrations as obtained from fitting the corresponding ACF with single- or double-component diffusion model (eq.1 with $m = 1$ or 2). Consistently, the size of the non-bound polymers without antibody presence was determined to be around 1.8 nm, independent of the respective polymer's GME content. After mixing with anti-PEG IgG, negligible interactions were observed at an antibody concentration of 0.05 μM for all polymers, as no size increase could be detected in any of the samples. This is expected, since this concentration is well below K_d for most of the polymers. At an anti-PEG IgG concentration of 0.5 μM , PEG started to form aggregates ($R_h = 9$ nm) in addition to the presence of individual, free polymer chains that are still visible. Raising the concentration of antibodies to 5 μM , where the saturation of interaction should be reached, the aggregate size increased to 37 nm. A similar interaction pattern was observed for $\text{rPEG}_{98}^{0.17}$. It is noteworthy that both PEG and $\text{rPEG}_{98}^{0.17}$ exhibited substantial interactions at 0.5 μM and 5 μM , with a consistent increase in R_h with increasing antibody concentration. This observation aligns with the determined binding affinities, as a concentration of 0.5 μM is already above K_d for both polymers. However, for $\text{rPEG}_{89}^{0.27}$, the anti-PEG antibody binding effect was found to be reduced. At an anti-PEG IgG concentration of 0.5 μM , negligible aggregate formation is observed ($R_h = 2$ nm), while at an anti-PEG IgG concentration of 5 μM , an increase in aggregate formation is evident ($R_h = 10$ nm). This finding aligns with the observation that 0.5 μM anti-PEG IgG falls within the K_d range for $\text{rPEG}_{89}^{0.27}$. In the case of $\text{rPEG}_{91}^{0.42}$, negligible aggregation was observed, implying a lack of effective binding. Similarly, $\text{rPEG}_{76}^{0.52}$ exhibited no measurable alterations in R_h even at high anti-PEG IgG concentrations, indicating no discernible binding effects for $\text{rPEG}_{91}^{0.42}$ and $\text{rPEG}_{76}^{0.52}$. These results collectively highlight the critical role of polymer composition in dictating antibody binding behavior. In particular, the lack of interaction seen with polymers $\text{rPEG}_{91}^{0.42}$ and $\text{rPEG}_{76}^{0.52}$ suggests that the high GME content within the polymer backbone substantially hinders antibody recognition and binding. To further verify compatibility with a physiological setting, FCS measurements of all polymers in human citrate plasma with average anti-PEG IgG concentration ($c = 9.6 \pm 0.8 \mu\text{g mL}^{-1}$ corresponding to 0.064 μM , as reported by

Deuker et al.[16]) were performed. The results are shown in Figure S64. For all polymers, no size increase was observed, indicating that no significant interactions with plasma components took place. However, it has to be noted that the detection of aggregates with anti-PEG IgG was not expected at this APA concentration following the results reported in **Figure 3** but might rather occur in samples collected from individuals with ~ 10 times higher APA levels. Still, we can exclude any other undesired effects, suggesting safe application in biological environment. In the future, interactions need to be investigated under physiological conditions also at higher clinically relevant APA levels.

Conclusion

The binding of APAs to PEGylated drugs can present a significant problem, as it can induce the ABC effect or trigger immune reactions in patients. Consequently, alternatives to PEG are intensely discussed. In this study, we investigated the interaction between anti-PEG IgG antibodies and a series of random copolymers of EO and GME (rPEG) in relation to the respective molar fraction of GME. It should be emphasized that any copolymer of EO and GME is a structural isomer of PEG, irrespective of its composition.

The MST analysis revealed a decrease in the normalized fluorescence signal (ΔF_{norm}) with increasing GME content in the rPEGs. Accordingly, the dissociation constants K_d of the copolymers increased analogously, revealing a decrease in the binding affinity of anti-PEG IgG. Further studies relying on FCS demonstrated that polymer-antibody aggregate formation depended on the GME content in the polymer. At an antibody concentration of 0.05 μM , no significant interaction was detected. At higher concentrations, an increase in the hydrodynamic radii R_h was observed for all polymers, except for rPEG₇₆^{0.52}. The extent of aggregation decreased as the GME content increased, confirming that GME units in the polymer chain impede antibody binding and suppress aggregate formation.

Lai *et al.*³³ found 16 consecutive EO units to be a prerequisite for backbone-specific APA binding to the polymer. Our simulations demonstrate that this type of chain segment manifests less frequently in chains with higher molar GME content and is virtually absent in polymers exceeding 50 mol% GME. The experimental data aligns closely with the simulation outcomes, as evidenced by an analogous decrease in the interaction between APAs and rPEGs during MST measurements. Consequently, our results prove that EO-GME copolymers possess the capacity to mitigate backbone-specific APA interactions and that incorporating 50 mol% GME into the polymer chains effectively prevents the binding of anti-PEG IgG antibodies. Since key properties such as aqueous solubility and cell viability of the polymer remain unaltered³², rPEG can be regarded as a superior alternative to PEG. Furthermore, we postulate that due to the substantial variation in the statistical composition of the copolymer chains, the adaptation of the immune system and its interaction with rPEG could be significantly impeded compared to PEG. Of course, this hypothesis has to be examined through *in vivo* studies by repeated injections to investigate the effect of long-term

exposure. This will be the subject of future research. By understanding the polymer-antibody interactions, we aim to contribute to the development of more effective and safer PEG alternatives, albeit based on PEG-derived structures.

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Supporting Information

Overcoming PEG Antigenicity: Statistical PEG Isomers

Reduce Antibody Binding

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1. Materials and Methods

1.1. Materials

Ethylene oxide was purchased from *Air Liquide Deutschland*. All solvents were obtained from *Thermo Fisher Scientific*. Compounds were purchased from *TCI Chemicals*, *Sigma-Aldrich*, and *Thermo Fisher Scientific*. All materials were used as received.

Dulbecco's phosphate buffered saline (PBS, Thermo Fisher Scientific, USA). Chimeric human anti-PEG IgG (clone no. c3.3 IgG, product no. 3.3-PABG-A) was purchased from IBMS Academia Sinica (Taipei, Taiwan) and used without further purification.

To obtain pooled plasma, blood was taken from 10 healthy donors after obtaining informed consent and pooled subsequently. All experiments containing human blood plasma from these donors were approved by the ethics committee of the Landesärztekammer Rheinland-Pfalz, Mainz No. 837.439.12 (8540-F). Accordingly, all experiments involving human material were performed in compliance with all relevant laws and guidelines.

After collection, the citrate plasma was centrifuged at 20,000 g for 1 h at room temperature to remove residual protein precipitates and stored at – 80 °C until further use.

1.2. Proton nuclear magnetic resonance spectroscopy (^1H NMR)

^1H NMR was conducted on a Bruker Avance II HD 400 (400 MHz, 25 °C) and analyzed using *MestReNova v14.2* by *Mestrelab Research SL*. DOSY NMR of the polymer samples were measured in D_2O at a concentration of 3.5 mg mL^{-1} . DOSY measurements were conducted on a *Bruker Avance III HD* (400 MHz) with a 5 mm nitrogen-cooled BBO-cryoprobe-head (BB+H+F) with *z*-gradient, automated tune and match (ATM) and autosampler SampleXPress 60. Deuterated chloroform and deuterated water were purchased from *Deutero GmbH*.

1.3. Size exclusion chromatography (SEC)

Measurements were conducted using an Agilent 1100 series HPLC system, which included a degasser, isocratic pump (G1310A), autosampler (G1313A), column oven (G1316A), and detectors for refractive index (RI) (G1310A) and variable wavelength (VWD) (G1314A). Separations were carried out employing a four-column set-up (*MZ-Analysentechnik GmbH*) connected sequentially:

- i. HEMA-40 guard column (40 Å pore size, 10 µm particle size, 50x8.0 mm)
- ii. HEMA-40 analytical column (40 Å pore size, 10 µm particle size, 300x8.0 mm)
- iii. HEMA-100 analytical column (100 Å pore size, 10 µm particle size, 300x8.0 mm)
- iv. HEMA-300 analytical column (300 Å pore size, 10 µm particle size, 300x8.0 mm)

The eluent consisted of DMF (Fisher Chemical) with 1 mg/mL anhydrous LiBr (Acros Organics), using a flow rate of 1 mL/min. Both the column oven and RI detector cell were maintained at 50 °C. Calibration was performed using well-defined poly(ethylene glycol)s from PSS (PSS Standards Kit) with molar mass values (M_p) ranging from 106 to 42700 g mol⁻¹. Samples were dissolved in DMF (with 1 mg mL⁻¹ anhydrous LiBr) at a concentration of 1 mg mL⁻¹ with the addition of 1 drop of toluene. The injection of 100 µL of the stock solutions was carried out via the autosampler, with a measurement duration of 45 min. Elution times were referenced using toluene as an internal standard. RI traces were analyzed using the *PSS WinSEC Unichrom V8.31* software.

1.4. Matrix-assisted laser desorption ionization using time-of-flight detection (MALDI TOF)

MALDI TOF MS measurements were carried out at a *Bruker autoflex maX MALDI TOF/TOF* using a Smartbeam-II solid-state laser with a wavelength of 337 nm. Spectra were recorded using the software *Bruker flexControl 3.4* and analyzed using *Bruker flexAnalysis 3.4* and *Bruker polytools 1.31*. The potassium salt of trifluoroacetic acid (KTFA) and trans-2-[3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) were utilized as ionization salt and

matrix, respectively. For sample preparation, the polymers were dissolved in chloroform at 10 mg mL^{-1} . $20 \text{ }\mu\text{L}$ of this solution was combined with $20 \text{ }\mu\text{L}$ of a 10 mg mL^{-1} solution of the matrix in chloroform. $5 \text{ }\mu\text{L}$ of a 0.1 M solution of the salt in methanol were added and $1 \text{ }\mu\text{L}$ of the resulting mixture was spotted onto an *MTP 384 ground steel target plate*. The solvents were allowed to evaporate completely before the measurement.

In some cases, fragmentation reactions were observed in the MALDI TOF spectra. In the mass spectra of the azide-functionalized polymers (**Figure S34**, **Figure S39**, **Figure S44**, **Figure S49**, **Figure S54**), fragmentation of nitrogen can be observed. This reaction during the measurement is also reported in literature.¹ A so far not determined fragmentation reaction could be observed in particular spectra of the FITC-functionalized polymers (**Figure S41** and **Figure S51**). Although the exact reaction remains unknown, the functionalization of all polymers could be verified via 2D DOSY NMR (**Figure S7**, **Figure S13**, **Figure S19**, **Figure S25**, **Figure S31**) and the SEC curves of the FITC-functionalized polymers (**Figure S61**) showed no increase in dispersity. Despite several purification steps – including extraction, dialysis, and preparative column chromatography – trace amounts of unreacted FITC could be observed in the 2D DOSY NMR spectra and confirmed via FCS measurements. However, because of the significant difference in diffusion coefficients compared to the polymers, unreacted FITC was determined to not impede the FCS and MST measurements in any way.

1.5. Microscale Thermophoresis (MST)

Solutions of labeled polymers (mPEG-FITC and rPEG-FITC) were prepared with a concentration of 40 nM in PBS buffer. The stock solution of the anti-PEG antibody (anti-PEG IgG c3.3, $c = 10 \text{ }\mu\text{M}$ in PBS) was to produce a series of 16 1:1 dilutions with PBS buffer, producing antibody concentrations ranging from 0.306 nM to $10 \text{ }\mu\text{M}$. For the measurement, each ligand dilution was mixed with an equal volume of labeled polymer solution, resulting in a final polymer concentration of 20 nM and final ligand concentrations ranging from 0.153 nM to $5 \text{ }\mu\text{M}$. After 10 min incubation time, the samples were loaded into *Monolith NT.115 premium-coated capillaries* (NanoTemper Technologies). MST was measured using a *Monolith NT.115* instrument (NanoTemper Technologies) at room temperature, with a blue excitation laser (488 nm) and medium MST power.

Data from three independent measurements were analyzed using the MO. *Affinity Analysis Software version 2.2.4*, utilizing the signal from an MST-on time of 10 s for rPEG-FITC and 1.5 s for PEG-FITC. Dose-response curves were generated by fitting the averaged normalized fluorescence change (ΔF_{norm}) to yield the dissociation constants (K_d values).

1.6. Fluorescence Correlation Spectroscopy (FCS)

The stock solution of the anti-PEG antibody was diluted to three different concentrations with PBS buffer: 0.1 μM , 1 μM and undiluted. 20 μL of the labeled polymers (PEG-FITC and rPEG-FITC, 40 nM) were mixed with 20 μL of each anti-PEG antibody concentration. For measurements in human plasma, the labeled polymers (PEG-FITC and rPEG-FITC) were dissolved at a concentration of 200 nM in PBS. These polymer solutions were then mixed with human citrate plasma in a 1:10 ratio, resulting in a final polymer concentration of 20 nM.

FCS experiments were performed on a commercial device, *LSM 880* (Carl Zeiss, Jena, Germany). The excitation was done with the 488 nm line of an Argon laser focused into the studied samples through a *C-Apochromat 40 $\times$ /1.2 W water immersion objective* (Carl Zeiss, Jena, Germany). The emission light was collected with the same objective and after passing through a confocal pinhole, directed to a spectral detection unit (*Quasar, Carl Zeiss*) in which a detection range of 500 – 550 nm was selected. Eight-well polystyrene chambered cover glasses (*NuncTM Lab-TekTM, Thermo Fisher Scientific*, Waltham, MA, USA) were used as sample cells for the studied solutions. The confocal volume was positioned 20 μm above the cover glass. Each FCS experiment included ten technical replicates per sample, acquired at 10-second intervals. All replicates were successful and showed consistent diffusion times and hydrodynamic radius values.

The recorded autocorrelation functions were fitted with the analytical expression for an ensemble of m different types of freely diffusing fluorescence species:²

$$G(\tau) = 1 + \left[1 + \frac{f_T}{1-f_T} e^{-\tau/\tau_T} \right] \frac{1}{N} \sum_{i=1}^m \frac{f_i}{\left[1 + \frac{\tau}{\tau_{Di}} \right] \sqrt{1 + \frac{\tau^2}{s^2 \tau_{Di}^2}}} \quad (\text{eq S1})$$

Here, N is the average number of diffusing fluorescence species in the observation volume, f_T and τ_T are the fraction and the decay time of the triplet state, τ_{D_i} is the diffusion time of the i -th type of species, f_i is the fraction of component i , and S is the so-called structure parameter $S = z_0/r_0$, where z_0 and r_0 represent the axial and radial dimensions of the confocal volume, respectively. Furthermore, the diffusion time τ_{D_i} is related to the respective diffusion coefficient D_i through $\tau_{D_i} = \frac{r_0^2}{4D_i}$. The fits yielded the corresponding diffusion times, and subsequently the diffusion coefficients of the fluorescent species. As the value of r_0 depends strongly on the specific characteristics of the optical setup, calibration experiments were performed using a fluorescent tracer with a known diffusion coefficient, i.e., *Alexa Fluor 488* in water. Finally, the hydrodynamic radii of the studied fluorescent species were evaluated from the respective diffusion coefficients using the Stokes-Einstein (SE) relation:

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (\text{eq S2})$$

Here, k_B is Boltzmann's constant, T is the temperature and η is the viscosity of water.

1.7. Analysis of microstructure based on simulations

Simulation of the comonomer sequence is based on the code written by Philip Dreier for our previous work.³ There, polymers of constant chain lengths were investigated. To accommodate for the varying chain lengths of the polymers investigated in this work, calculations were adapted accordingly.

In brief, the rationale of a living anionic polymerization was taken as a foundation which implies the absence of chain termination or chain transfer reactions and a Poisson distribution of the chain lengths. The copolymerization reaction can be described by the four rate constants: The homopropagation rate constants k_{11} and k_{22} as well as the cross-propagation rate constants k_{12} and k_{21} . The copolymerization kinetics was assumed to be perfectly random, implying

$$k_{11} = k_{22} = k_{12} = k_{21} \quad (\text{eq S3})$$

As a result, it was assumed in the simulations that EO and GME monomers possess the same probability to be incorporated into the chain. For each polymerization, 10^4 chains were simulated. The simulations were run with a targeted quantitative conversion and an overall degree of polymerization of 114. Molar ratios of 15 mol% up to 50 mol% of GME with a stepwise increase were presumed. In **Figure 1b** the four simulated polymerizations and the respective proportional occurrence of 16 consecutive EO units are listed. To visualize the results of the simulations, 5 exemplary chains each for GME contents of 15 mol% and 50 mol% are shown in **Figure 1c**. All simulations were performed using *MathWorks Matlab R2019a*.

2. Preparative procedures

2.1. Synthesis of random copolymers of ethylene oxide (EO) and glycidyl methyl ether (GME)

The statistical copolymerization of EO and GME was performed as described in literature.³

The synthesis is given exemplary for rPEG₈₉^{0.27} with a molecular weight of 5 kDa. All other polymer synthesis were performed identically with varying monomer equivalents.

GME was freshly dried before every polymerization by stirring over calcium hydride overnight in a flame-dried flask and subsequently cryo-transferred immediately before use.

Diethylene glycol monomethyl ether (34.1 mg, 280 μ mol, 1 eq) was dissolved in benzene (5 mL) and transferred via syringe into a flame-dried flask equipped with a stirrer bar, stop cock, and septum. Potassium *tert*-butoxide (28.7 mg, 260 μ mol, 0.9 eq) was dissolved in freshly distilled THF (3 mL) and added to the solution in the flask via syringe. The mixture was stirred for 20 min before the solvents were slowly removed under vacuum. The resulting initiator salt was dried at 60 °C under high vacuum overnight. The flask was sealed and the initiator salt was dissolved in dry DMSO (5 mL). The flask was subsequently cooled to – 90 °C with an ethanol/nitrogen cooling bath. GME (555 mg, 6.30 mmol, 23 eq) was added via syringe into the cooled flask. Ethylene oxide (800 μ L, 18.9 mmol, 67 eq) was condensed into a graduated ampule at – 90 °C and then transferred to the flask. After sealing the flask, the cooling bath was removed and the mixture was allowed to reach room temperature. After stirring for 48 h at room temperature the polymerization was terminated by flushing the flask with argon and adding a mixture of 1M HCl and methanol (1:1, 1 mL). The reaction mixture was taken up in chloroform (20 mL) and extracted twice with deionized water and subsequently with brine. The organic phase was dried with magnesium sulfate, filtered, and the solvent was removed under reduced pressure. The polymer was dialyzed against methanol (regenerated cellulose, MWCO = 2000 g mol⁻¹) for 24 h, affording a pale-yellow viscous liquid (836 mg, 60%).

2.2. Mesylation

The synthesized copolymer (800 mg, 160 μmol , 1 eq) was dissolved in benzene (5 mL) and transferred into a flame-dried Schlenk flask under argon. Benzene was slowly removed under reduced pressure to azeotropically dry the polymer. Subsequently, the polymer was further dried at 60 °C under high vacuum overnight. After the flask was cooled down to room temperature the polymer was dissolved in dry DCM (10 mL) under an argon atmosphere. Triethylamine (130 mg, 177 μL , 1.28 mmol, 8 eq) was added and the solution was stirred for 10 min before methyl sulfonyl chloride (147 mg, 99.0 μL , 1.28 mmol, 8 eq) was added to the solution. The reaction mixture was stirred under argon atmosphere for 72 h at room temperature. Subsequently, the mixture was transferred to a separating funnel and washed two times with deionized water and once with brine, dried over magnesium sulfate, and filtered. After removal of the solvent 649 mg (81%) of mesylated polymer was isolated.

2.3. Azidation

The mesylated polymer (600 mg, 120 μmol , 1 eq) was dissolved in benzene (5 mL) and transferred into a flame-dried Schlenk flask under argon. Benzene was slowly removed under reduced pressure, and the polymer was dried at 60 °C under high vacuum overnight. After removing the heating bath, the polymer was dissolved in 5 mL of dry DMF under argon atmosphere. Sodium azide (62.4 mg, 960 μmol , 8 eq) was added under argon flow, and the mixture was stirred at 65 °C for 72 h under argon atmosphere. Subsequently, DMF was removed under reduced pressure, the residue taken up in DCM (5 mL), and the precipitate was removed by filtration. The remaining solution of the polymer in DCM was washed once with deionized water and once with brine, dried over magnesium sulfate, and the solvent was removed under vacuum to obtain 390 mg (64%) of polymer azide.

2.4. Reduction of the Azide

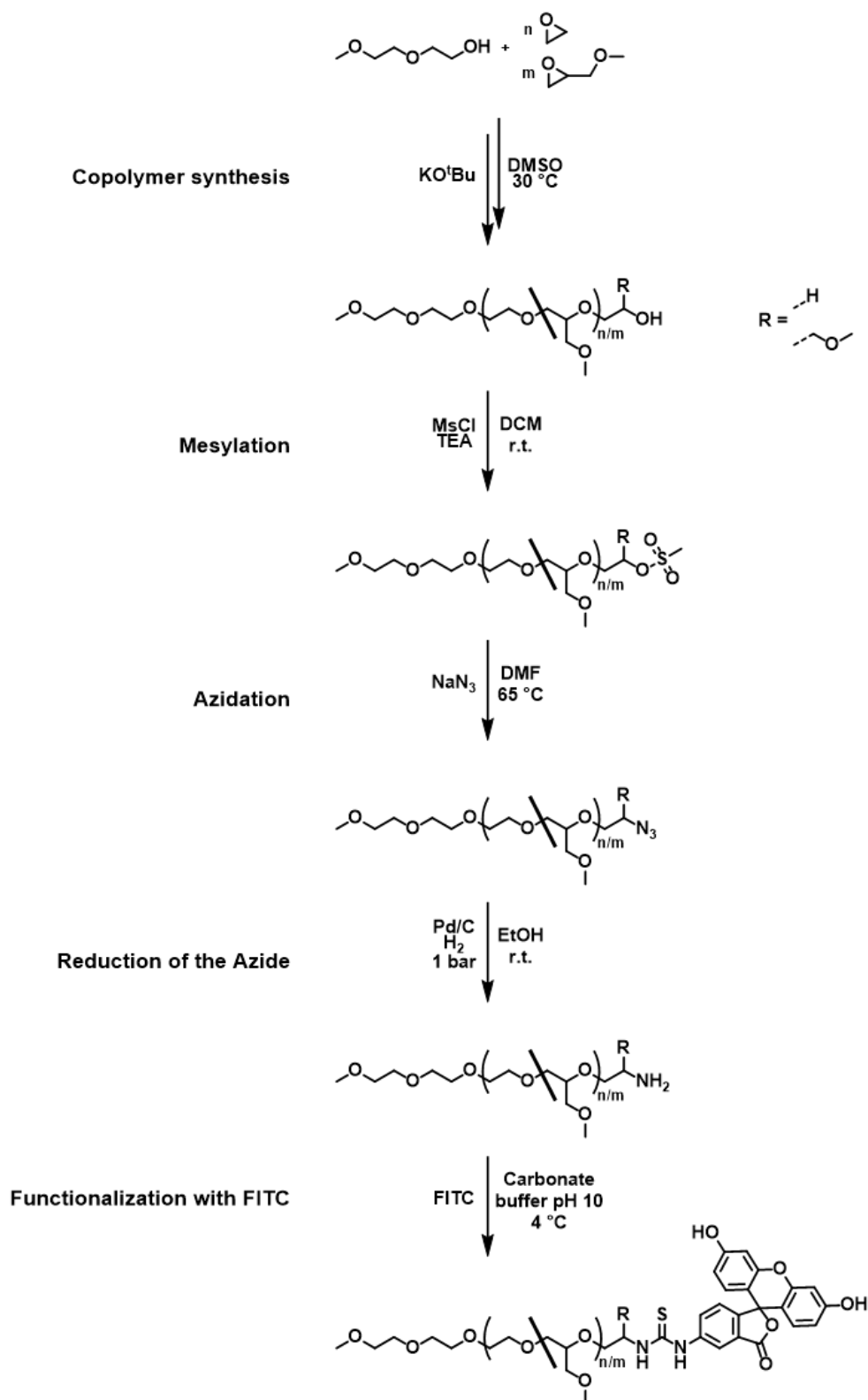
The polymer azide (300 mg, 60 μmol , 1 eq) was dissolved in ethanol (5 mL) and transferred into a Schlenk flask. 10 wt% Pd/C (39.4 mg, 40 μmol Pd, 0.66 eq) were suspended in 2 mL of ethanol and added to the polymer solution. The flask was sealed and a vacuum was applied until the solvent started to evaporate. The flask was flooded with argon again and this procedure was repeated two more times. Afterwards, a balloon filled with hydrogen gas was connected to the flask, and a vacuum was applied again until the solvent started to evaporate. This time the flask was flooded with hydrogen from the balloon. This procedure was also repeated two more times so the atmosphere in the flask was saturated with hydrogen gas. The mixture was stirred under hydrogen atmosphere for 48 h and the hydrogen balloon was refilled after 24 h as needed. The reaction mixture was subsequently filtered over Celite, the filter was washed twice with ethanol, and the solvent was removed under reduced pressure to afford 277 mg (92%) of polymer amine.

2.5. Functionalization with FITC

A carbonate-buffer solution with a concentration of 0.1 mol L^{-1} at pH10 was prepared by dissolving sodium bicarbonate (3.88 g, 46 mmol) and sodium carbonate (5.71 g, 54 mmol) in Millipore water (1000 mL).

The polymer amine (100 mg, 20 μmol , 1 eq) and fluorescein isothiocyanate (5-FITC, 15.6 mg, 40 μmol , 2 eq) were dissolved separately in the buffer solution (1.5 mL). Both solutions were combined and kept at 4 °C for 120 h under exclusion of light. Afterwards, the reaction mixture was transferred into a tainted glass flask and water was removed under reduced pressure. The residue was taken up in DCM (5 mL) and the precipitate was filtered off. The solution was washed once with the buffer solution and then with brine, dried over magnesium sulfate, and the solvent was removed again. The resulting polymer was dialyzed against the carbonate buffer solution (regenerated cellulose, MWCO = 2000 g mol^{-1}) under the exclusion of light. Finally, the dialyzed polymer solution was transferred to a tainted glass flask and the water was removed again under reduced pressure. The residue was taken up in chloroform (2 mL) and the precipitate was removed by filtration. The solution of the functionalized polymer in chloroform was purified via preparative SEC to yield 32 mg (30%) of FITC-functionalized polymer.

3. Reaction sequence: copolymer functionalization



Scheme S1. Reaction sequence: functionalization of the copolymers.

4. ^1H NMR spectra

The last repeating unit of the polymer chain is statistically either an EO unit ($\text{R} = -\text{H}$) or a GME unit ($\text{R} = -\text{CH}_2-\text{O}-\text{CH}_3$). This difference is visible in the spectra of the mesylated rPEGs, where distinct signals for the terminal methylene group of the EO unit and the methine group of the GME unit can be observed. In all other cases the signal of the methine group overlaps with the backbone signal and therefore is not visible in the spectrum.

The samples' composition is described with $\text{rPEG}_{D_p}^f$ where f is the molar fraction of GME and D_p is the total degree of polymerization of the sample.

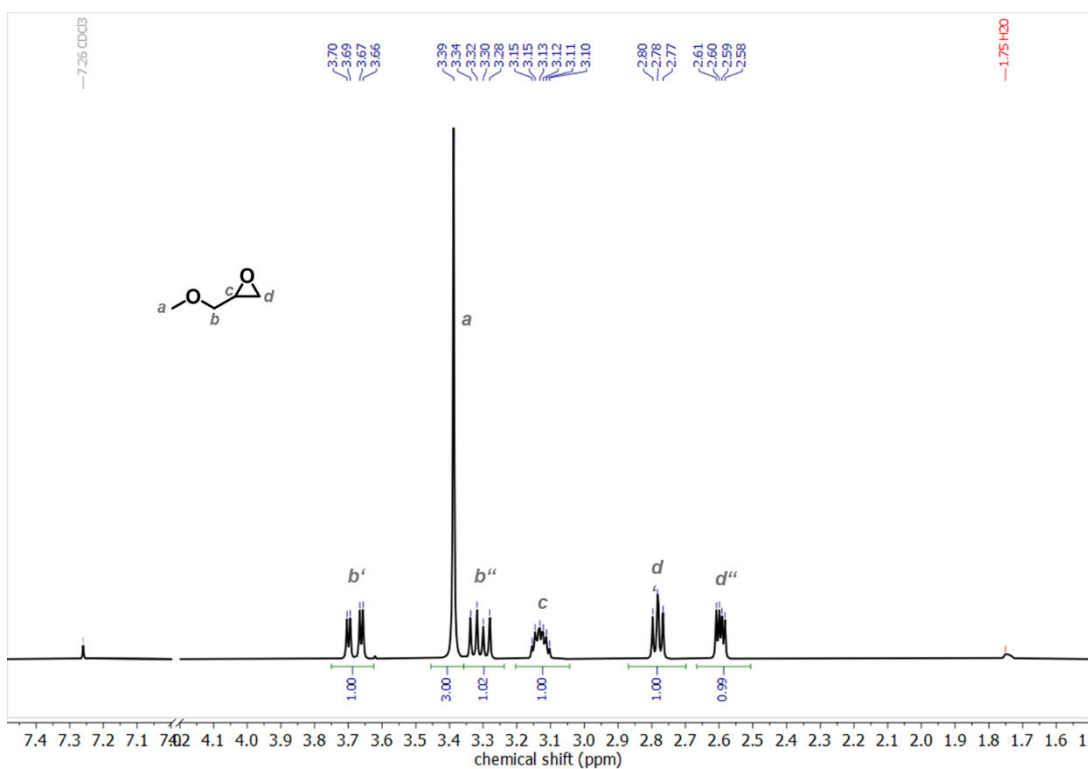


Figure S1. ^1H -NMR spectrum (CDCl_3 , 400 MHz) of glycidyl methyl ether (GME).

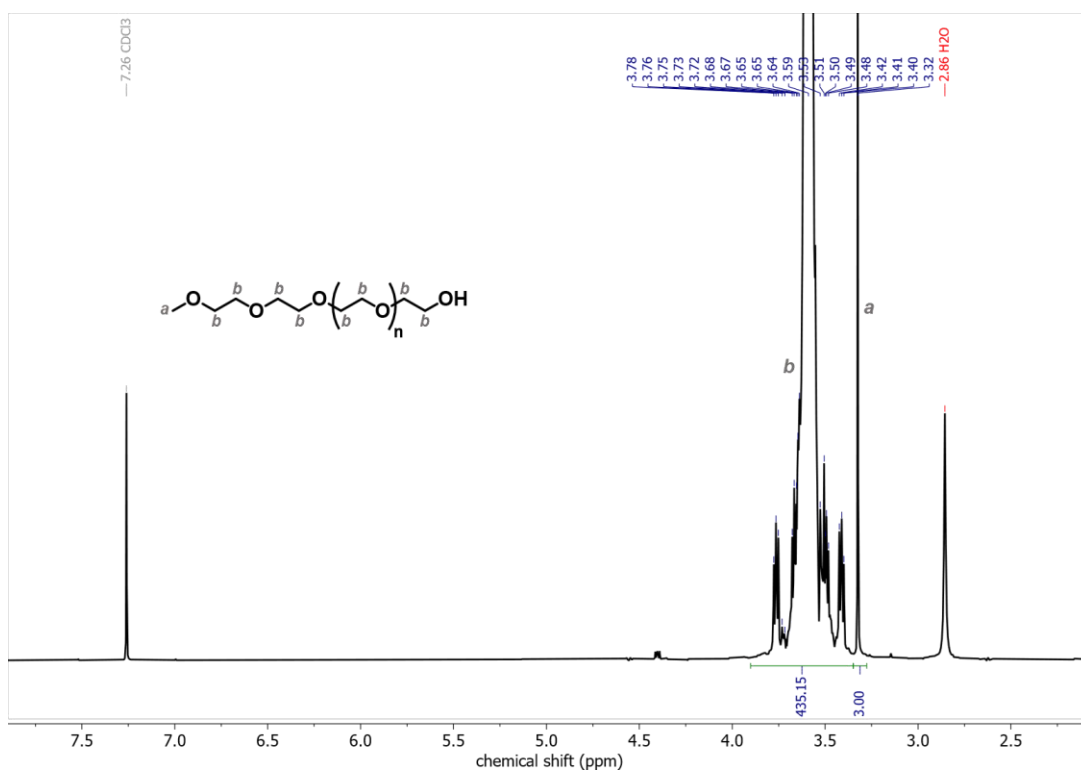


Figure S2. ¹H-NMR spectrum (CDCl₃, 400 MHz) of mPEG₁₁₄.

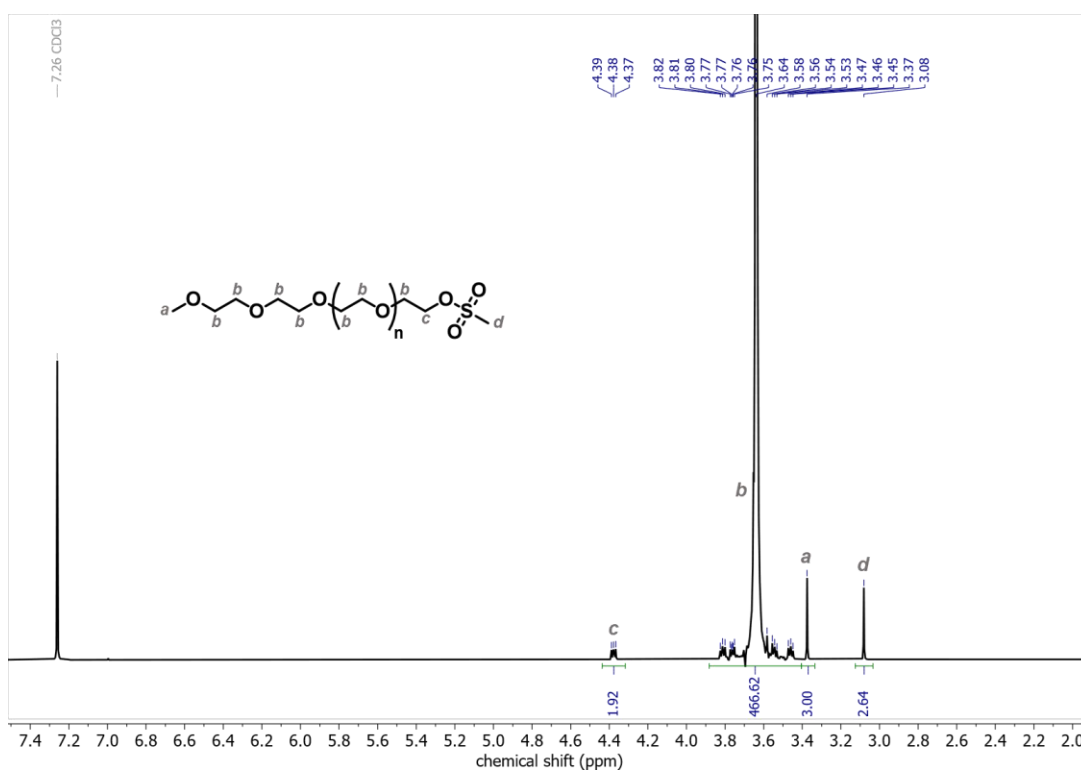


Figure S3. ¹H-NMR spectrum (CDCl₃, 400 MHz) of mPEG₁₁₄-mesylate.

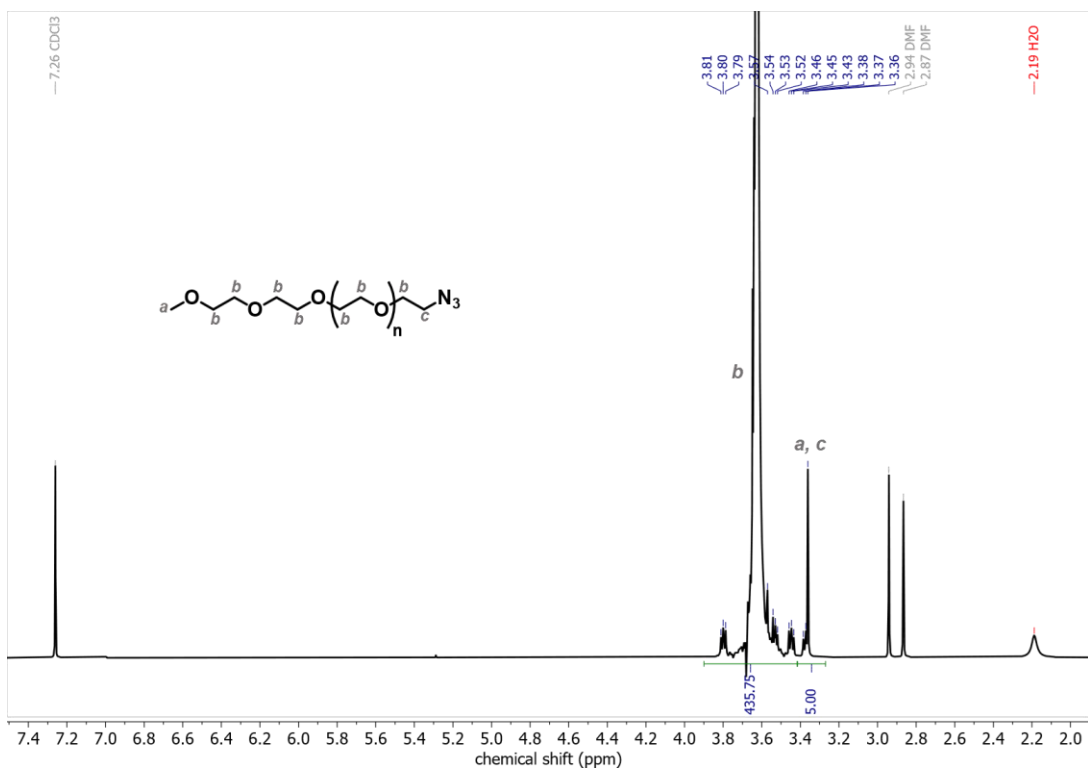


Figure S4. ¹H-NMR spectrum (CDCl₃, 400 MHz) of mPEG₁₁₄-azide.

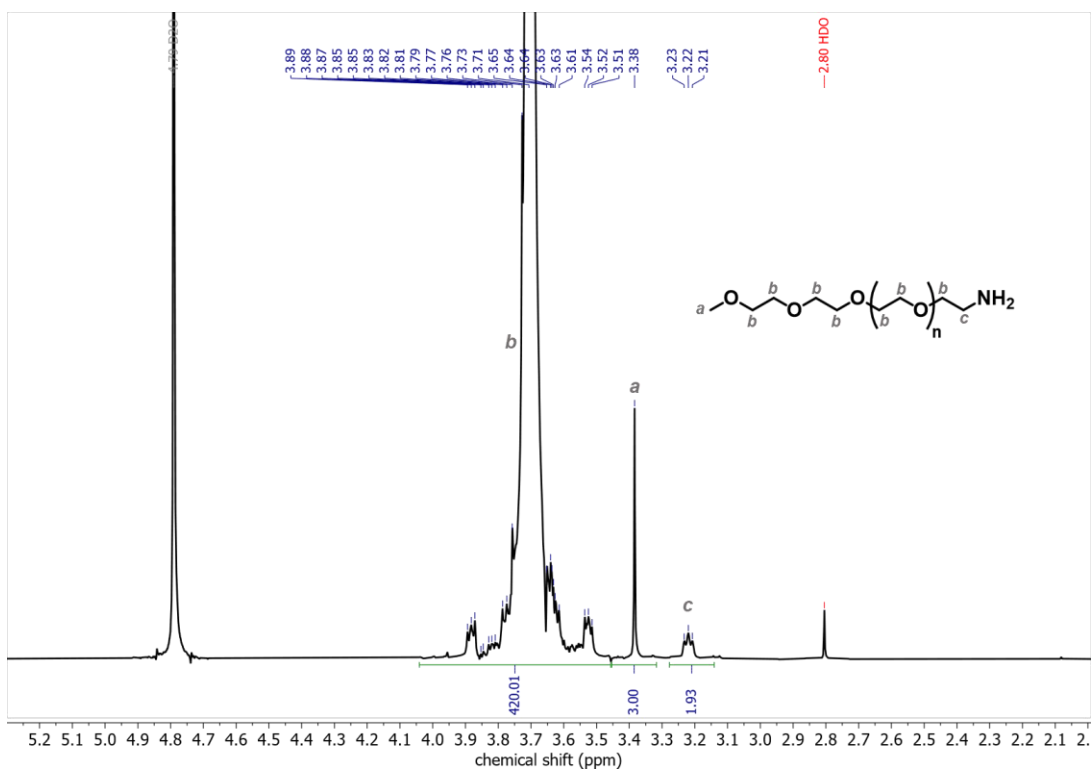


Figure S5. ¹H-NMR spectrum (D₂O, 400 MHz) of mPEG₁₁₄-amine.

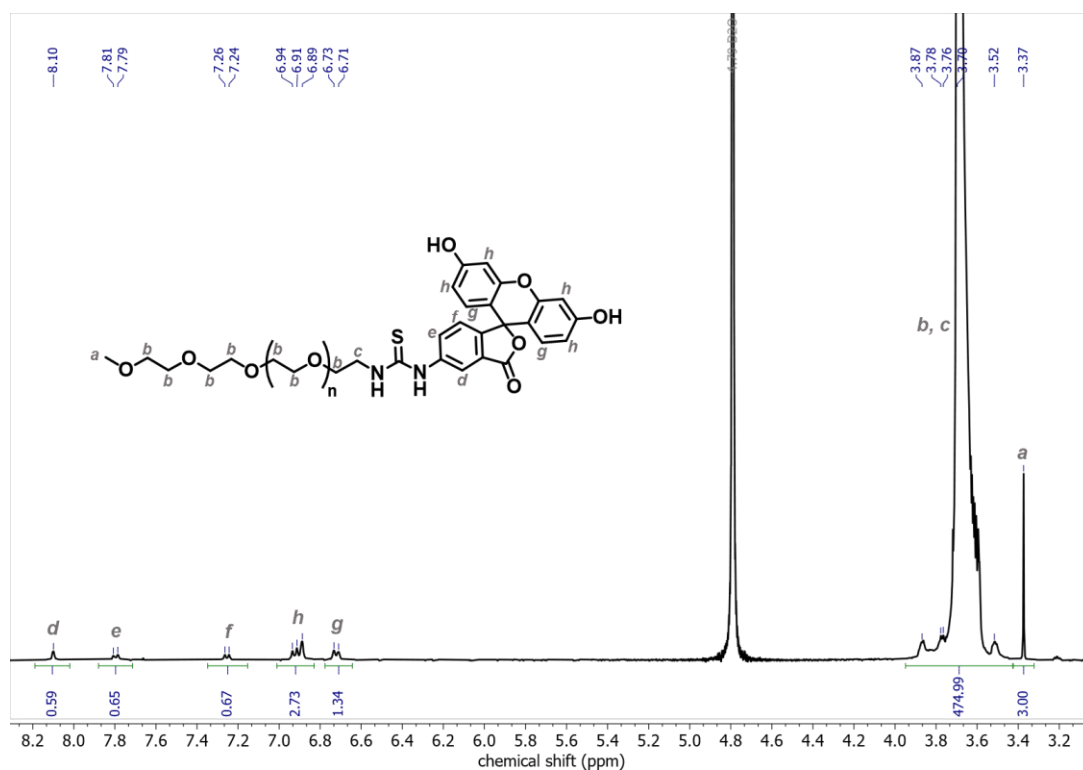


Figure S6. ¹H-NMR spectrum (D₂O, 400 MHz) of mPEG₁₁₄-FITC.

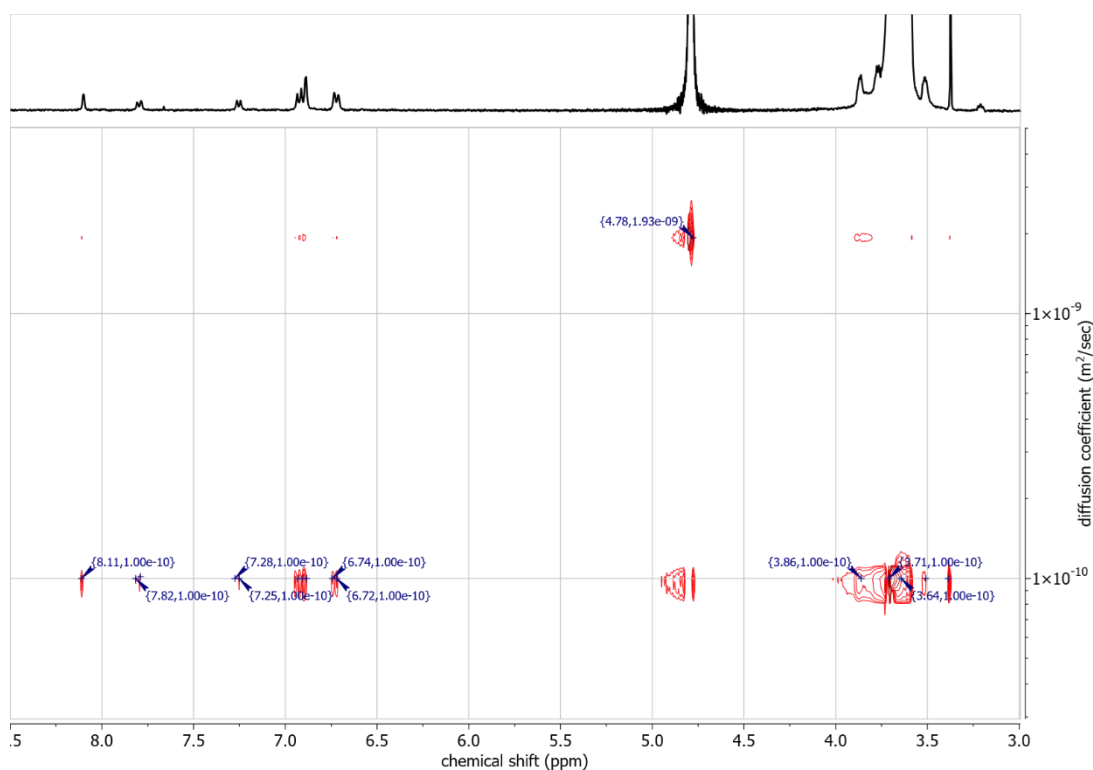


Figure S7. 2D DOSY-NMR spectrum (D₂O, 400 MHz) of mPEG₁₁₄-FITC.

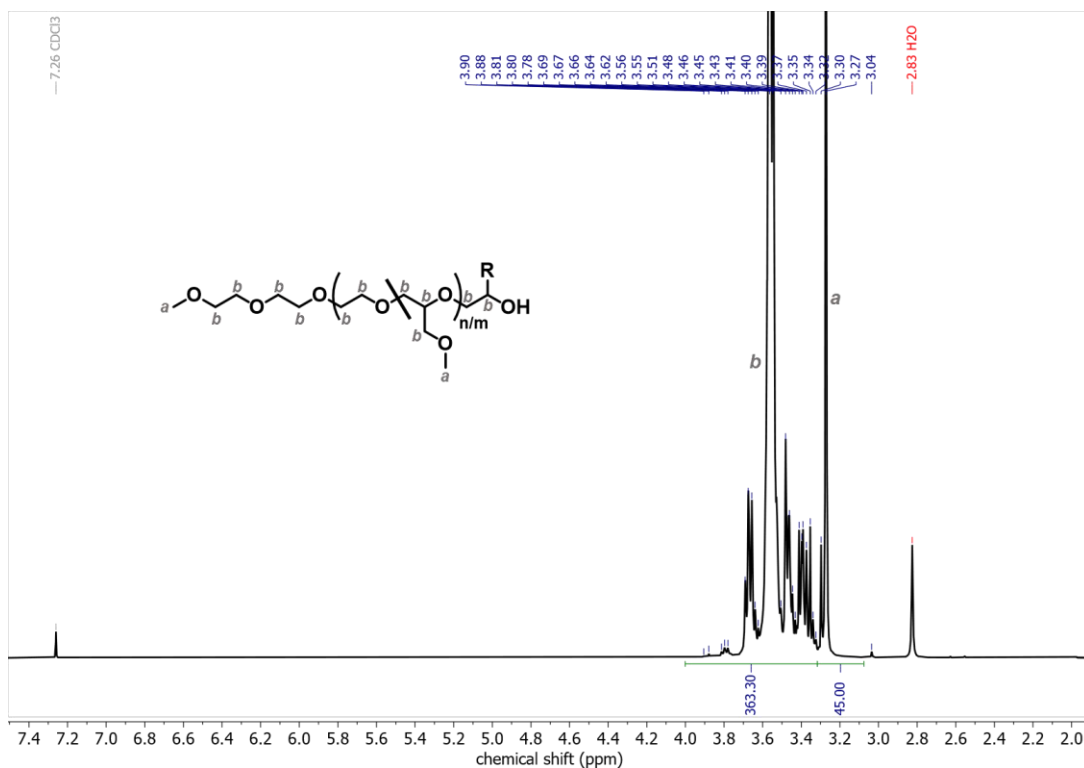


Figure S8. ¹H-NMR spectrum (CDCl₃, 400 MHz) of rPEG₉₈^{0.17}.

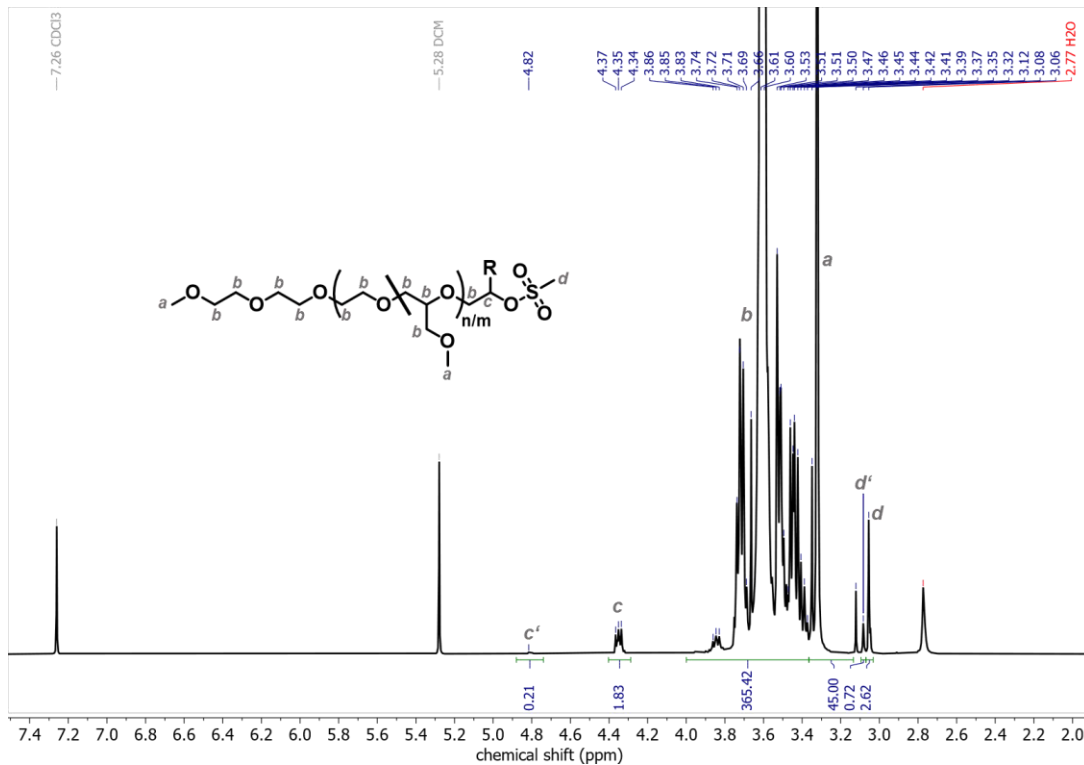


Figure S9. ¹H-NMR spectrum (CDCl₃, 400 MHz) of rPEG₉₈^{0.17}-mesylate
(c/d = terminal EO unit, c'/d' = terminal GME unit).

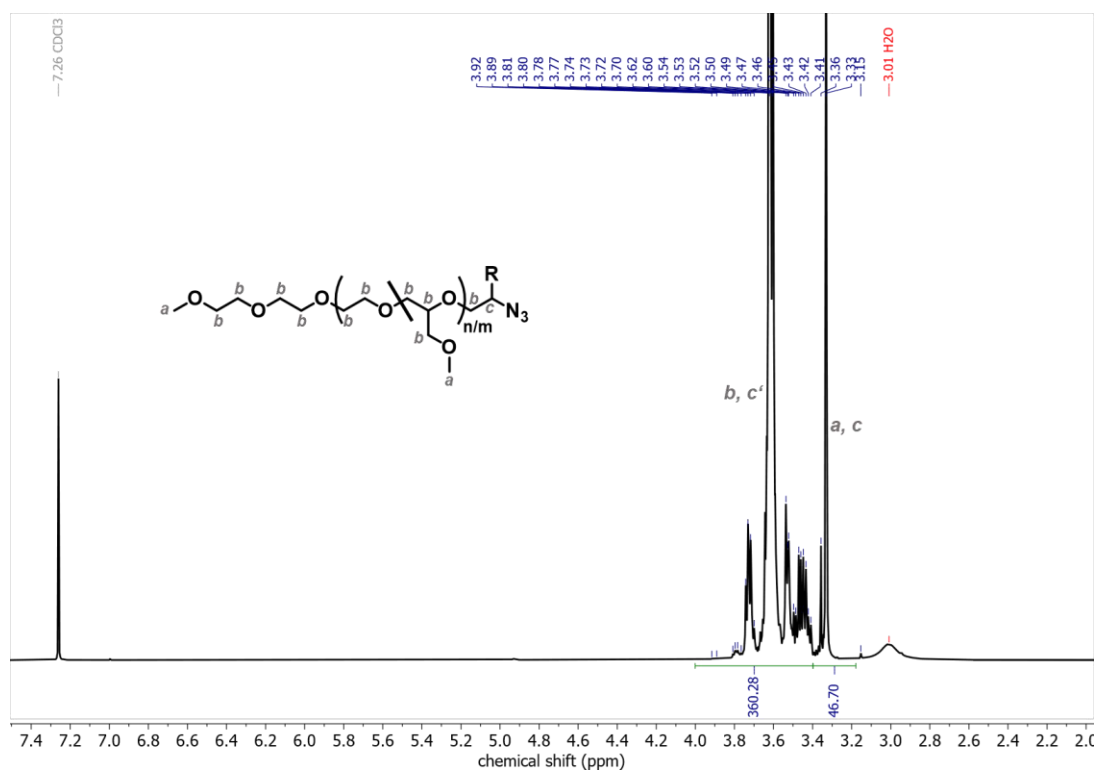


Figure S10. ^1H -NMR spectrum (CDCl_3 , 400 MHz) of $r\text{PEG}_{98}^{0.17}\text{-azide}$
(c = terminal EO unit, c' = terminal GME unit).

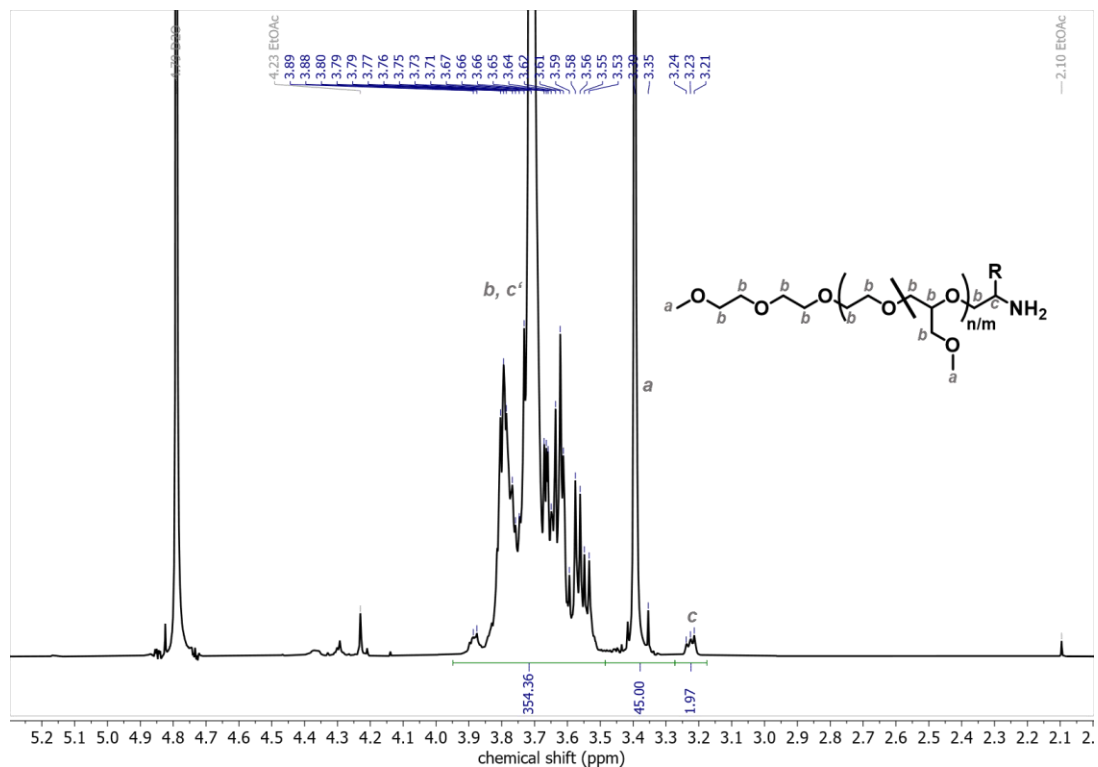


Figure S11. ^1H -NMR spectrum (D_2O , 400 MHz) of $r\text{PEG}_{98}^{0.17}\text{-amine}$
(c = terminal EO unit, c' = terminal GME unit).

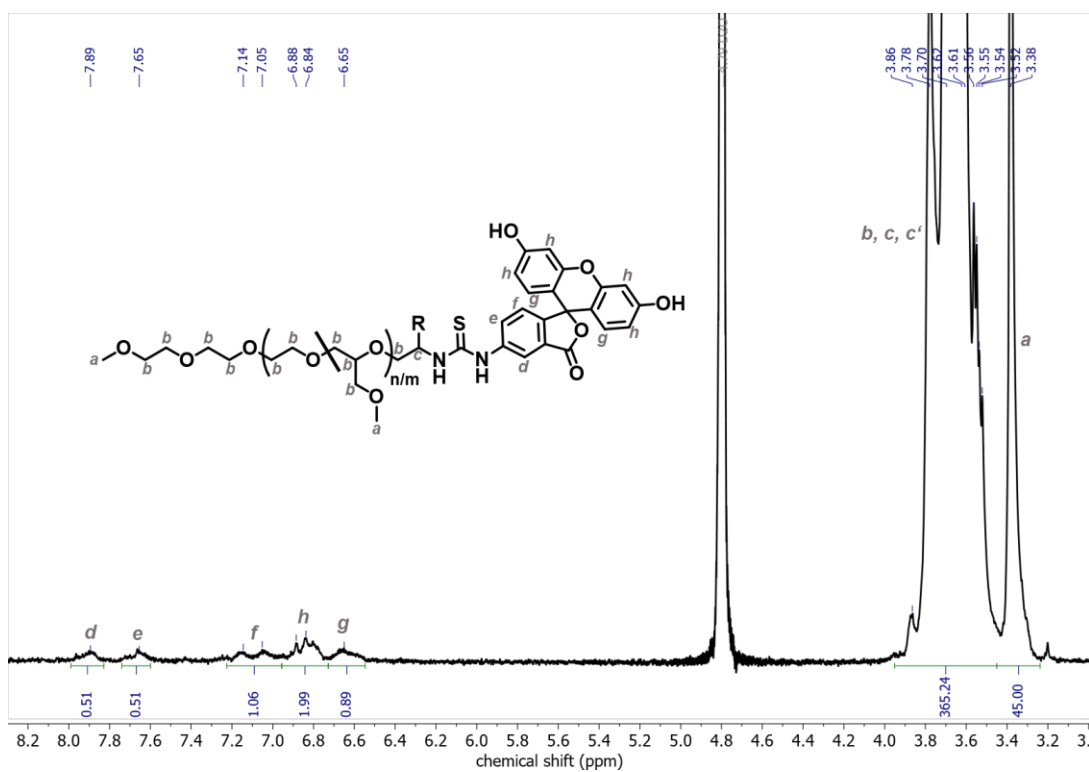


Figure S12. ^1H -NMR spectrum (D_2O , 400 MHz) of $r\text{PEG}_{98}^{0.17}\text{-FITC}$
(*c* = terminal EO unit, *c'* = terminal GME unit).

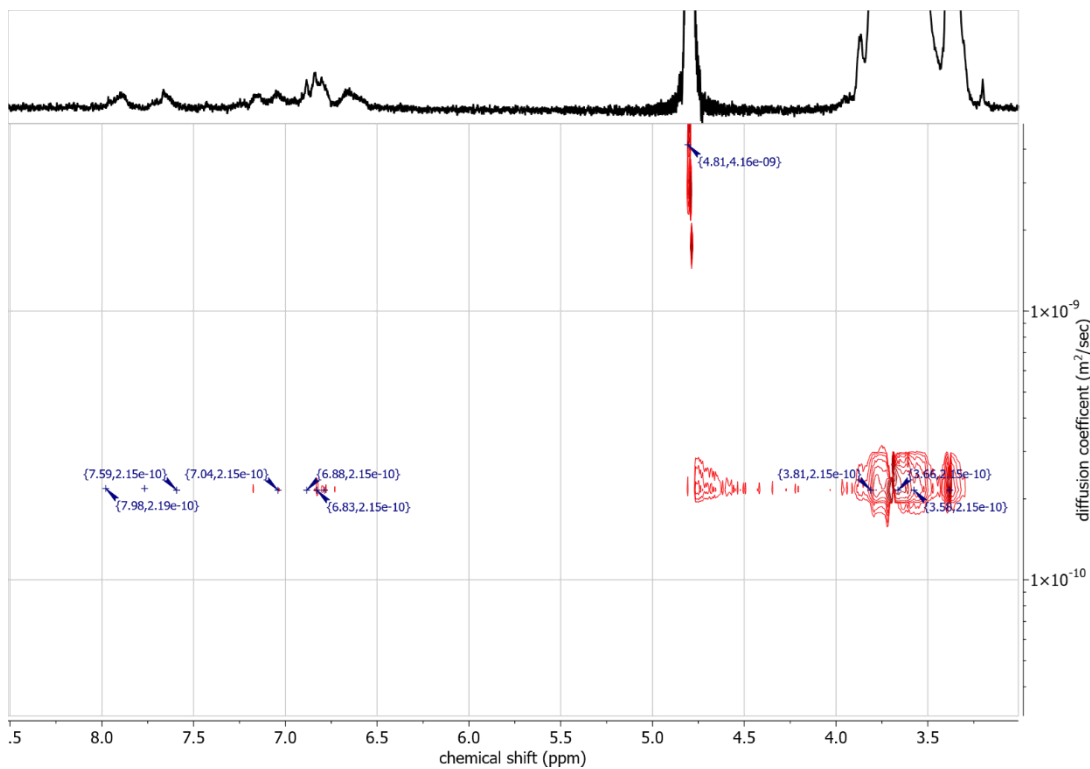


Figure S13. 2D DOSY-NMR spectrum (D_2O , 400 MHz) of $r\text{PEG}_{98}^{0.17}\text{-FITC}$.

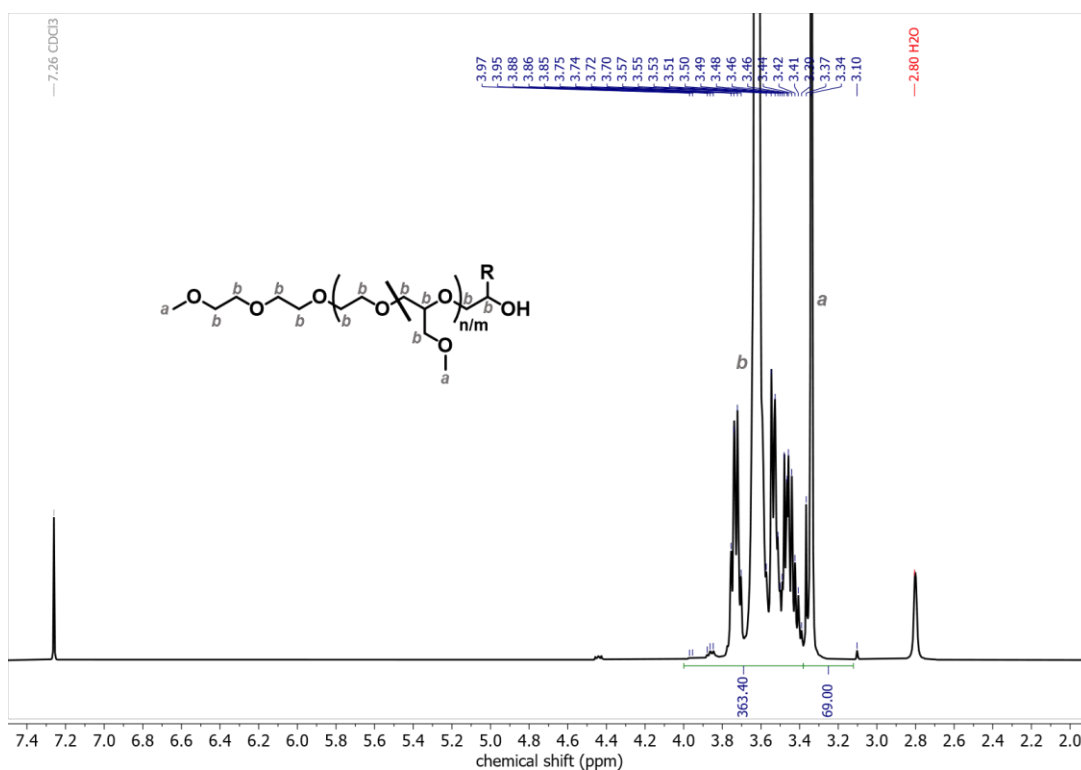


Figure S14. ¹H-NMR spectrum (CDCl₃, 400 MHz) of rPEG₈₉^{0.27}.

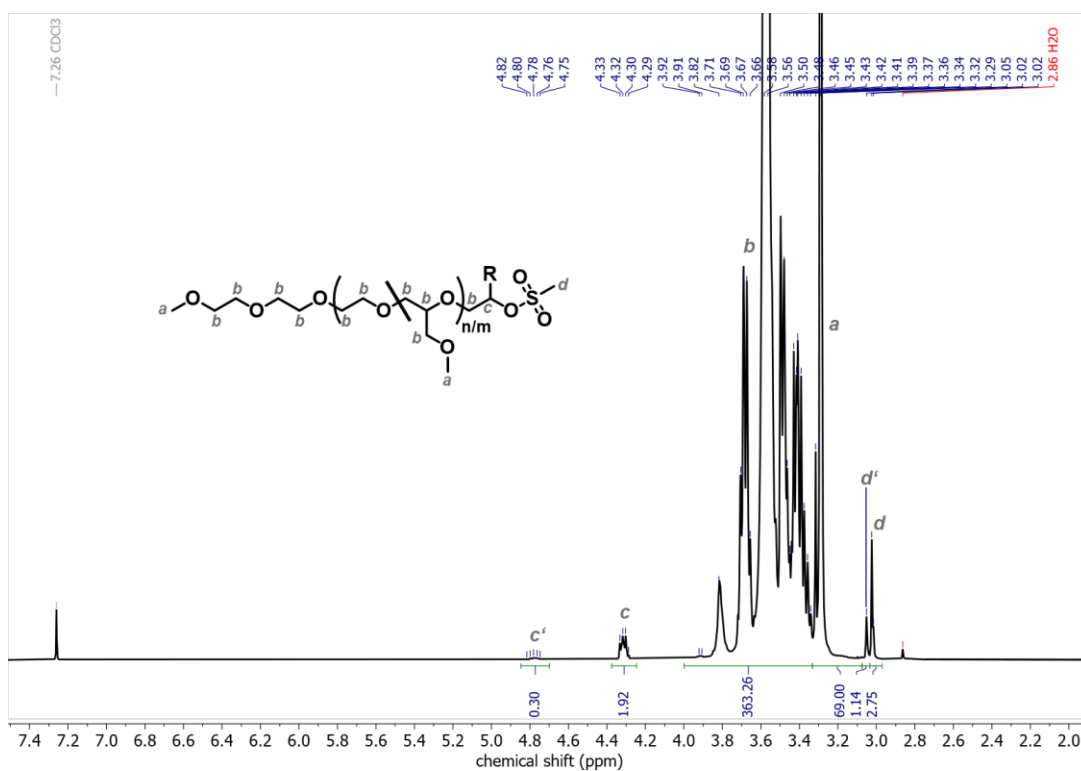


Figure S15. ¹H-NMR spectrum (CDCl₃, 400 MHz) of rPEG₈₉^{0.27}-mesylate
(c/d = terminal EO unit, c'/d' = terminal GME unit).

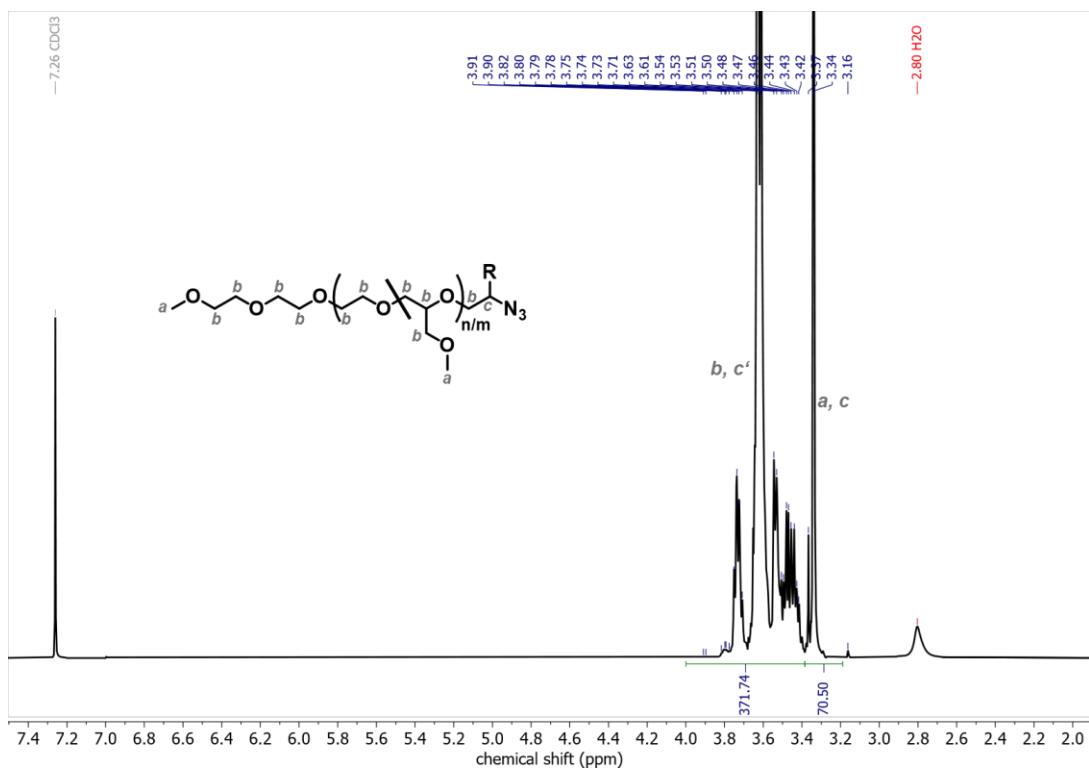


Figure S16. ¹H-NMR spectrum (CDCl₃, 400 MHz) of rPEG₈₉^{0.27}-azide
(*c* = terminal EO unit, *c'* = terminal GME unit).

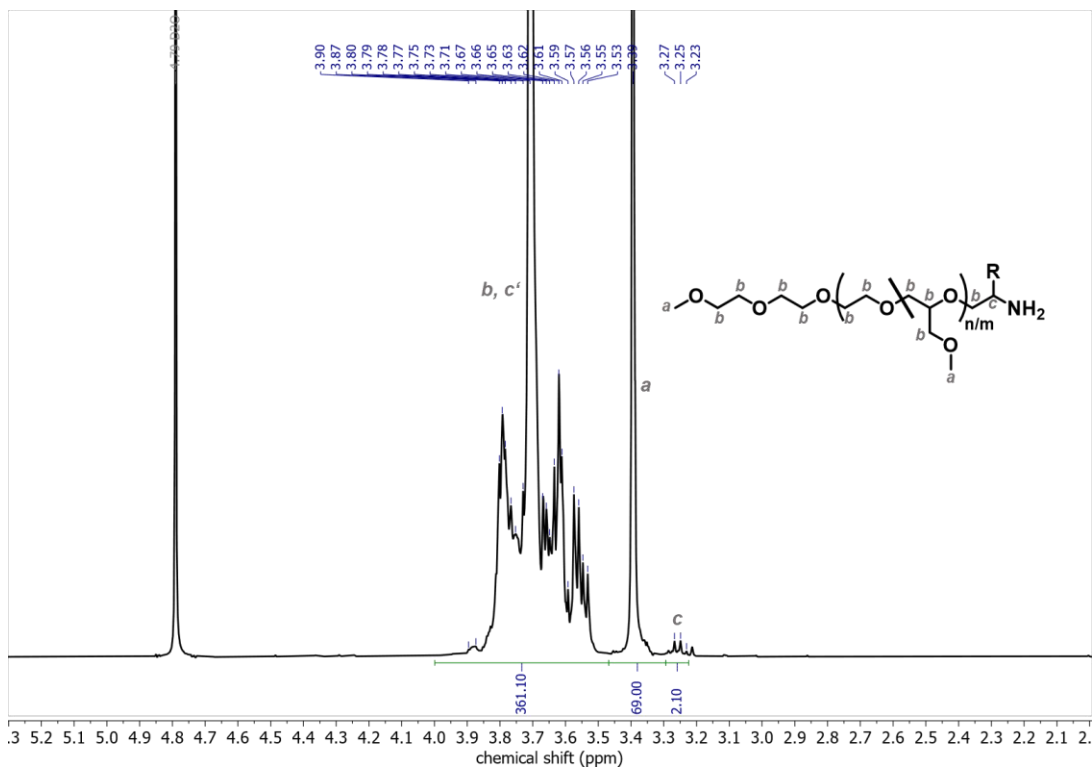


Figure S17. ¹H-NMR spectrum (D₂O, 400 MHz) of rPEG₈₉^{0.27}-amine
(*c* = terminal EO unit, *c'* = terminal GME unit).

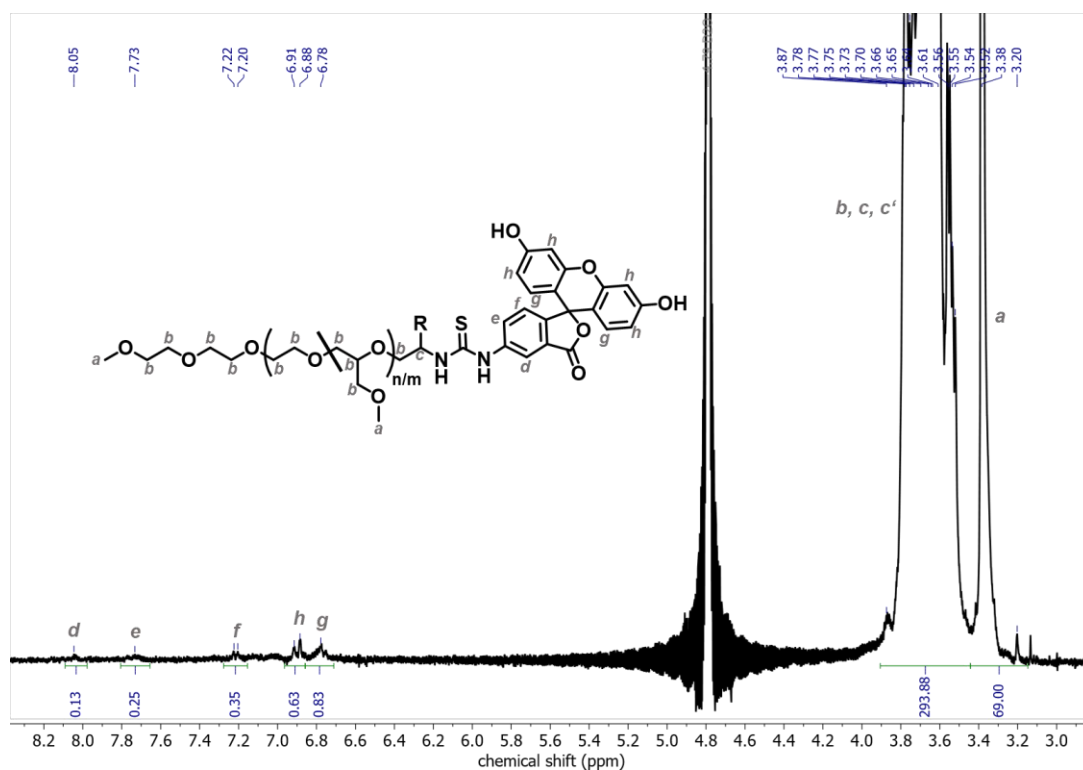


Figure S18. ¹H-NMR spectrum (D₂O, 400 MHz) of rPEG₈₉^{0.27}-FITC
(*c* = terminal EO unit, *c'* = terminal GME unit).

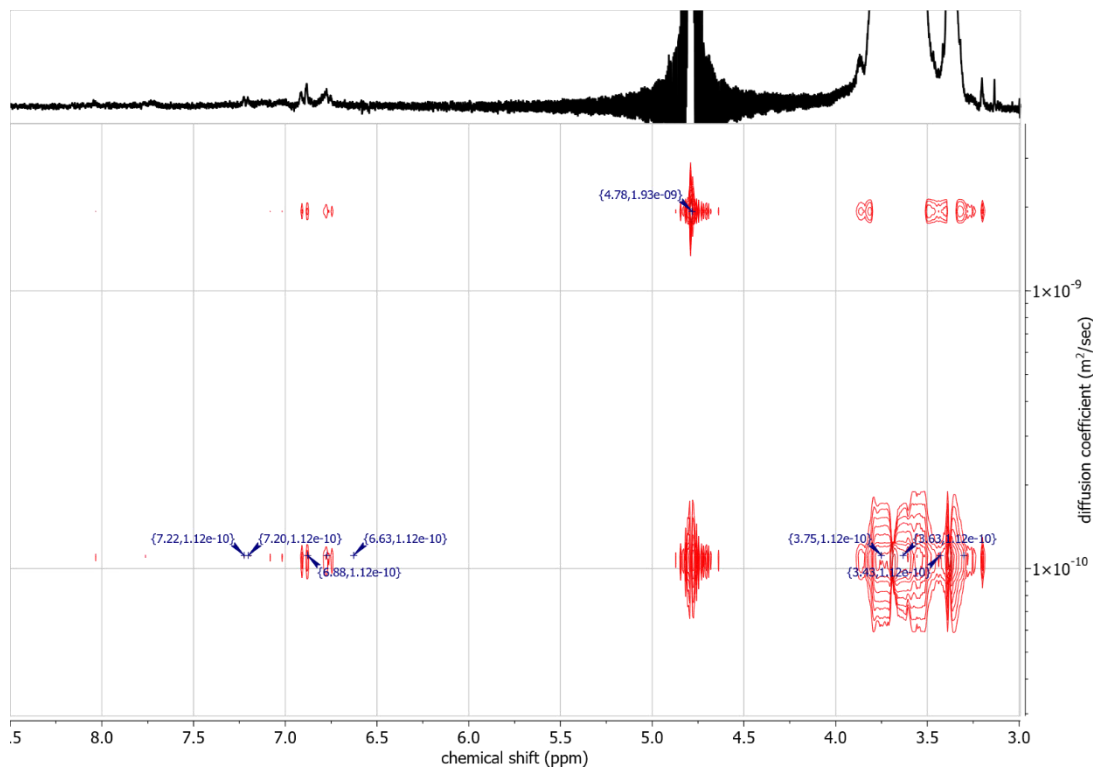


Figure S19. 2D DOSY-NMR spectrum (D₂O, 400 MHz) of rPEG₈₉^{0.27}-FITC.

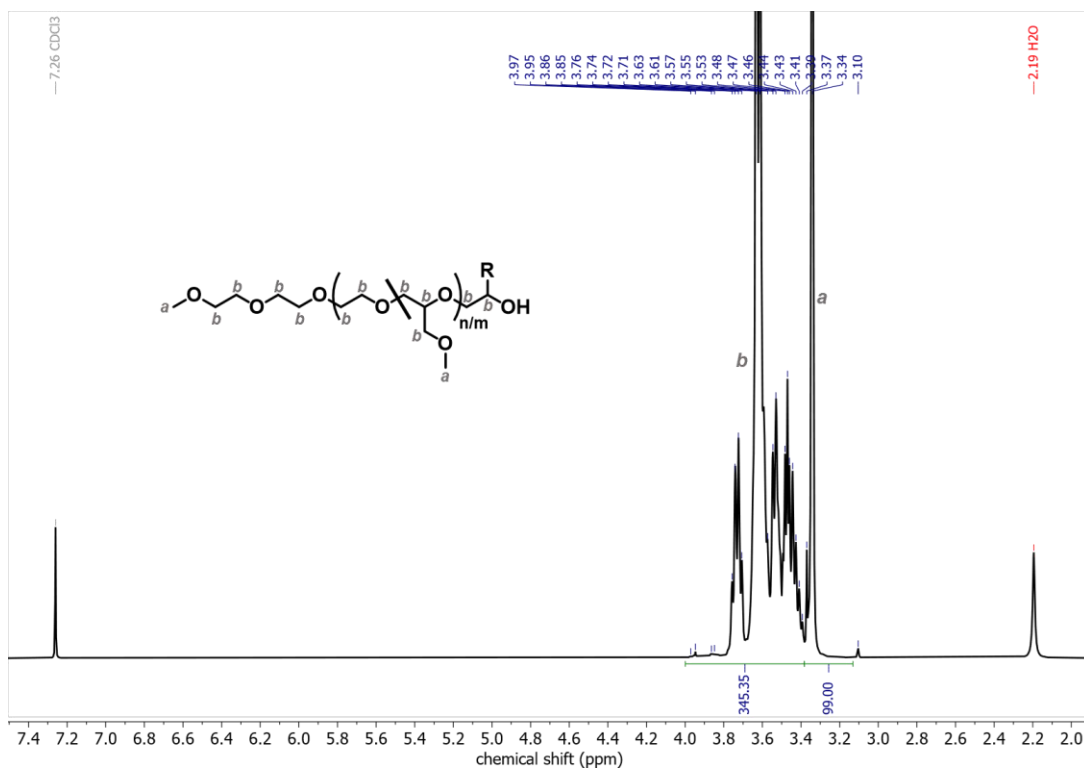
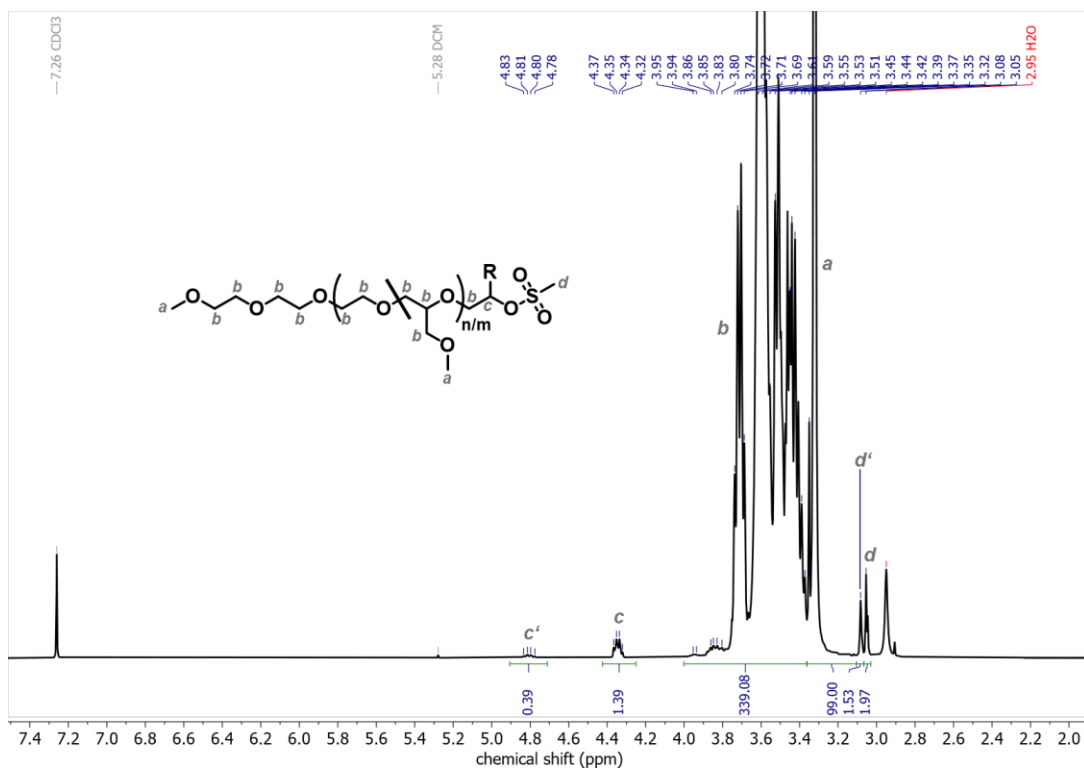
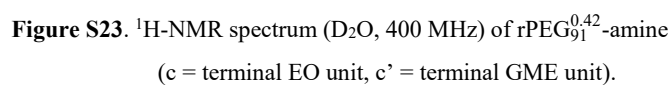
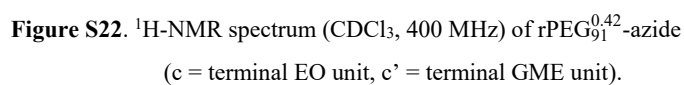


Figure S20. ¹H-NMR spectrum (CDCl₃, 400 MHz) of rPEG₉₁^{0.42}.



(c/d = terminal EO unit, c'/d' = terminal GME unit).



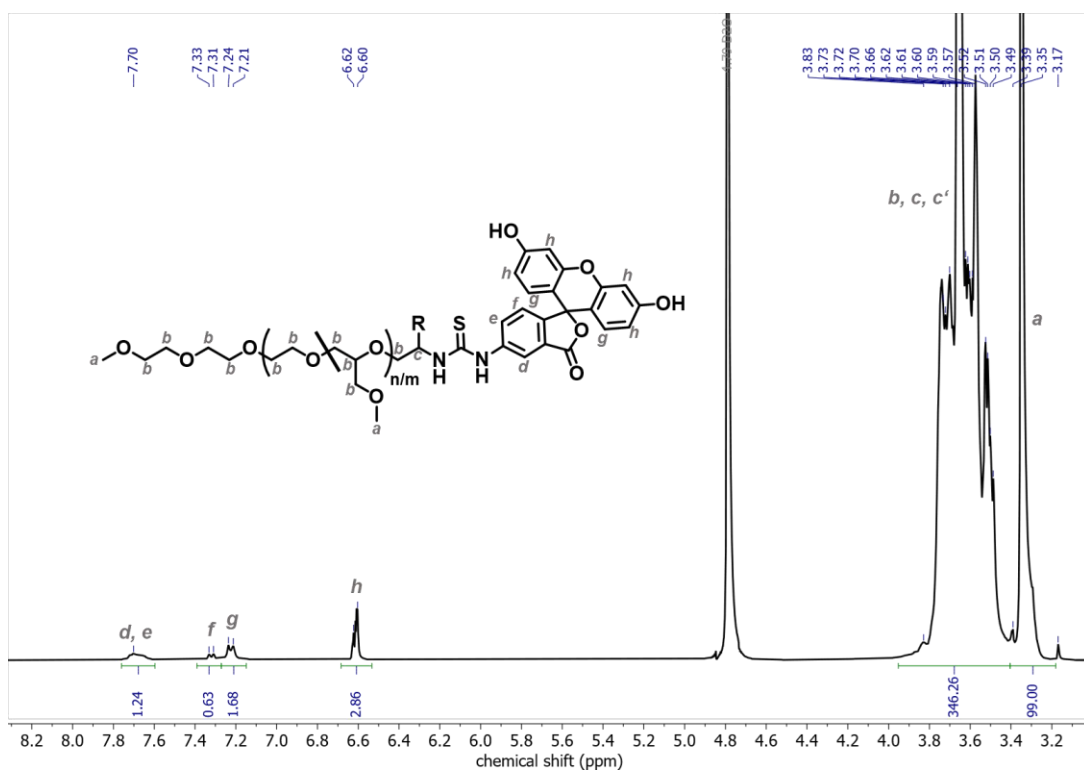


Figure S24. ¹H-NMR spectrum (D₂O, 400 MHz) of rPEG₉₁^{0.42}-FITC
(c = terminal EO unit, c' = terminal GME unit).

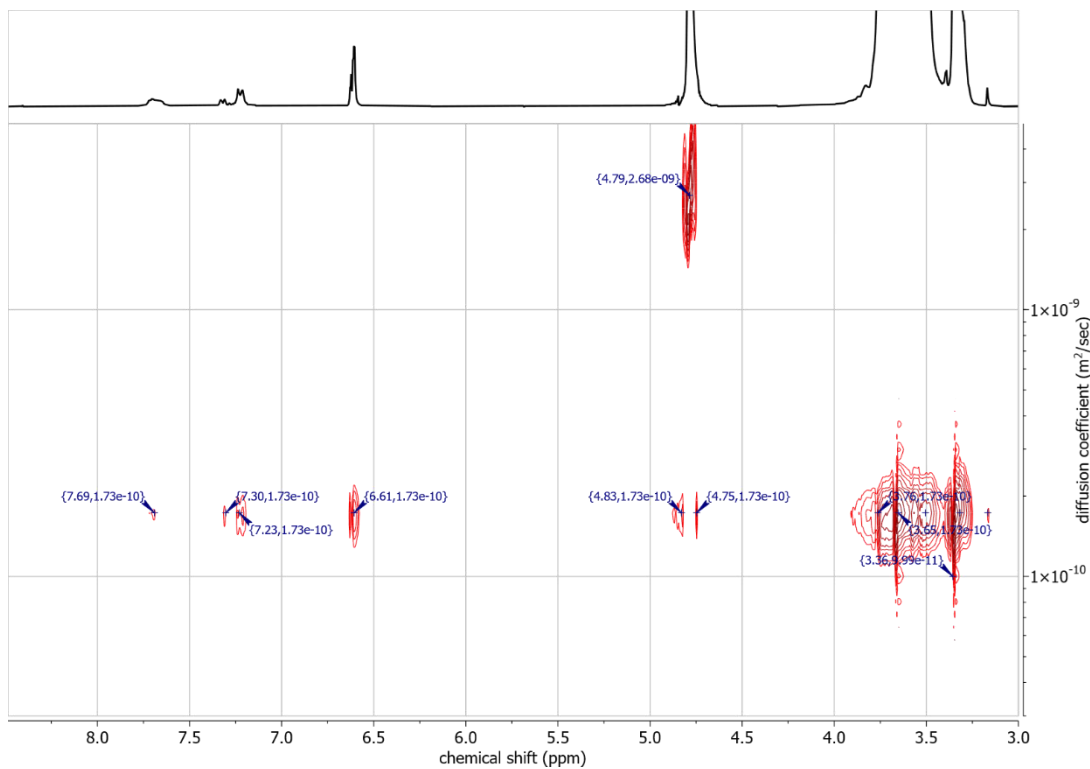
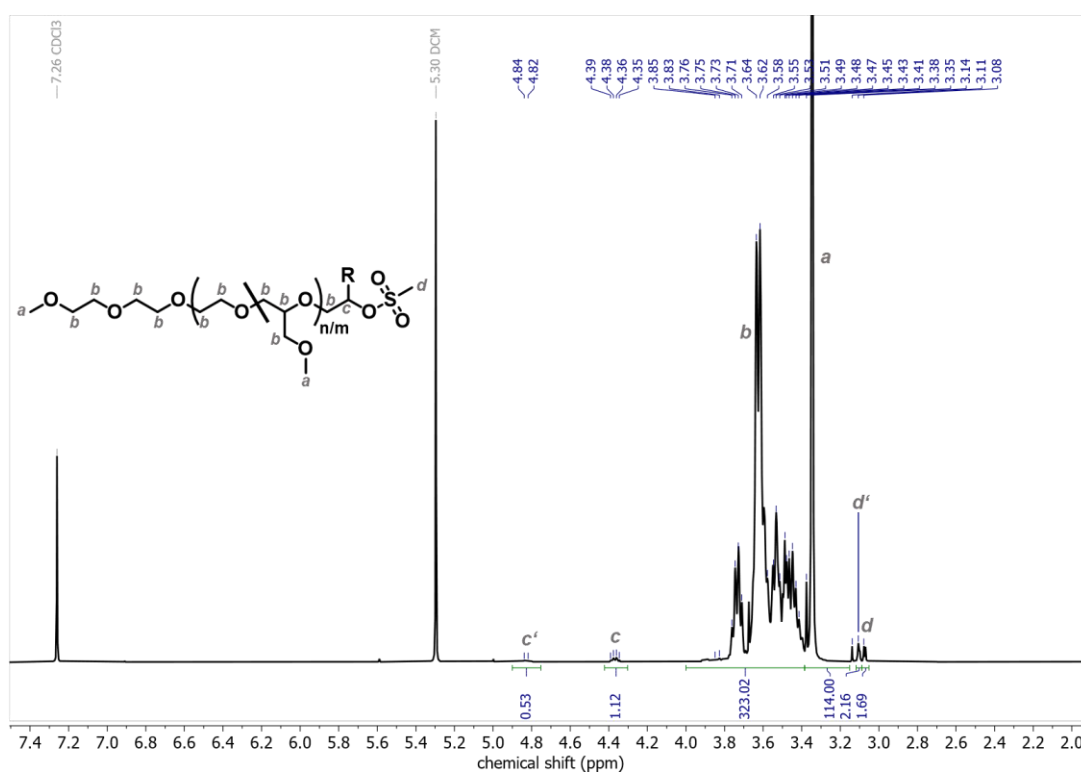
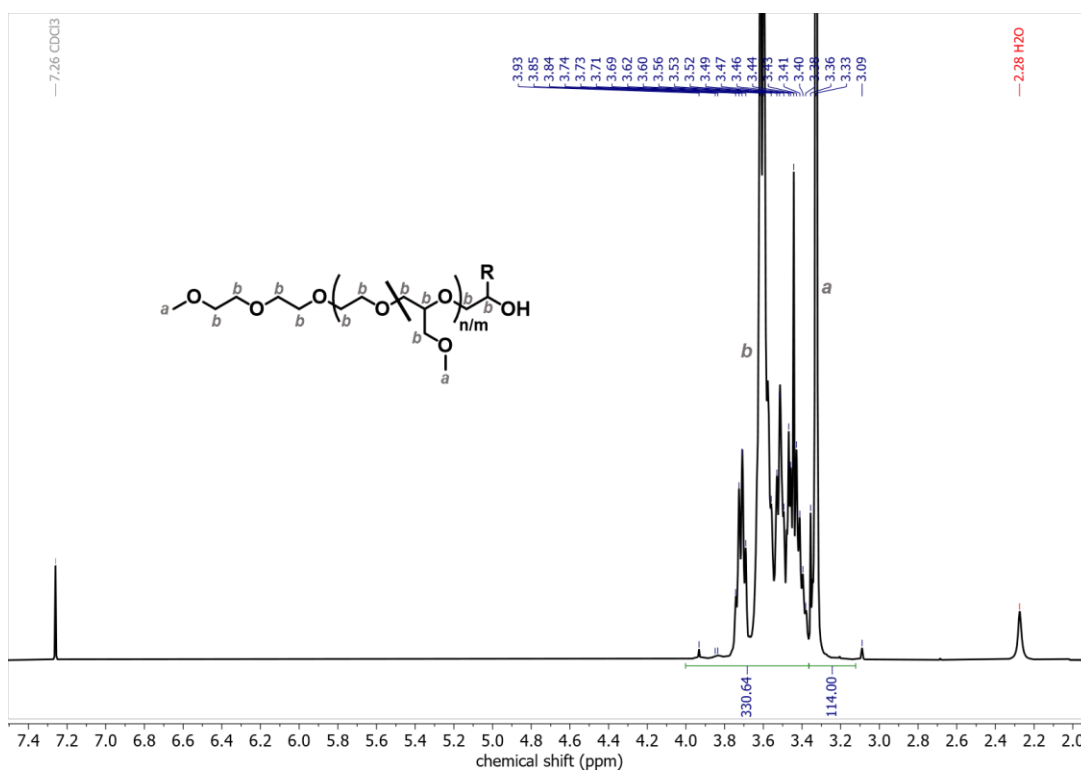


Figure S25. 2D DOSY-NMR spectrum (D₂O, 400 MHz) of rPEG₉₁^{0.42}-FITC.



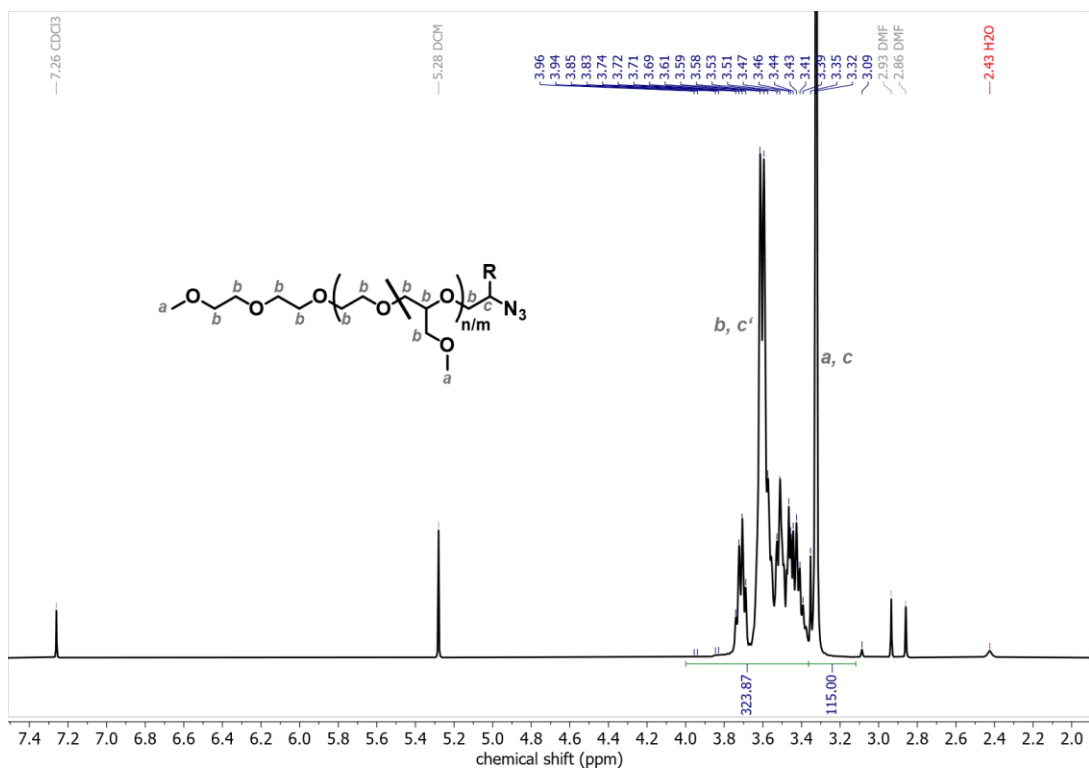


Figure S28. ¹H-NMR spectrum (CDCl₃, 400 MHz) of rPEG₇₆^{0.52}-azide
(*c* = terminal EO unit, *c'* = terminal GME unit).

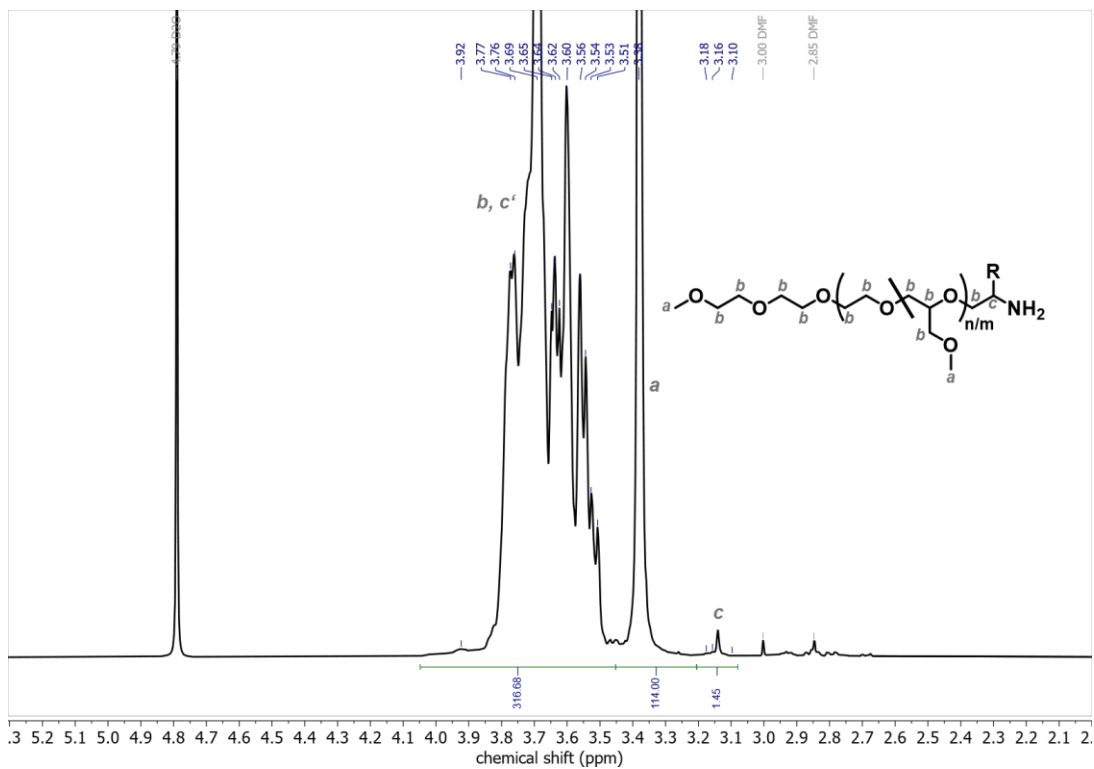


Figure S29. ¹H-NMR spectrum (D₂O, 400 MHz) of rPEG₇₆^{0.52}-amine
(*c* = terminal EO unit, *c'* = terminal GME unit).

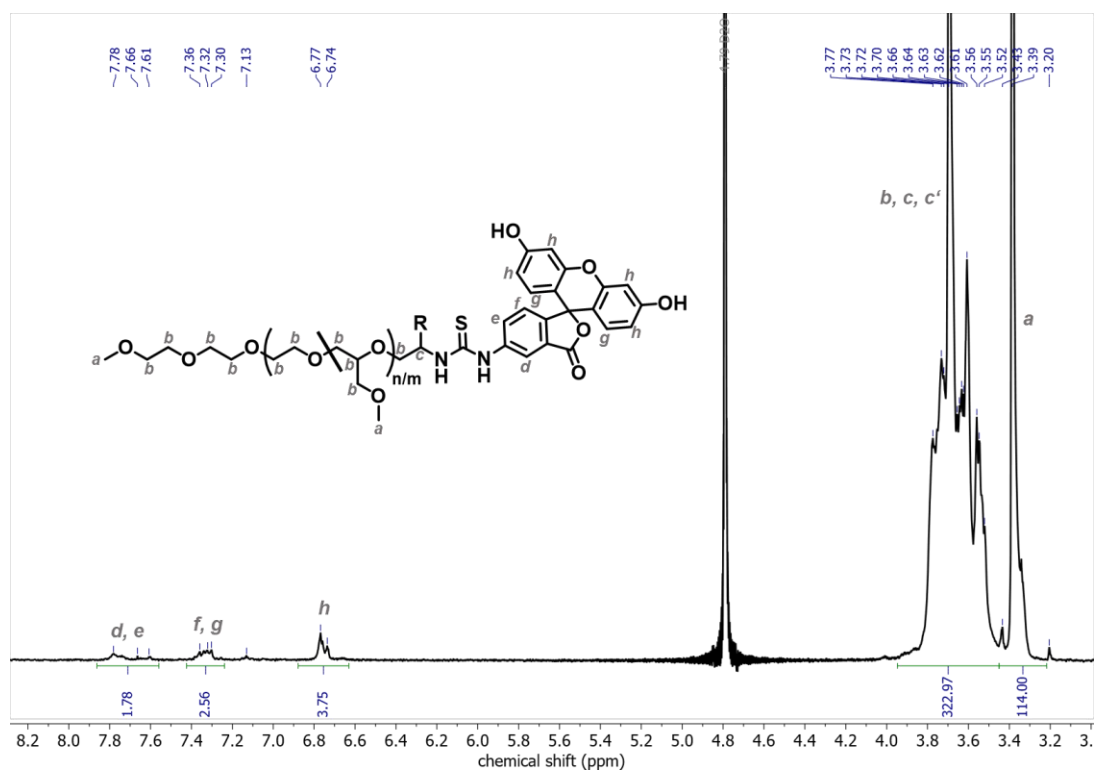


Figure S30. ¹H-NMR spectrum (D₂O, 400 MHz) of rPEG₇₆^{0.52}-FITC

(c = terminal EO unit, c' = terminal GME unit).

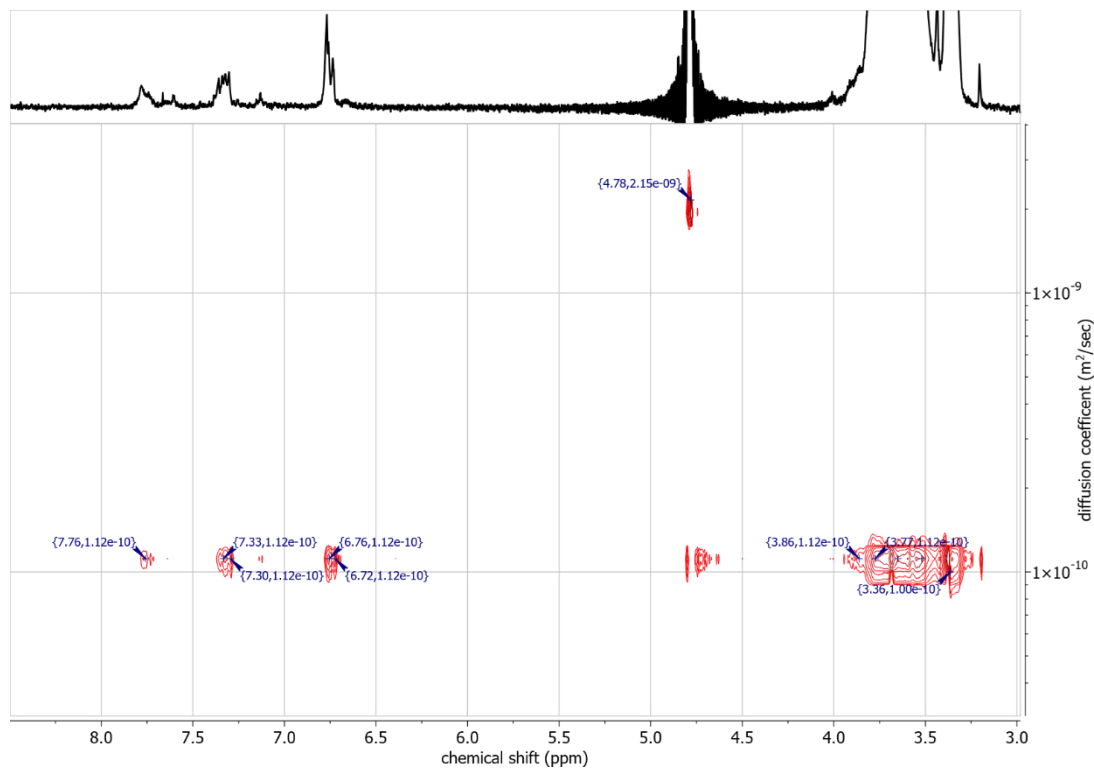


Figure S31. 2D DOSY-NMR spectrum (D₂O, 400 MHz) of rPEG₇₆^{0.52}-FITC.

5. MALDI TOF mass spectra

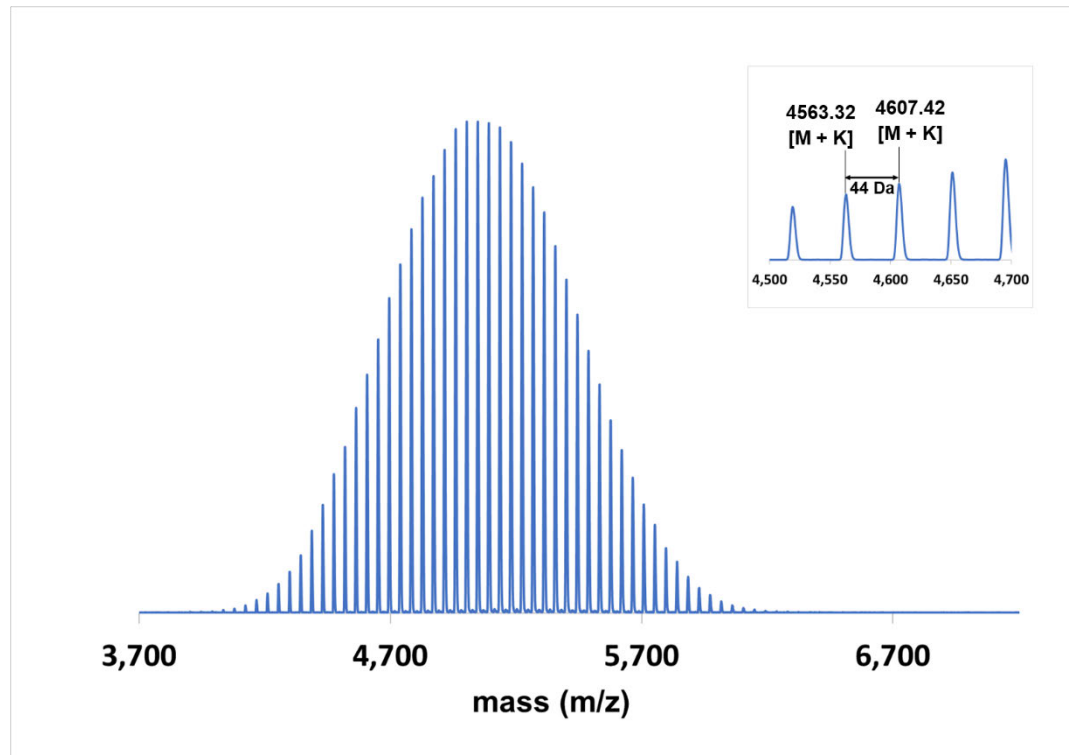


Figure S32. MALDI TOF mass spectrum of mPEG₁₁₄.

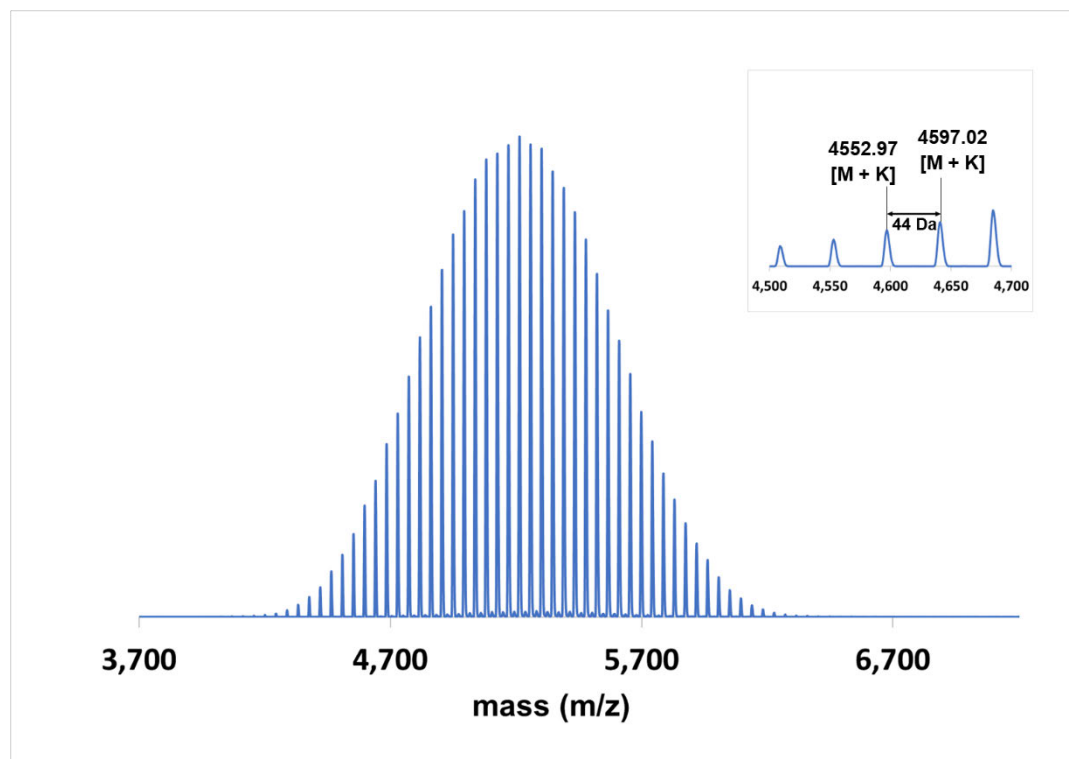


Figure S33. MALDI TOF mass spectrum of mPEG₁₁₄-mesylate.

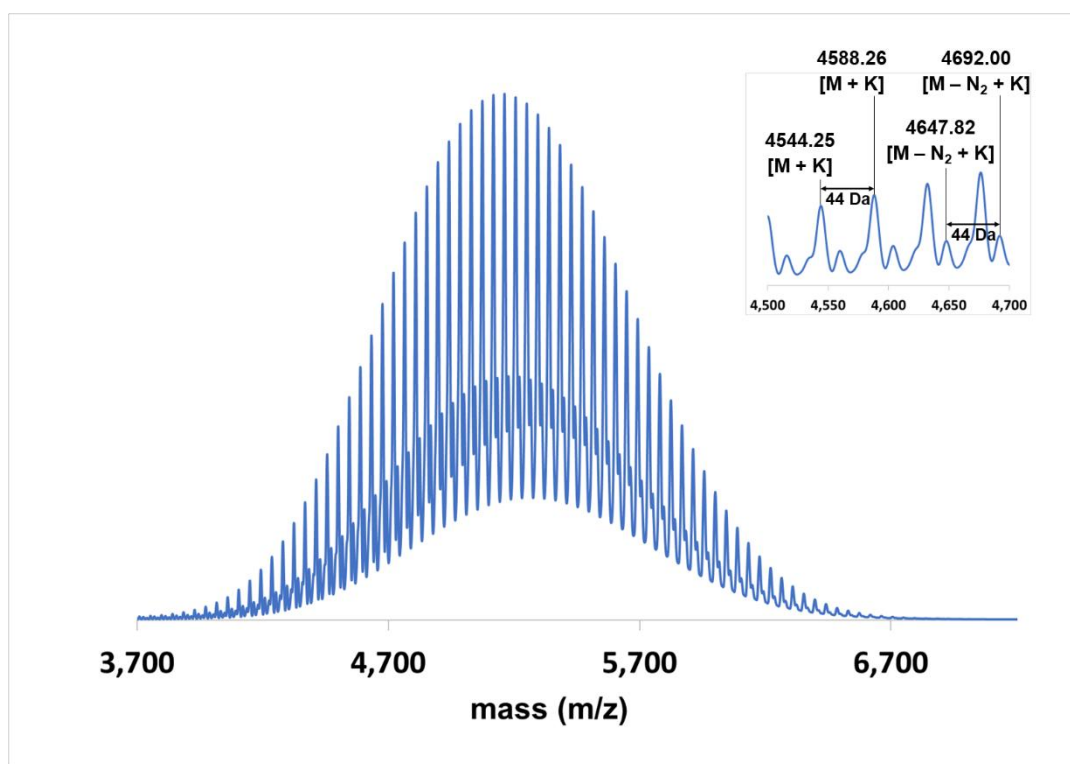


Figure S34. MALDI TOF mass spectrum of mPEG₁₁₄-azide.

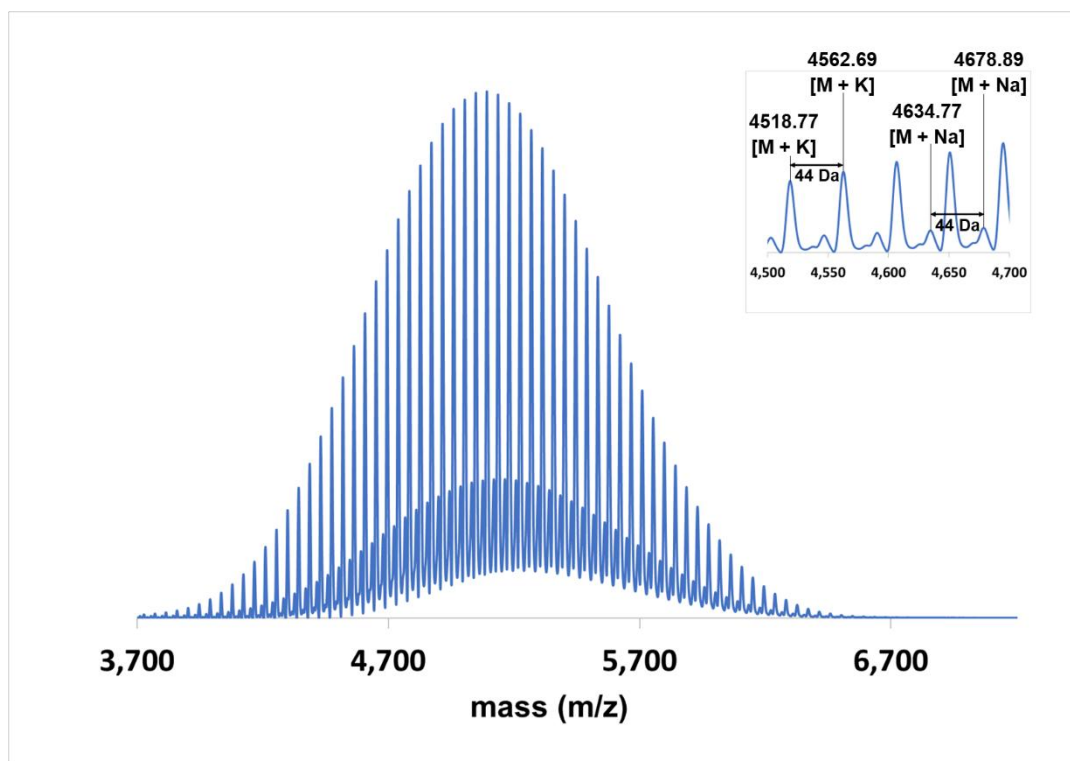


Figure S35. MALDI TOF mass spectrum of mPEG₁₁₄-amine.

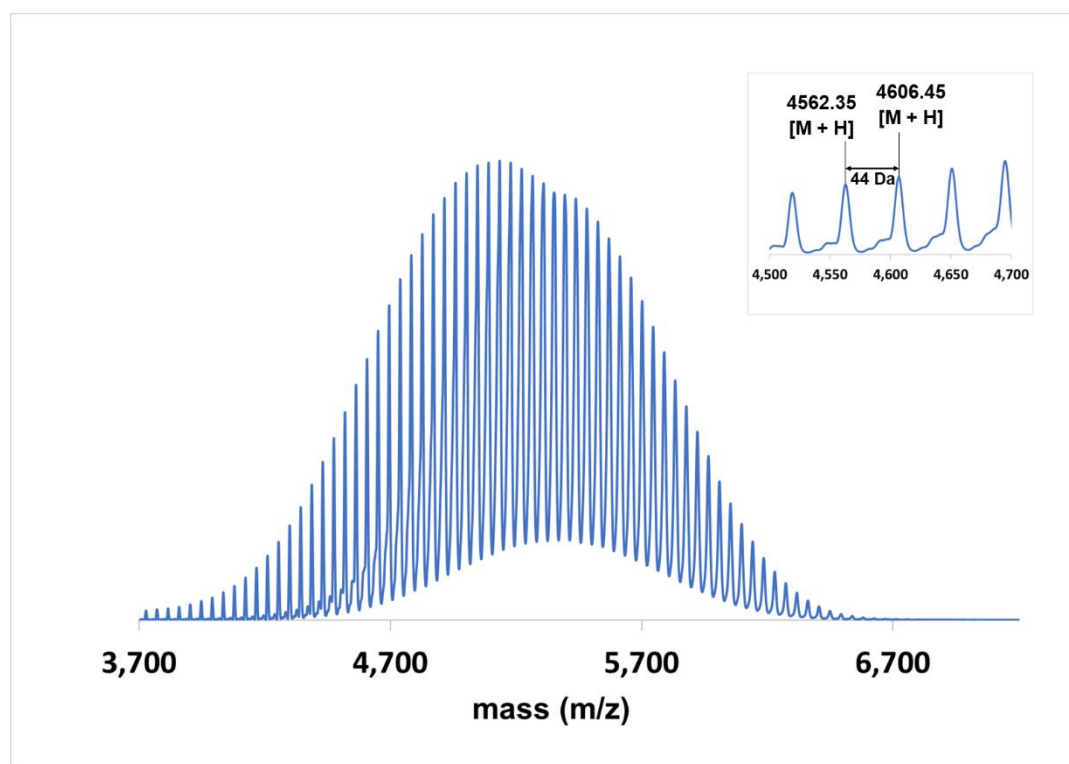


Figure S36. MALDI TOF mass spectrum of mPEG₁₁₄-FITC.

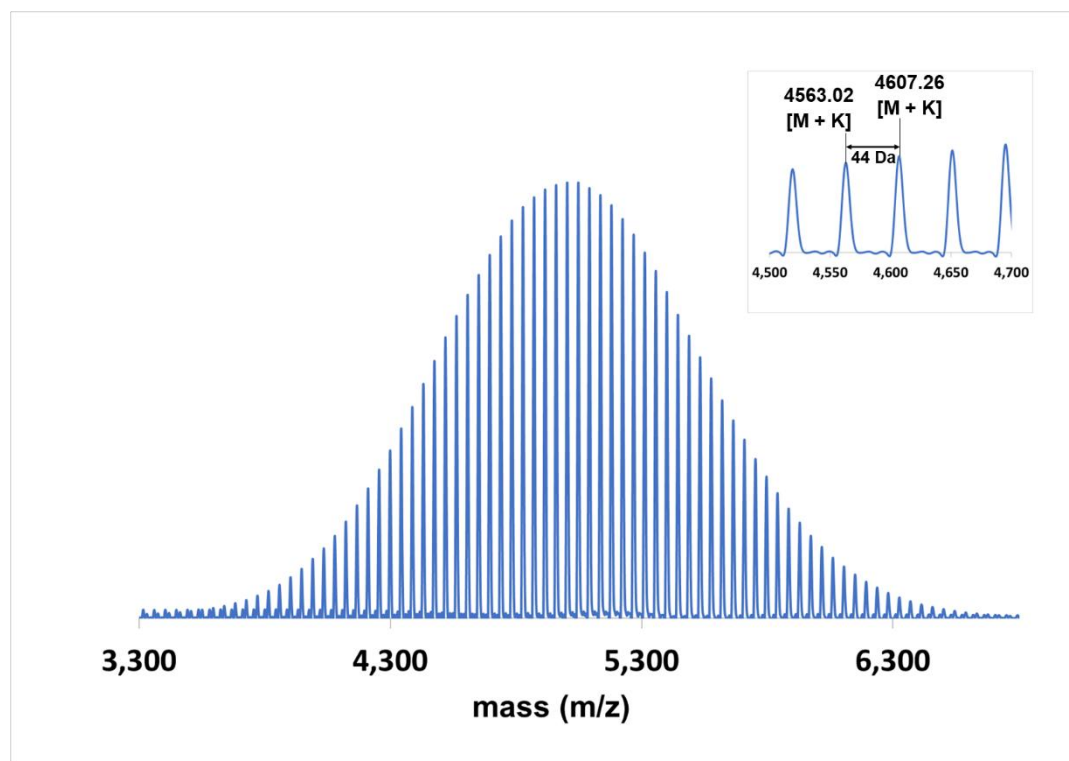


Figure S37. MALDI TOF mass spectrum of rPEG₉₈^{0.17}.

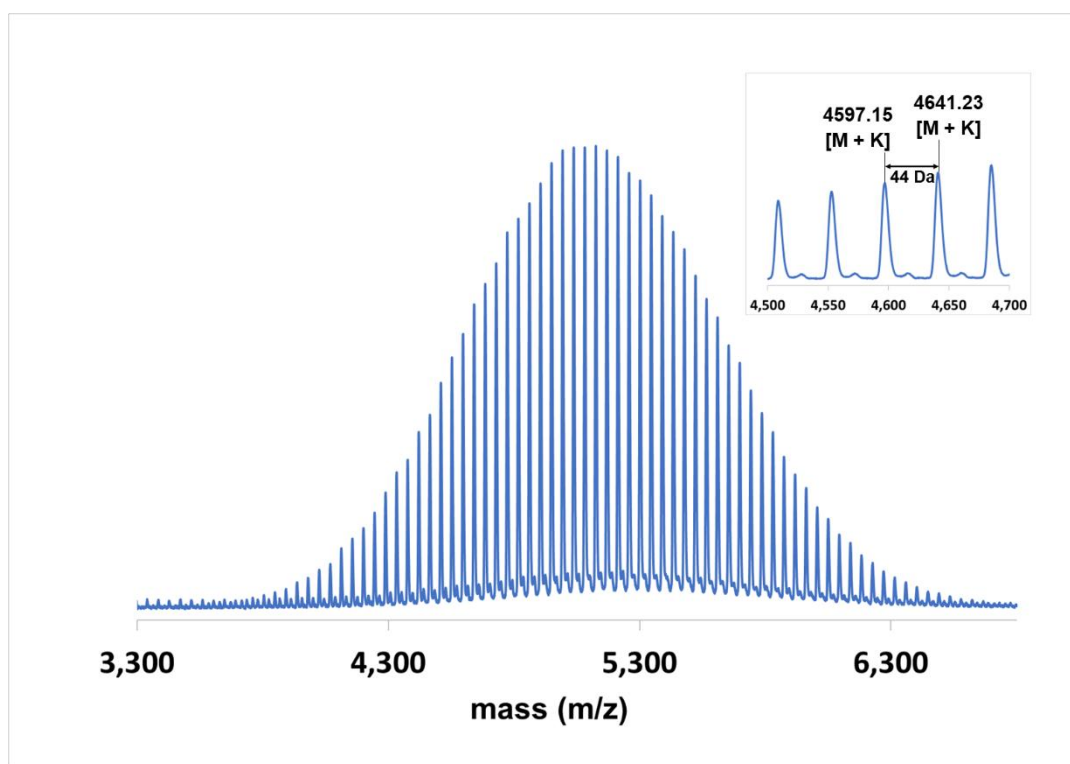


Figure S38. MALDI TOF mass spectrum of rPEG₉₈^{0.17}-mesylate.

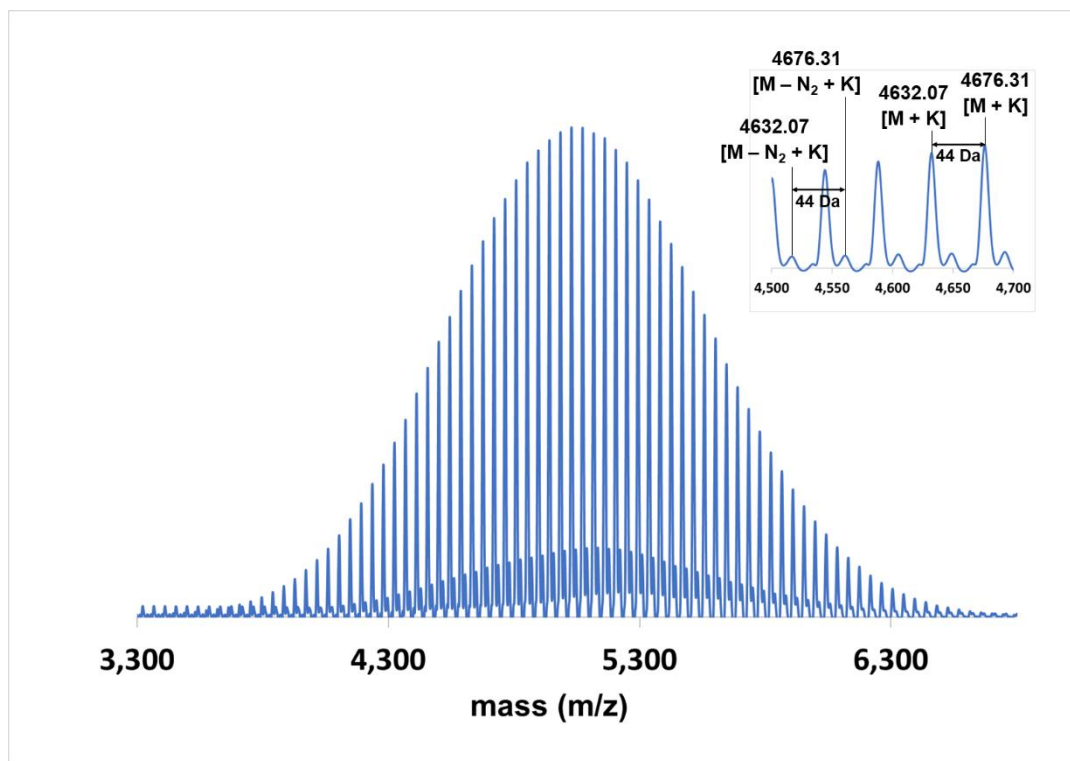


Figure S39. MALDI TOF mass spectrum of rPEG₉₈^{0.17}-azide.

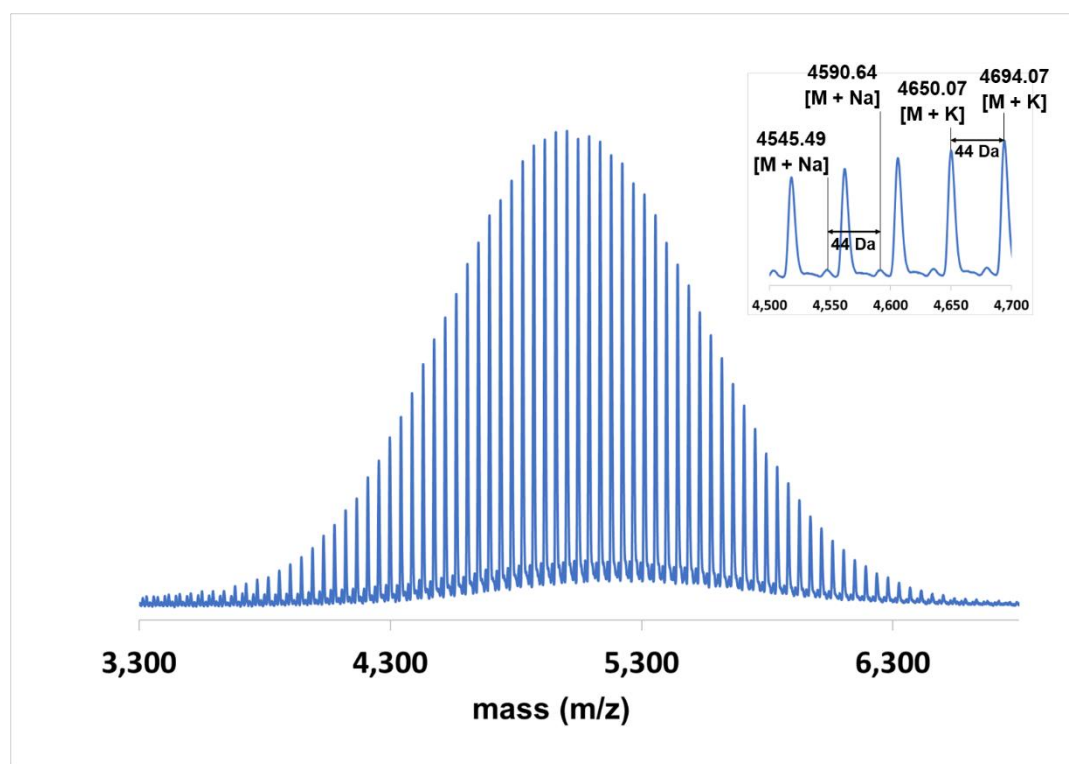


Figure S40. MALDI TOF mass spectrum of rPEG₉₈^{0.17}-amine.

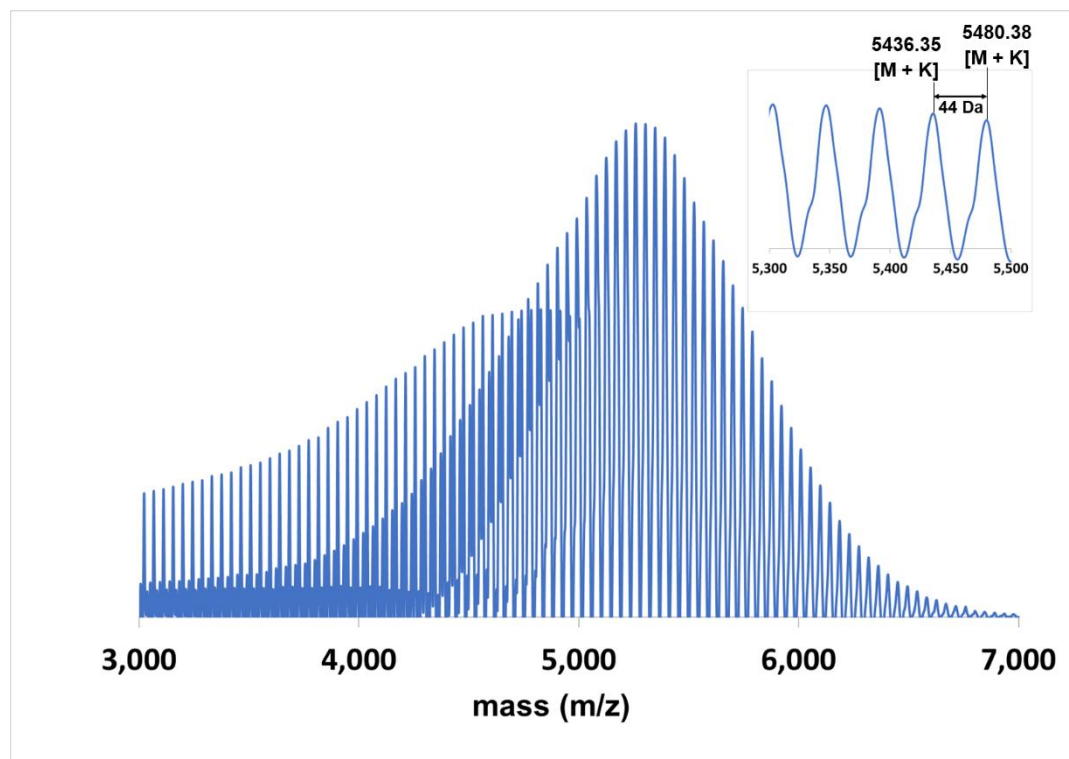


Figure S41. MALDI TOF mass spectrum of rPEG₉₈^{0.17}-FITC.

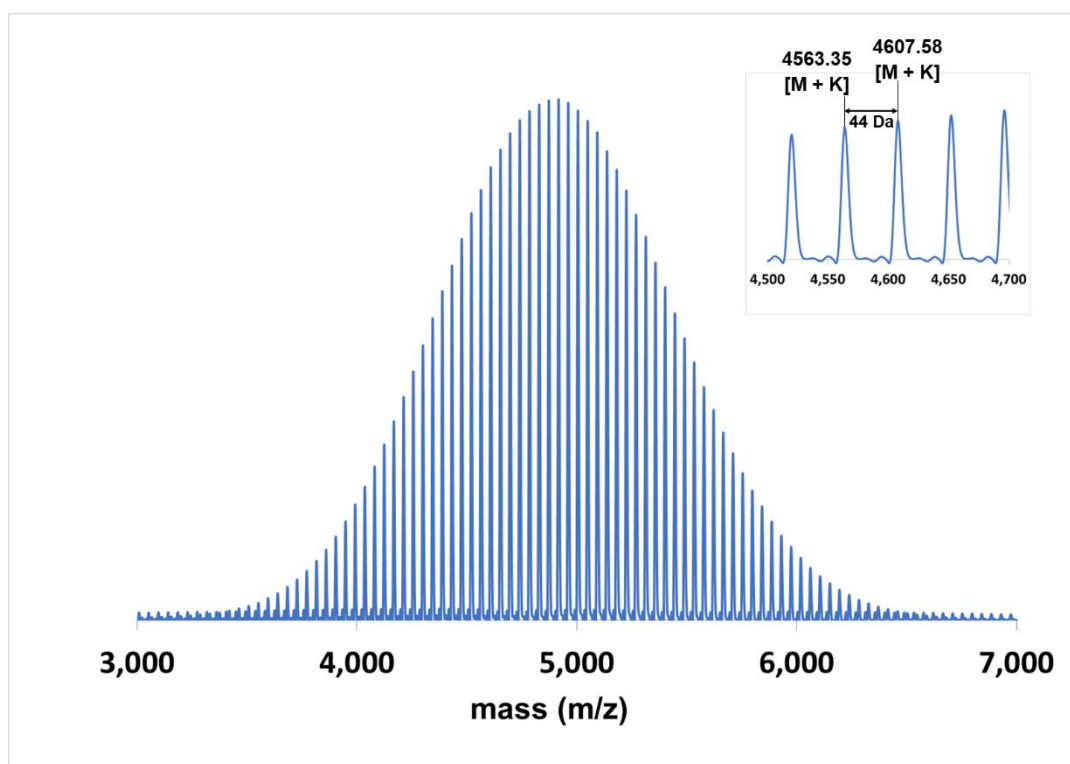


Figure S42. MALDI TOF mass spectrum of rPEG₈₉^{0.27}.

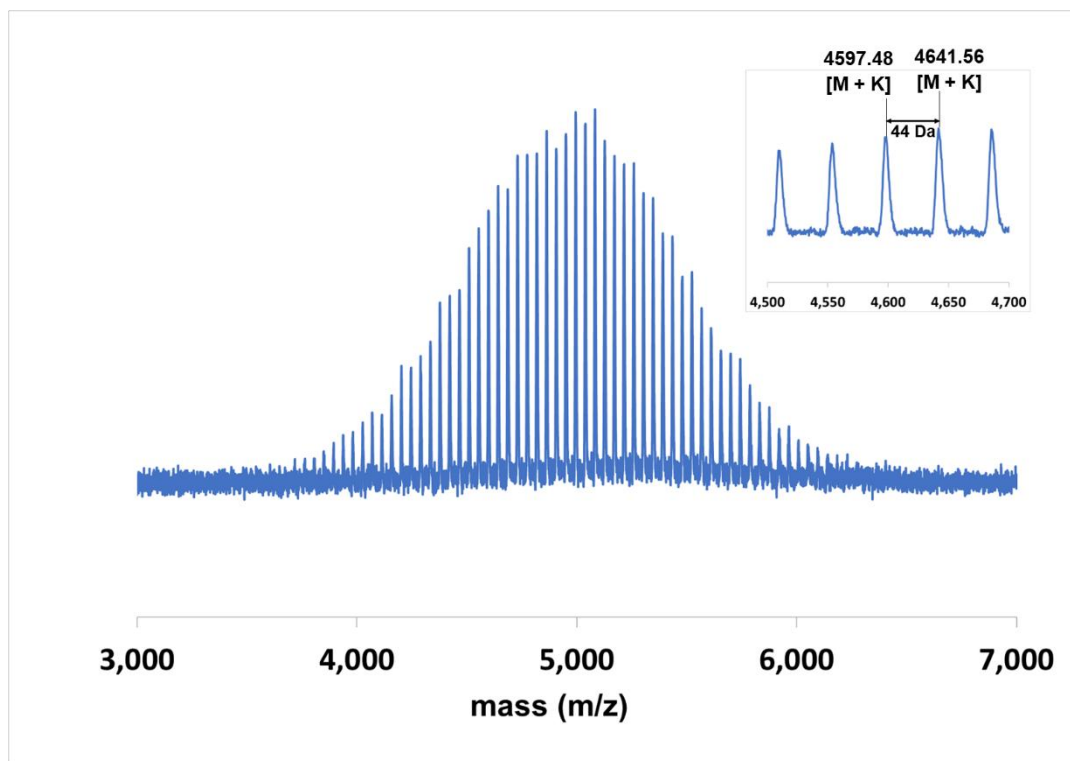


Figure S43. MALDI TOF mass spectrum of rPEG₈₉^{0.27}-mesylate.

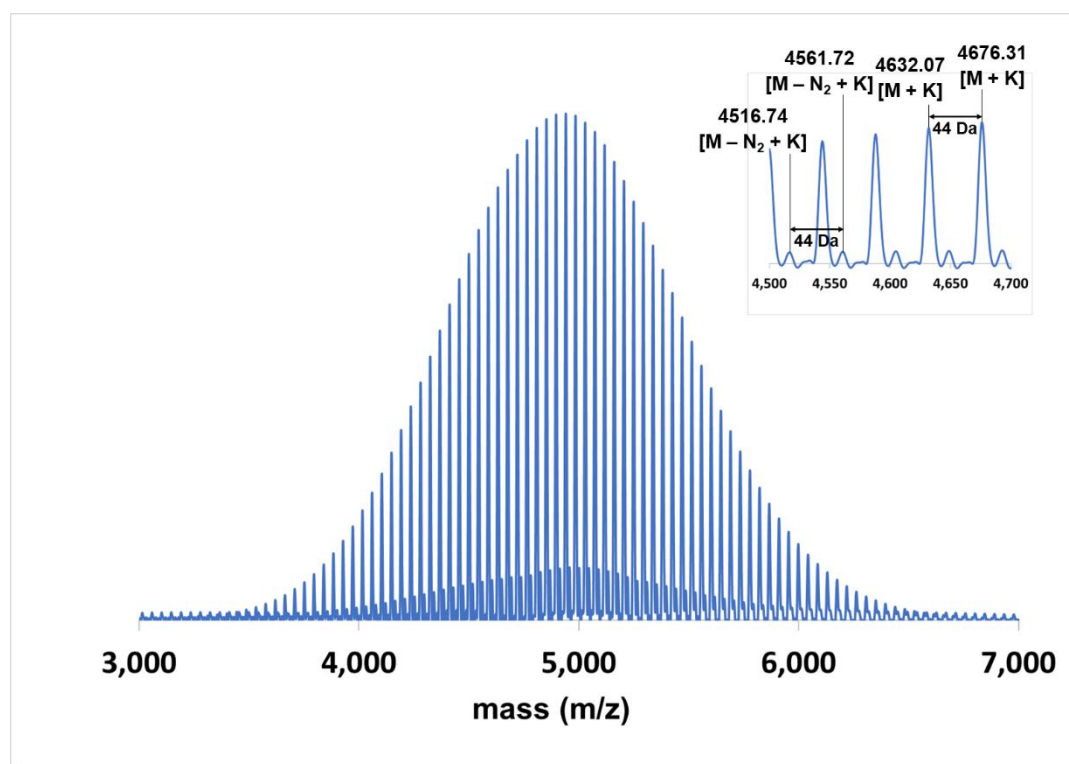


Figure S44. MALDI TOF mass spectrum of rPEG₈₉^{0.27}-azide.

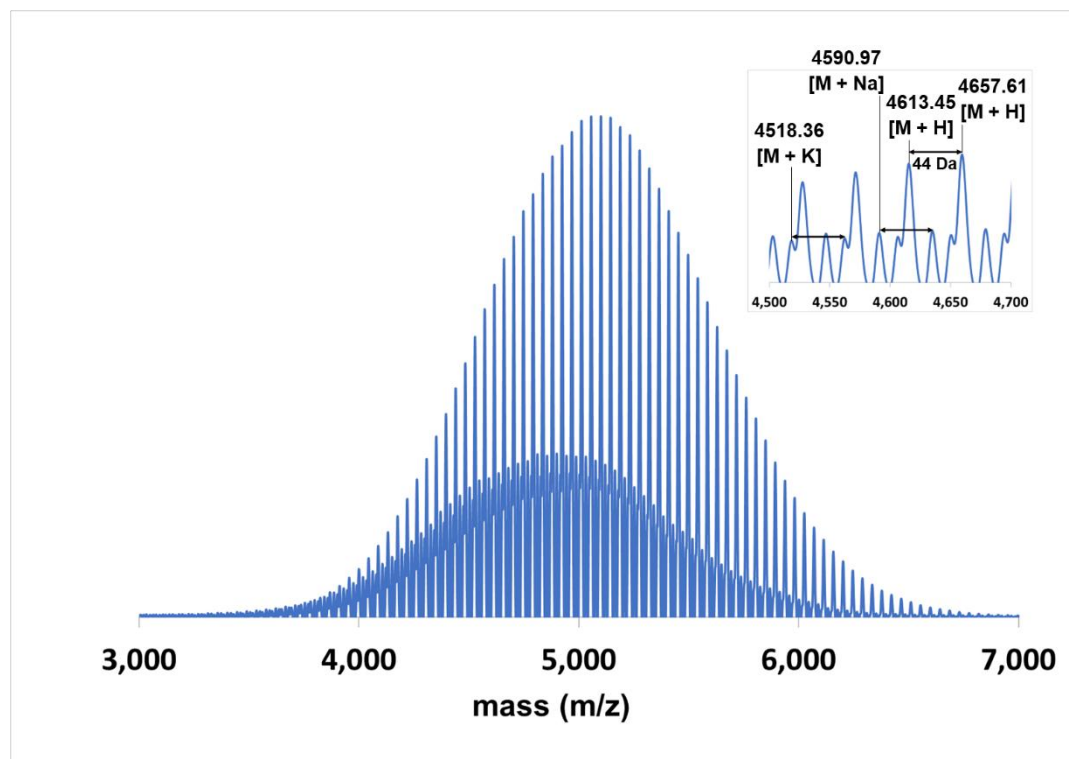


Figure S45. MALDI TOF mass spectrum of rPEG₈₉^{0.27}-amine.

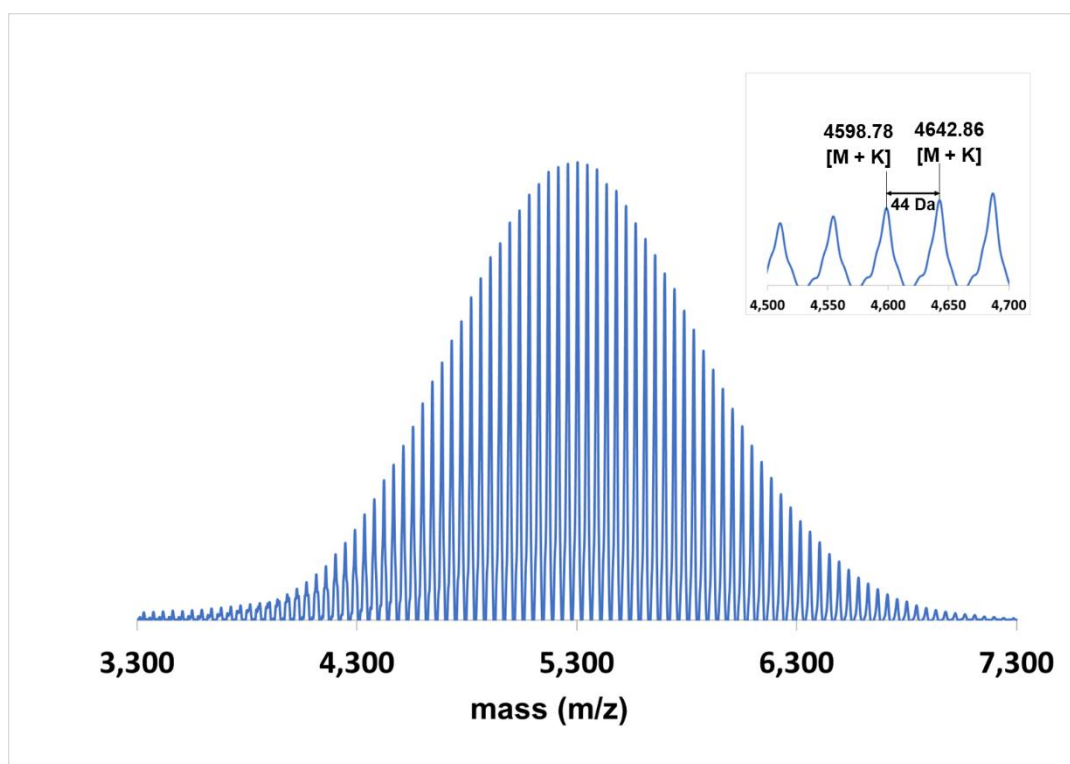


Figure S46. MALDI TOF mass spectrum of rPEG₈₉^{0.27}-FITC.

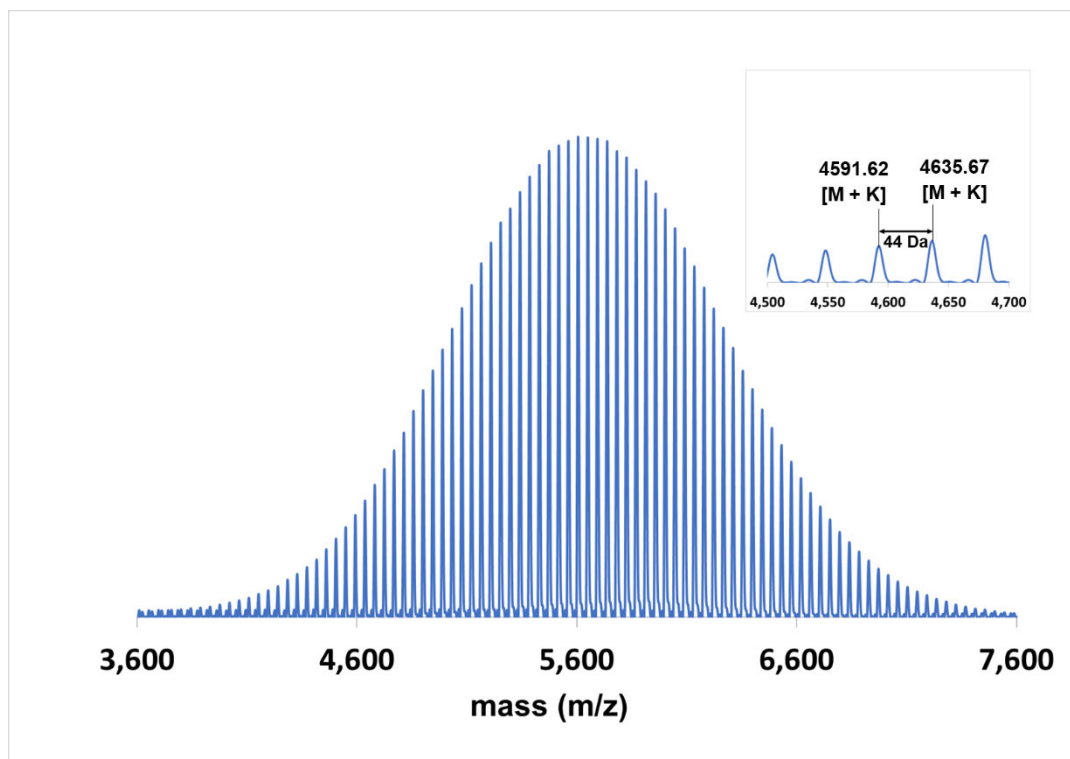


Figure S47. MALDI TOF mass spectrum of rPEG₉₁^{0.42}.

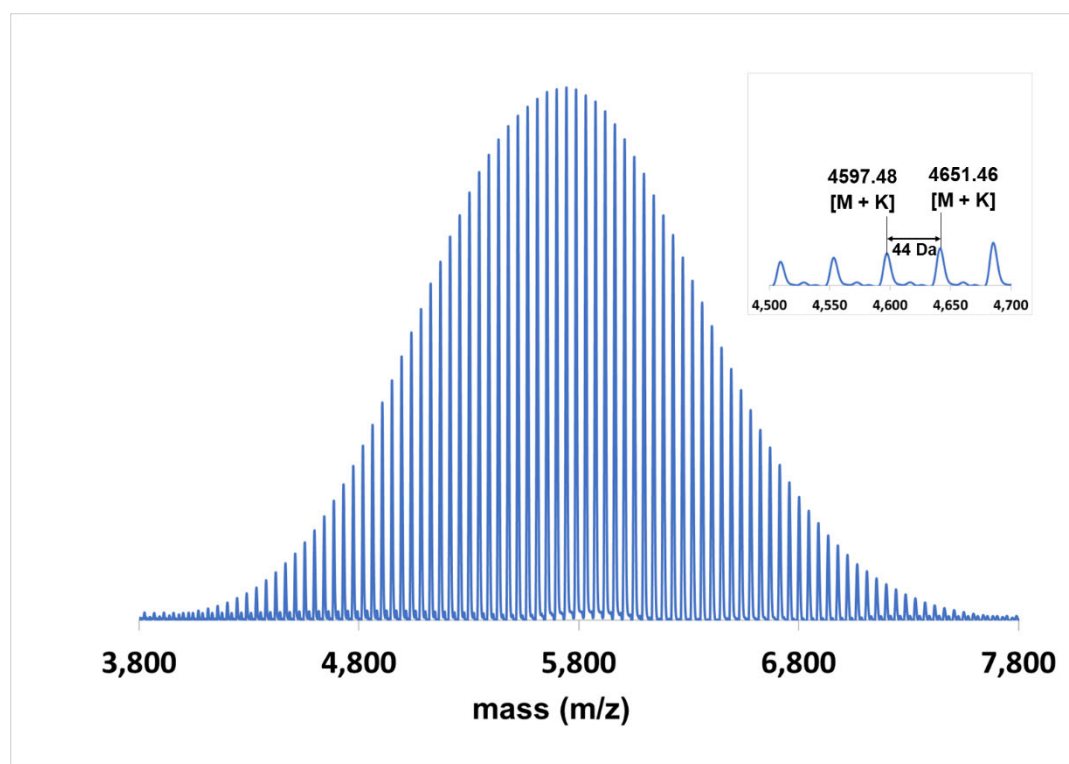


Figure S48. MALDI TOF mass spectrum of rPEG₉₁^{0.42}-mesylate.

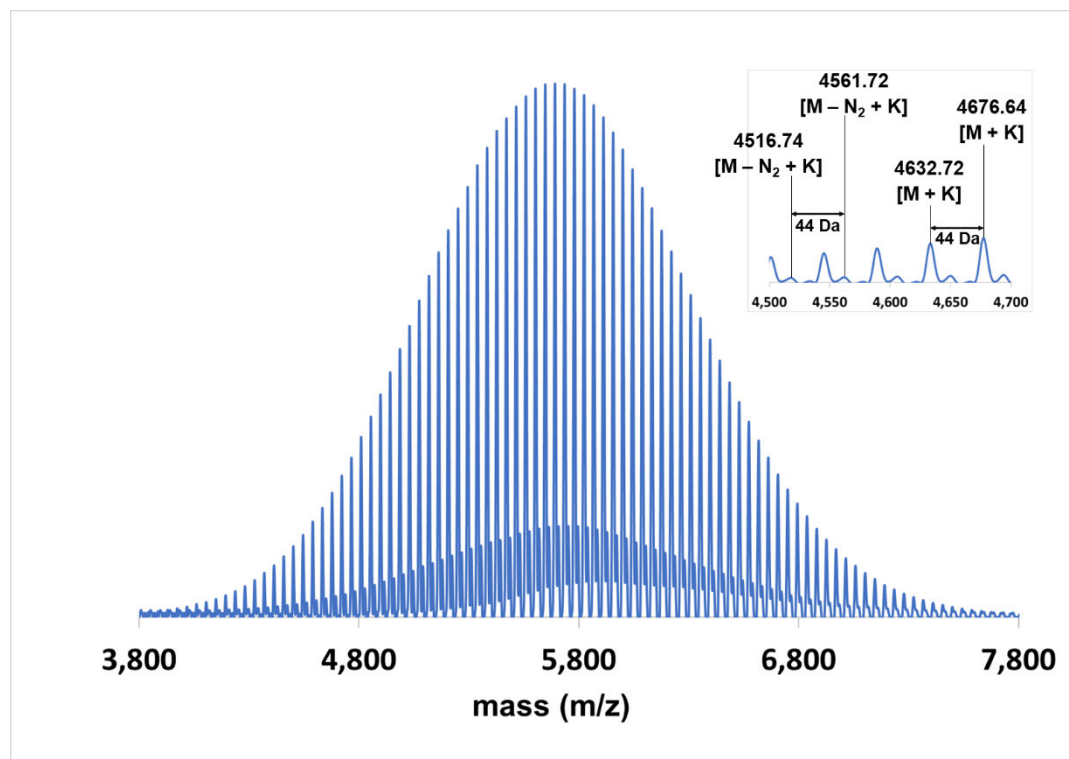


Figure S49. MALDI TOF mass spectrum of rPEG₉₁^{0.42}-azide.

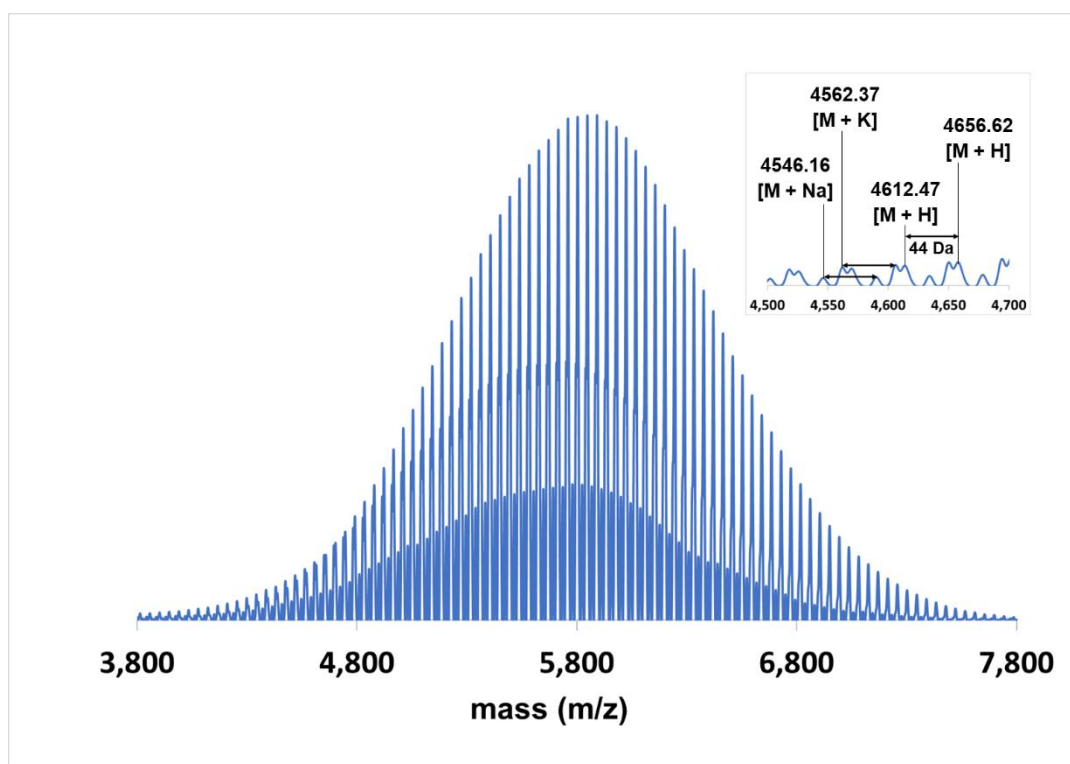


Figure S50. MALDI TOF mass spectrum of rPEG₉₁^{0.42}-amine.

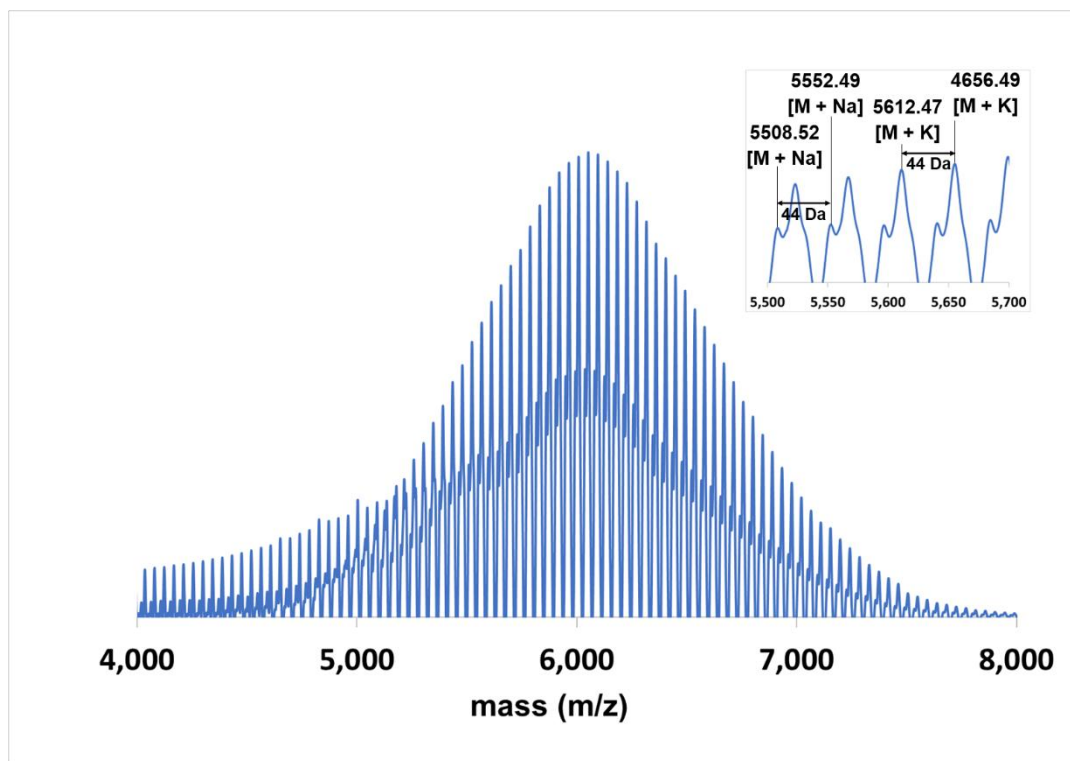


Figure S51. MALDI TOF mass spectrum of rPEG₉₁^{0.42}-FITC.

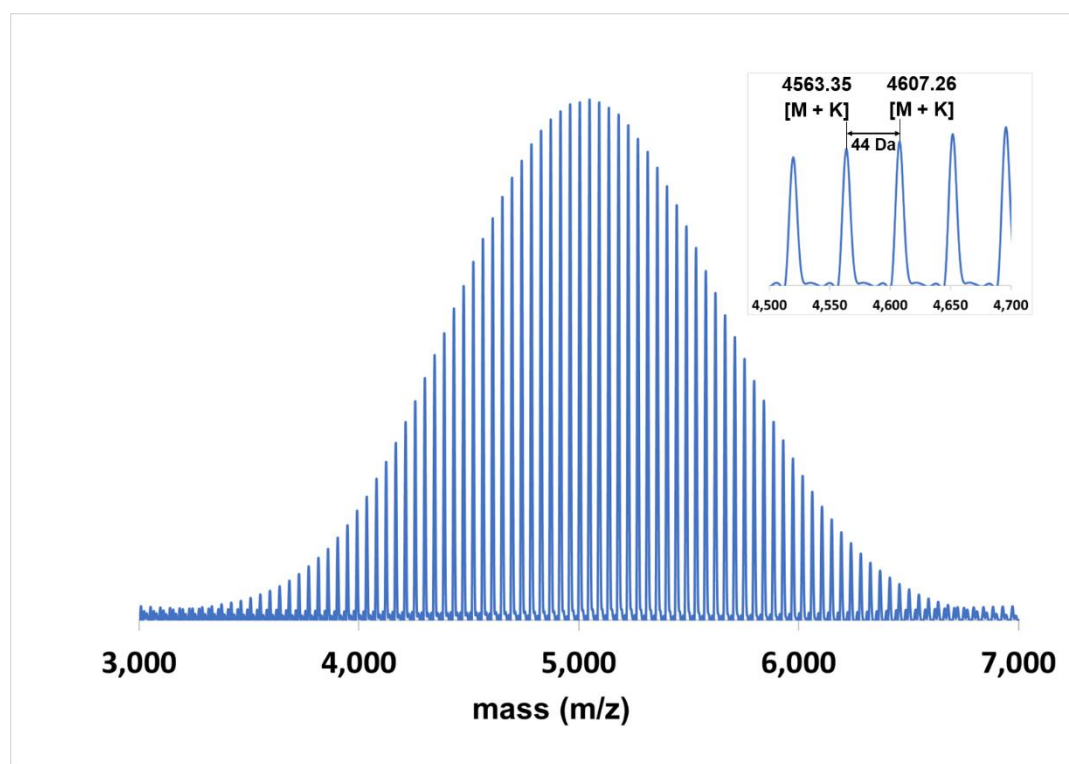


Figure S52. MALDI TOF mass spectrum of rPEG₇₆^{0.52}.

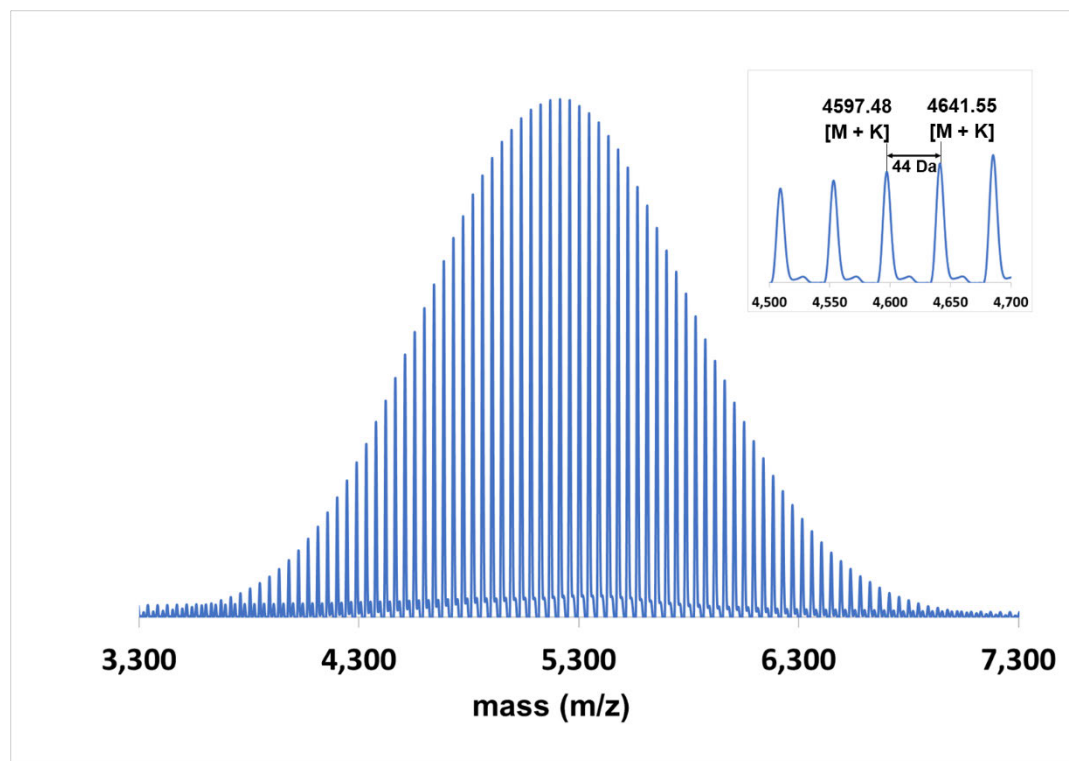


Figure S53. MALDI TOF mass spectrum of rPEG₇₆^{0.52}-mesylate.

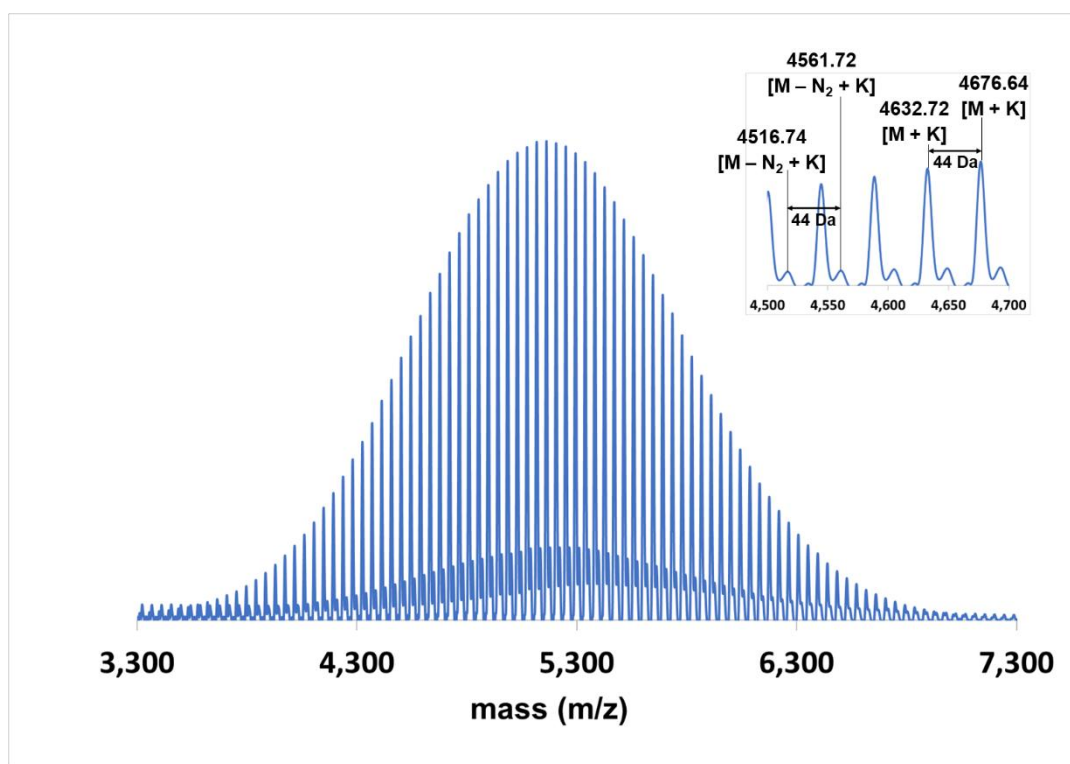


Figure S54. MALDI TOF mass spectrum of rPEG₇₆^{0.52}-azide.

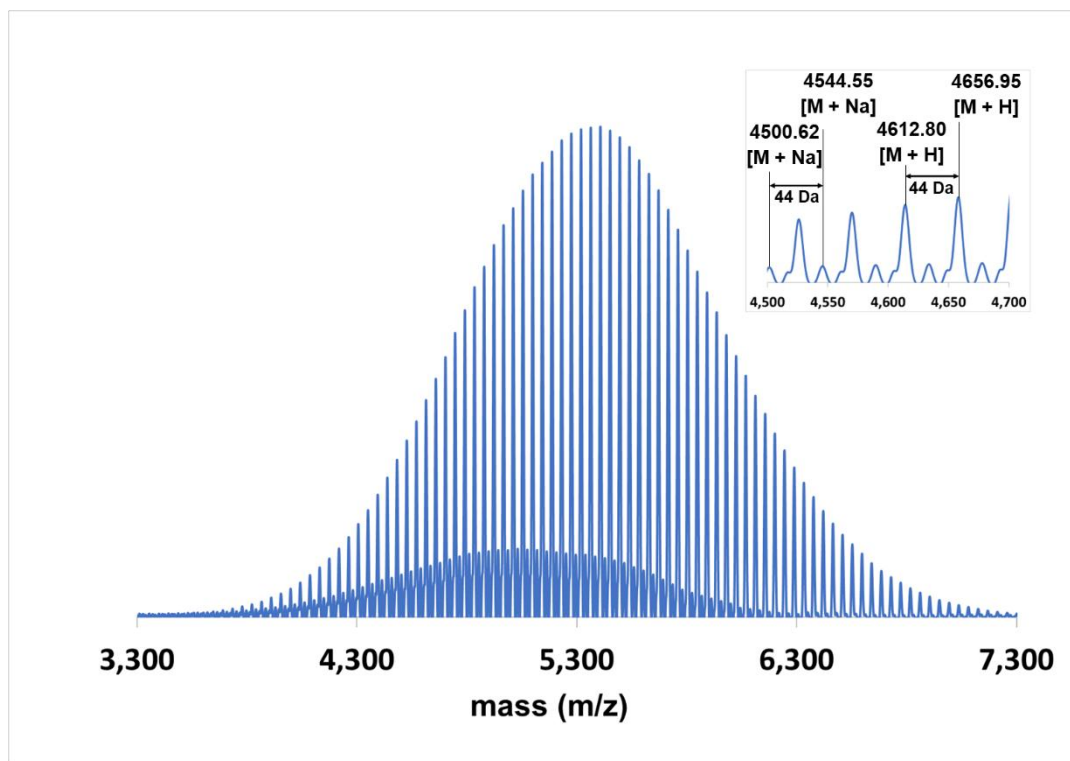


Figure S55. MALDI TOF mass spectrum of rPEG₇₆^{0.52}-amine.

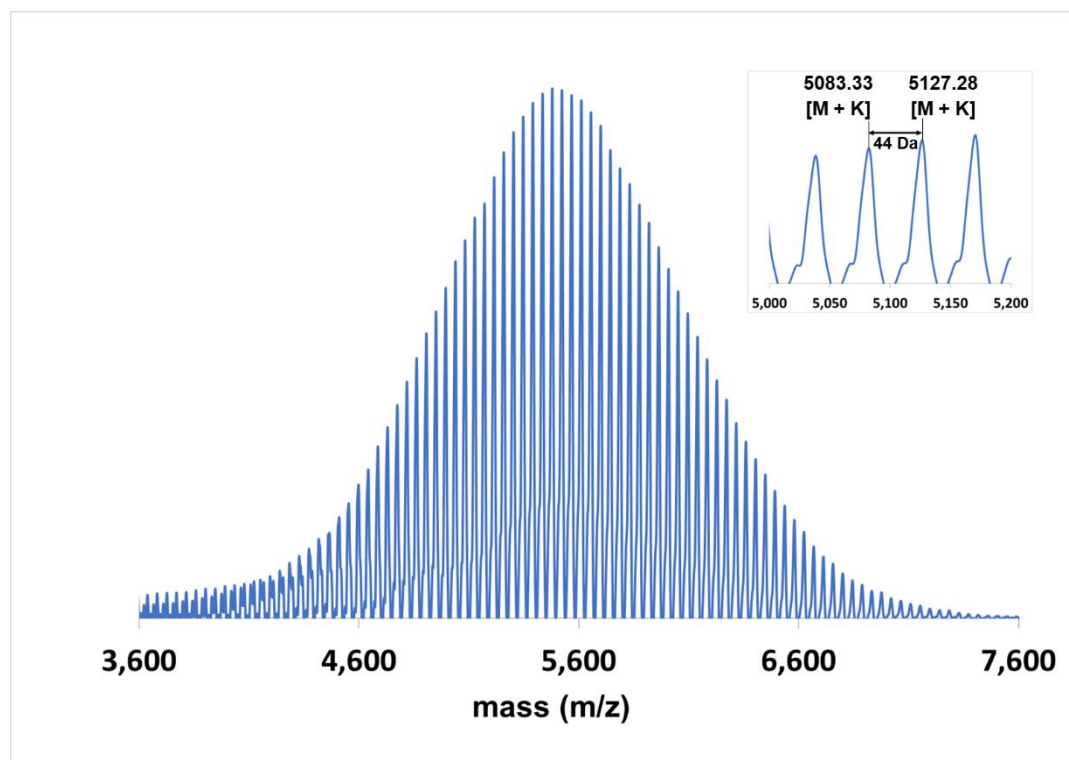


Figure S56. MALDI TOF mass spectrum of rPEG₇₆^{0.52}-FITC.

6. SEC curves

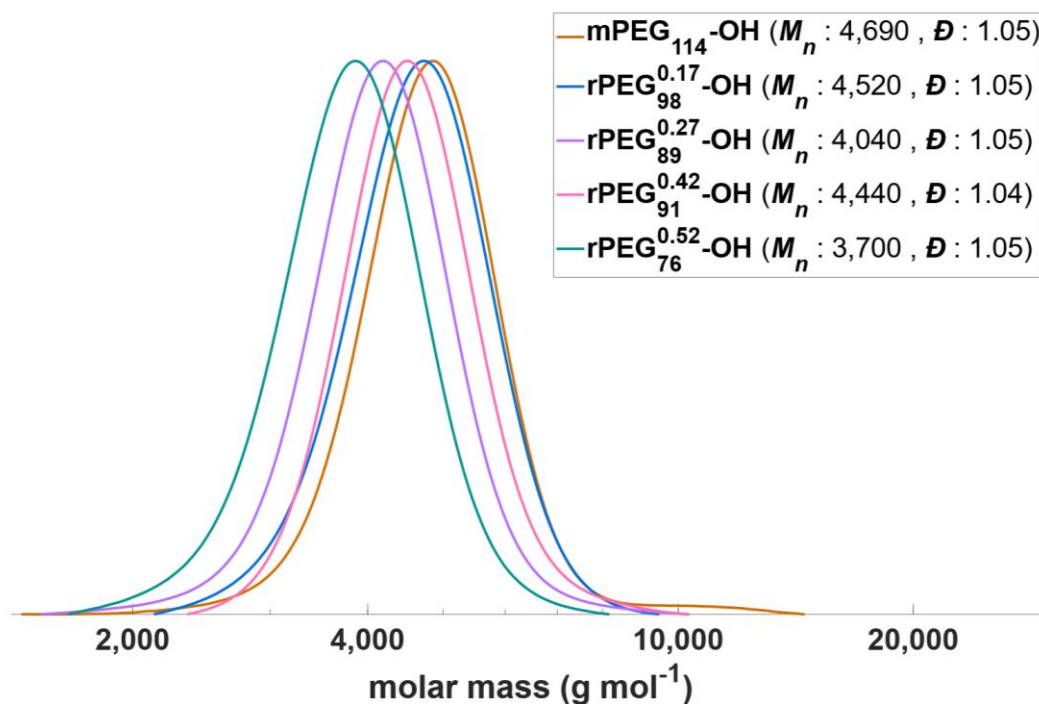


Figure S57. SEC curves of the unfunctionalized polymers (average molar mass M_n in g mol⁻¹ and dispersity D given; eluent: DMF, calibration: PEG, detector: RI).

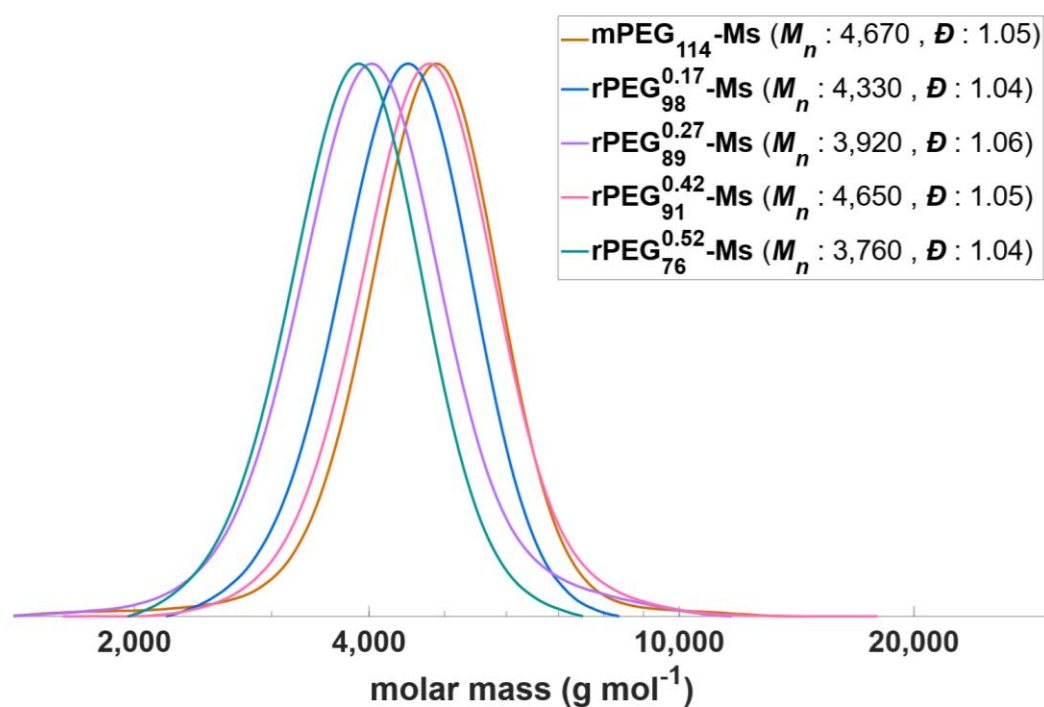


Figure S58. SEC curves of the mesylate-functionalized polymers (average molar mass M_n in g mol^{-1} and dispersity $\mathcal{D}$ given; eluent: DMF, calibration: PEG, detector: RI).

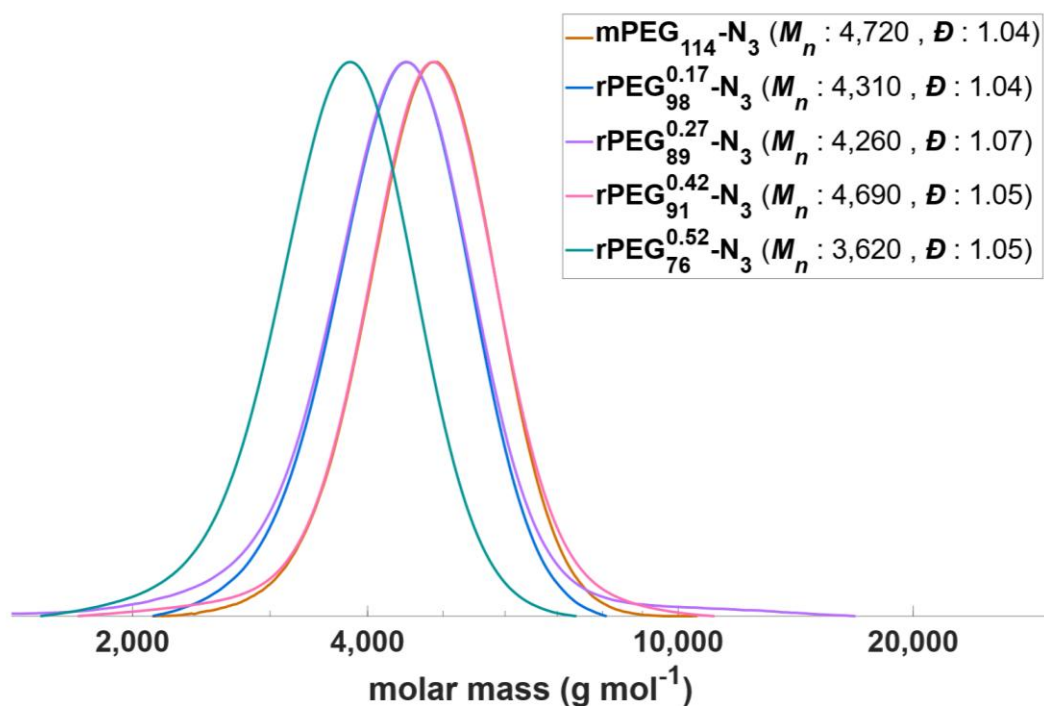


Figure S59. SEC curves of the azide-functionalized polymers (average molar mass M_n in g mol^{-1} and dispersity $\mathcal{D}$ given; eluent: DMF, calibration: PEG, detector: RI).

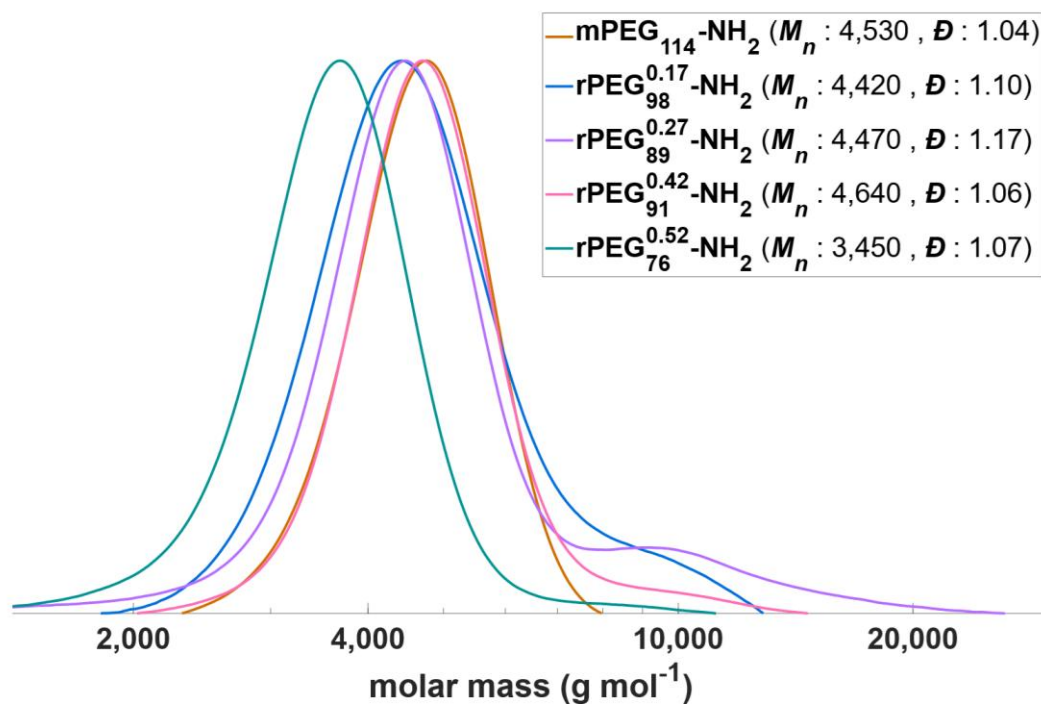


Figure S60. SEC curves of the amine-functionalized polymers (average molar mass M_n in g mol⁻¹ and dispersity D given; eluent: DMF, calibration: PEG, detector: RI).

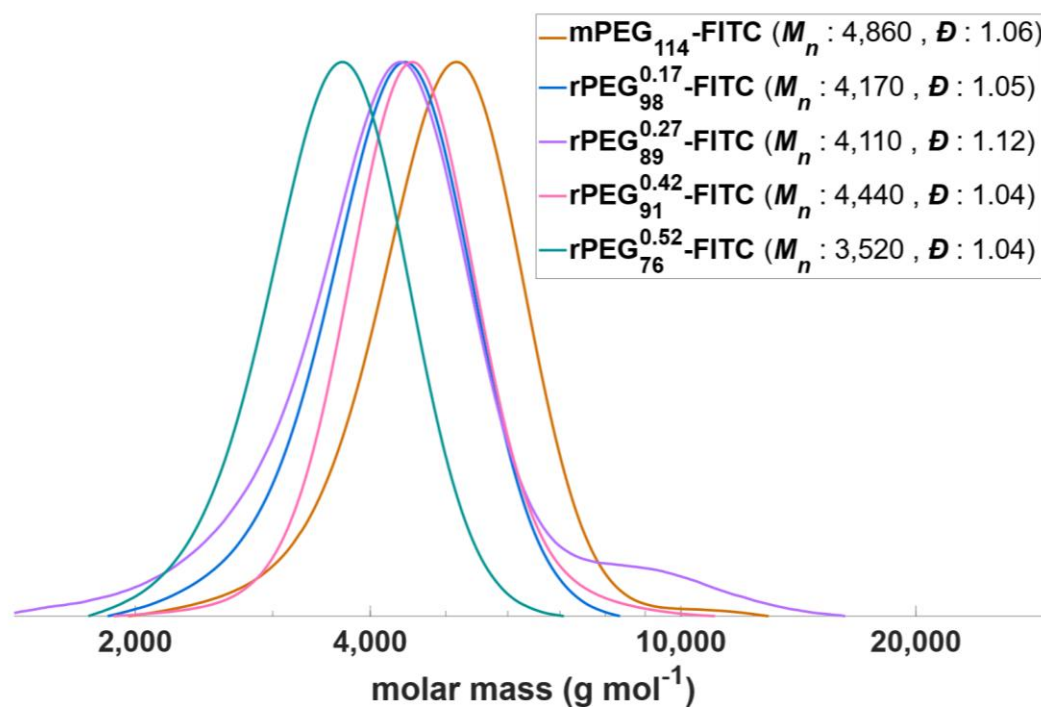


Figure S61. SEC curves of the FITC-functionalized polymers (average molar mass M_n in g mol⁻¹ and dispersity D given; eluent: DMF, calibration: PEG, detector: RI).

7. MST curves

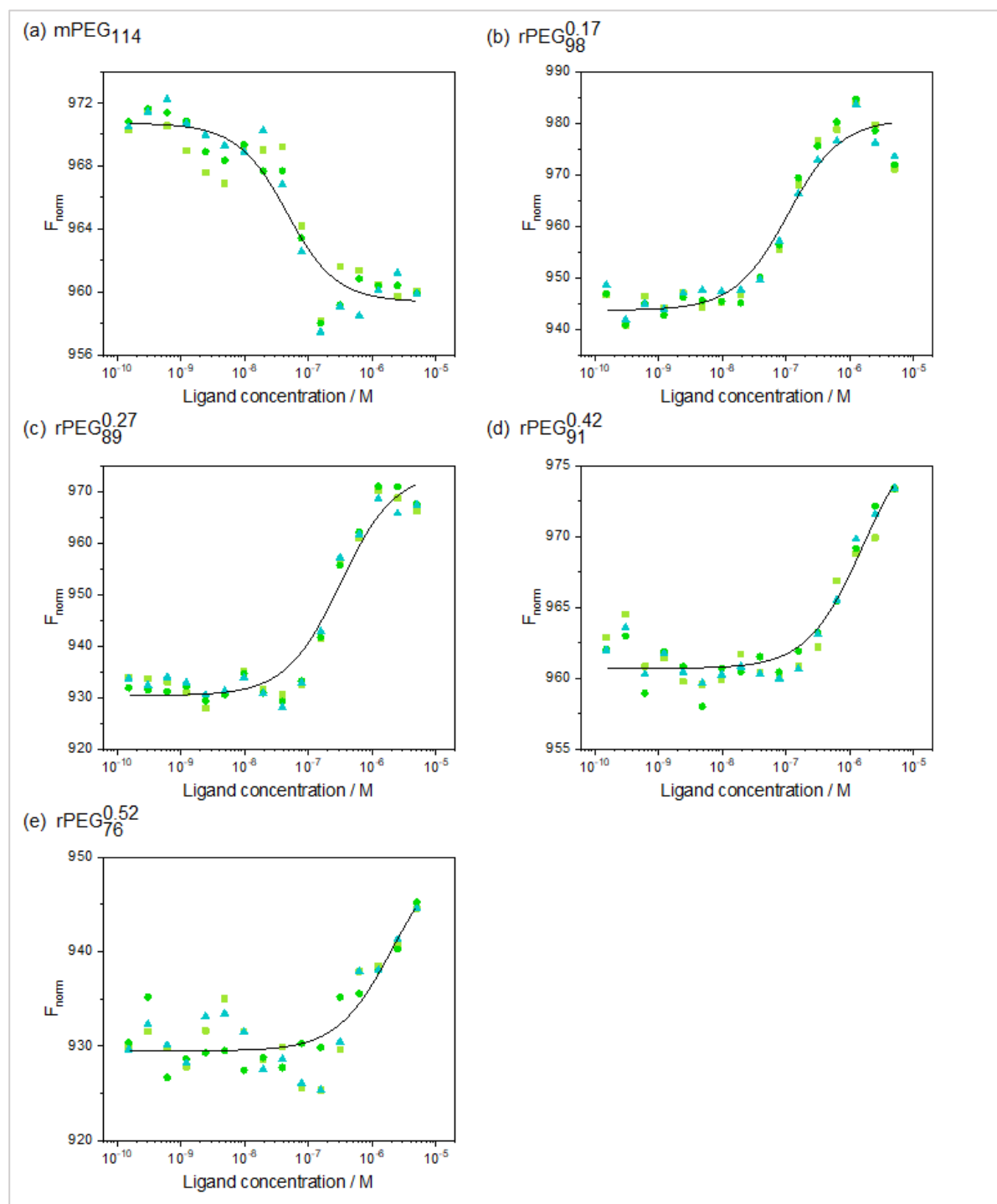


Figure S62. MST data displaying individual data points of technical triplicates (measurement of three capillaries).

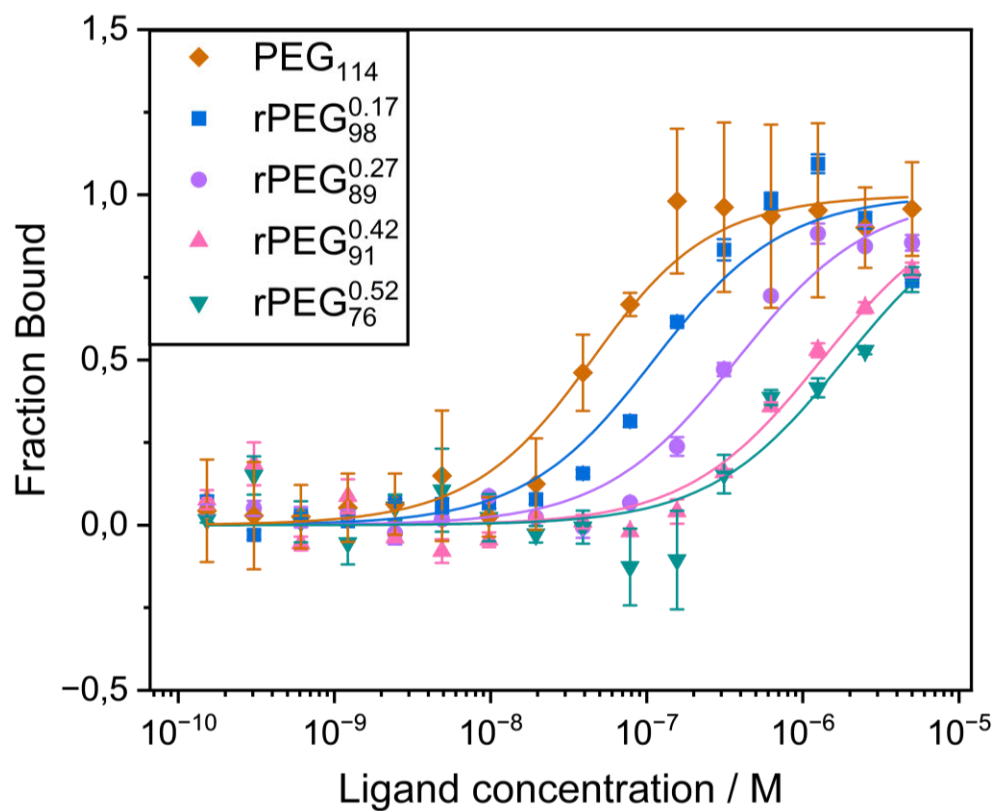


Figure S63. MST measurement results plotted as fraction bound depending on ligand (polymer) concentration (mean $\pm$ S.D., n = 3 technical replicates).

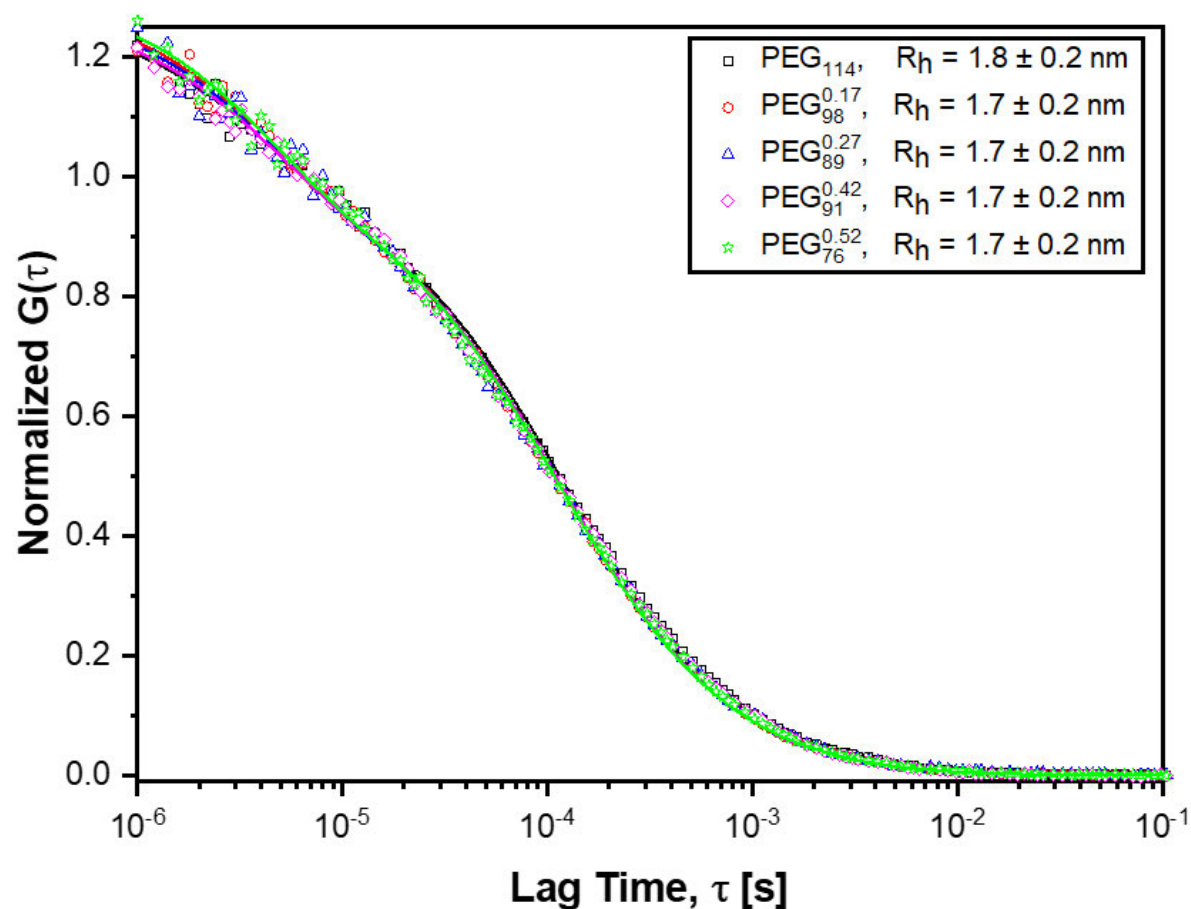


Figure S 64. Autocorrelation functions (ACFs) derived from FCS measurements of all polymers ($c = 20$ nM) in human citrate plasma, shown together with the corresponding fits (double-component diffusion models, where the small species refers to free dye and larger species refers to labeled polymer). The hydrodynamic radius is reported for the largest diffusing species in each sample. Error refers to the typical experimental error of $\pm 10\%$ for fluorescence correlation spectroscopy. Each FCS experiment included ten technical replicates per sample, acquired at 10-second intervals.

Supporting References

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- (2) Rigler, R.; Elson, E. *Fluorescence correlation spectroscopy: Theory and applications*; Springer Series in Chemical Physics, Vol. 65; Springer, **2001**.
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Chapter 4

Effect of surface functionalization with lipidated random poly(ethylene glycol – glycidyl methyl ether) copolymers (Lipid-rPEGs) on the formation of the protein corona of silica nanocapsules

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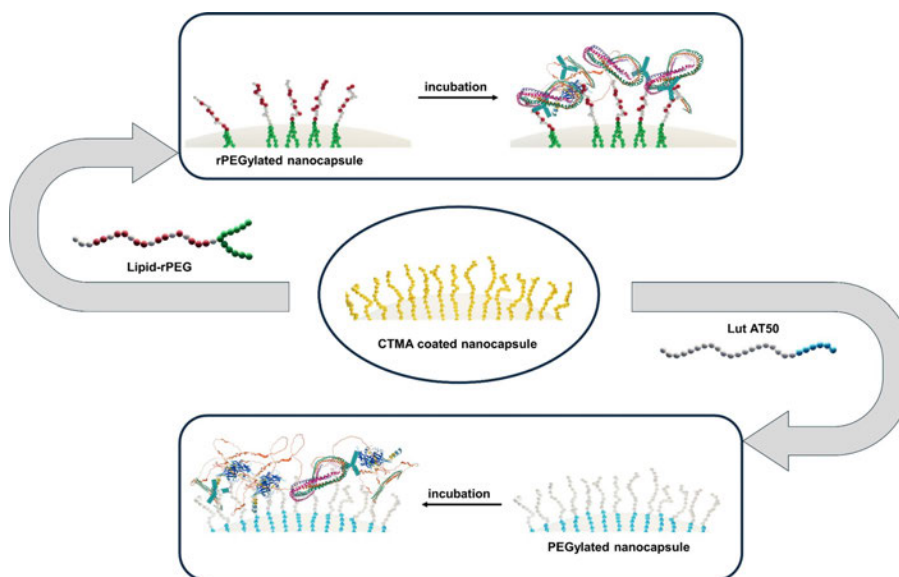
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Graphical Abstract



ABSTRACT

The protein corona of nanomaterials, which is formed when exogenous substances encounter proteins in the human blood stream, is an essential factor in how these materials interact with the immune system. The composition of the corona depends on parameters like hydrophilicity, surface charge or size of the nanomaterials. Past investigations have shown that PEGylation – the functionalization of a nanomaterial surface with poly(ethylene glycol) (PEG) – is able to reduce the adsorption of proteins by steric repulsion through its hydration shell and therefore prolong circulation time in the blood stream (stealth effect). To counteract the issue of rising prevalence of anti-PEG antibodies in the general population however, we have developed structural isomers of PEG with randomly distributed side chains in the polyether backbone (rPEGs) to preserve the biocompatibility and hydrophilicity of PEG while reducing the binding affinity to antibodies through steric hindrance. In this work we investigated the influence of rPEGs of varying amounts of side chains on the formation and composition of the protein corona of silica nanocapsules. Lipid-functionalized rPEGs were successfully synthesized under highly controlled conditions and utilized for surface coating of the nanocapsules. Protein corona composition was determined by Pierce assay, ELISA and LC-MS after incubation with human citrate plasma and indicated a large contribution of different lipid anchors on the formation of the corona as well as varying levels of opsonins and dysopsonins for different compositions of the polyether chains. The exact quantity to which these properties contribute to the changes in protein adsorption could not be determined so far. Therefore, further investigations will be necessary to assess the parameters dictating the protein corona formation for these rPEGylated nanomaterials.

Introduction

Nanomaterials have been a staple in biomedical research for well over two decades.¹ The potential to encapsulate active pharmaceutical ingredients (APIs) and deliver them to targeted areas of the body increases their effectiveness^{2–4}, reduces side effects^{4,5} and shields them against adverse immune reactions.^{6,7} Additionally, through the process of PEGylation^{8,9}, in which the surface of nanocarriers or APIs themselves are functionalized with poly(ethylene glycol) (PEG), these components can be “hidden” from the immune system by reducing their unspecific interaction with proteins in the blood. This phenomenon was originally discovered by Davis and Abuchowski in 1977¹⁰ and is referred to as the “stealth effect” of PEG.^{6,11} Since then, it has been utilized to prolong circulation times of APIs in the bloodstream manifold.¹²

However, the existence of anti-PEG antibodies (APAs) is raising concerns about the diminishing effectiveness of PEGylation^{13,14}. While at the time of their original discovery in 1983 by Richter and Åkerblom¹⁵ anti-PEG antibodies were only estimated to be prevalent in 0.2 % of the general population, later studies reported increasing numbers up to 72 %.^{16–18} A recent study conducted in the German general population found anti-PEG IgG or IgM prevalent in 83 % of serum samples.¹⁹ Adverse reactions can range from accelerated blood clearance²⁰, in which case the antibody-binding causes faster excretion of PEGylated APIs from the bloodstream and effectively negates the stealth effect, to complement activation-related pseudoallergy (CARPA).²¹ In rare cases of hypersensitivity, even life-threatening pseudo anaphylactic shocks have been reported.²²

To address this issue, several alternatives to PEG are currently subjects of intensive research as new stealth polymers.^{23,24} Among these polymers are poly(oxazolines)²⁵, poly(sarcosine)²⁶ and poly(vinyl pyrrolidone)²⁷, which share the low toxicity and high water solubility of PEG and therefore could be safely applied in pharmacological research and medical treatments. Although progress with these alternatives has been made, the formation of antibodies was also observed in immune reactions to homopolymers other than PEG²⁸, which raises the question if regularity in the polymer backbone presents a general issue regarding potential immunogenicity.

In a recently published study by Lai *et al*²⁹ the crystal-structures of two types of APAs were determined and showed that recognition of PEG is dominated by van der Waals interaction with the Fab paratope of the backbone-specific antibody, which requires a regular segment of 16

ethylene oxide (EO) units. Based on these findings, we aimed to design a polyether system that does not have a regular backbone structure while still exhibiting PEG-like biocompatibility. For this, we developed the ideal statistical copolymerization of EO and glycidyl methyl ether (GME) to receive randomized PEG (rPEG). Since GME is isomeric to precisely 2 molecules of EO, rPEGs are formally also structural isomers of PEG. They possess short, randomly distributed side chains along the backbone which are hypothesized to reduce the immune recognition of these copolymers compared to PEG since regularity in the PEG chain plays a crucial role in polymer-antibody binding. While low *in vitro* toxicity³⁰ and diminished antibody-binding³¹ have already been shown for the free polymers in our previous works (see **Chapter 3** and **Appendix Chapter 1** of this dissertation), detailed physicochemical properties in the blood have not been determined so far. To assess these properties, investigation of the protein corona of rPEGylated nanocarriers is imperative.

The protein corona is formed immediately after contact of nanomaterials with blood or other body fluids by adsorption of proteins on the particle surface.³² Critical properties like hydrodynamic size and aggregation behavior are determined by the protein corona³³ and therefore define the efficiency of the nanocarriers. The rate of blood clearance as well as the interaction between nanocarriers and cells is strongly affected by the proteins covering their surface, since these proteins are what is predominantly perceived by the cells.^{33–35} While opsonins like complement factor C3b enable uptake into monocytes³⁶ and therefore decrease retention time, dysopsonins like CD47 are able to inhibit uptake.³⁷ Factors like the surface structure of the nanocarrier, its electrostatic charge and its hydrophilicity strongly influence the formation of the protein corona. In regard to hydrophilicity, Mattoussi *et al*³⁸ showed that hydrophilic polymers like PEG severely reduced unspecific protein adsorption, while Ma *et al*³⁹ demonstrated that graphene surfaces functionalized with hydroxyl-groups reduced interaction with opsonins like human serum albumin (HSA) and Immunoglobulin E (IgE). Since rPEG possesses a similar hydrophilicity to PEG, the aim of this work is a detailed investigation of the composition of the protein corona formed around rPEGylated nanocarriers in the blood to gain insight into the effect of rPEG on the protein corona and hence on cells and the immune system.

Results and Discussion

Monomer synthesis

High purity GME was obtained by a 2-step synthesis. Hereby, epichlorohydrin was brought to reaction with methanol under the opening of the epoxide ring to receive 1-chloro-3-methoxypropan-2-ol. Subsequently, an intramolecular nucleophilic substitution under basic conditions was performed to receive GME. Analytics showed no impurities of epichlorohydrin in the product which are often found in commercially available GME. Further details about synthesis and analytics of GME can be found in the **Supporting Information**.

Initiator synthesis

Synthesis of the initiator was performed over three steps starting from benzyl glycidyl ether. After opening the epoxide ring via an acidic reaction in water, the resulting diol was brought to reaction with 1-bromooctadecane in a Williamson ether synthesis. Finally, the benzyl group was removed catalytically using hydrogen gas and a palladium catalyst to receive 1,2-*O*-dioctadecyl-*rac*-glycerol, which was used as initiator for the AROP. A detailed description of the initiator synthesis and the polymerization procedure are also given in the **Supporting Information**.

In our previous work³⁰, copolymerizations were performed in dimethyl sulfoxide (DMSO) as a solvent using a short-chained alcohol as an initiator. *In situ* kinetics of the copolymerizations using ¹H NMR were performed and showed ideal random behavior with reactivity ratios of $r_{EO}^{DMSO} \approx r_{GME}^{DMSO} \approx 1$ in DMSO while slight deviations from unity were observed in toluene and anisole. However, since 1,2-*O*-dioctadecyl-*rac*-glycerol shows low solubility in polar solvents like DMSO, a non-polar alternative solvent had to be considered. To still achieve nearly random incorporation of the monomers, a mixture of DMSO and toluene ($r_{EO}^{toluene} = 0.63$; $r_{GME}^{toluene} = 1.60$) was utilized for the copolymerization. The ratio of DMSO to toluene was intended to be maximized so that near ideal conditions could be applied. For this, solubility of the initiator was tested in different mixtures of DMSO and toluene at constant concentration of 1,2-*O*-dioctadecyl-*rac*-glycerol at room temperature and under heating. The results of these experiments are summarized in **Table 1**.

Table 1. Determination of solubility of 1,2-O-diocetadecyl-*rac*-glycerol in various mixtures of DMSO and toluene (Y = soluble, N = not soluble, p = partially dissolved, nt = not tested; optimal ratio for polymerization is highlighted in green).

volume fraction	total solvent volume	amount of initiator	conc.	dissolved	dissolved	dissolved	dissolved
DMSO / toluene	/ mL	/ mg	/ mmol mL ⁻¹	immediately	after 1h	after 2h	at heating ^a
8 / 1	0.500	5.9	0.020	N	N	N	N
4 / 1	0.500	6.0	0.020	N	N	N	N
2 / 1	0.500	5.7	0.019	N	N	N	N
1 / 1	0.500	5.8	0.019	N	p	p	Y ^b
1 / 2	0.500	5.8	0.019	N	p	p	Y
1 / 4	0.500	5.7	0.019	Y	Y	Y	nt
1 / 6	0.500	5.8	0.019	Y	Y	Y	nt
1 / 8	0.500	5.7	0.019	Y	Y	Y	nt
8 / 1	1.000	5.9	0.010	N	N	nt	N
4 / 1	1.000	6.0	0.010	N	N	nt	N
2 / 1	1.000	5.7	0.010	N	N	nt	Y ^b
1 / 1	1.000	5.8	0.010	N	N	nt	Y ^b

^a If not dissolved at room temperature after 2h on an orbital shaker, samples were heated to 60 °C

^b Sample dissolves in mixture while heating but reprecipitates after cooling to room temperature

Samples of the initiator were dissolved in the solvent mixtures and kept on an orbital shaker for 2h at room temperature while dissolution was monitored. If the samples were not dissolved after 2h they were removed from the shaker and cautiously heated to 60 °C. Initially, a concentration of approximately 0.020 mmol mL⁻¹ was chosen for all samples as this initiator concentration was targeted for the copolymerization. Some samples that did not dissolve at this concentration were tested again at 0.010 mmol mL⁻¹. The results showed that the sample dissolved in a mixture of DMSO-toluene of 1:2 (volume fraction) at approximately 45 °C and remained in solution after cooling the mixture to room temperature. Consequently, this solvent ratio was chosen for the subsequent AROP procedure.

Polymer synthesis

To analyze rPEGylated nanocarriers with focus on the protein corona, several copolymer lipids with varying molar ratios of EO and GME were designed. Analogous to a previously developed procedure⁴⁰, the lipid functionality was coated on the surface of core-shell silica nanocapsules to create a modified surface containing an rPEG layer that is able to interact with proteins and form a corona. Polymer synthesis was performed using anionic ring-opening polymerization (AROP). Instead of a post-polymerization reaction, a lipid initiator was used during polymerization to achieve a complete degree of functionalization of the copolymers.

Polymerization procedure

All polymers were synthesized with a targeted chain length of $D_p = 50$, equivalent to PEG with a molecular weight of 2,200 g mol⁻¹, typically applicable for PEGylation procedures. Different copolymers were synthesized with varying fractions of EO and GME in the backbone. For reference, a lipid-functionalized PEG was synthesized under identical conditions. The composition of the copolymers is described with Lipid-rPEG _{D_p} ^{f_{GME}} , where f_{GME} is the relative molar content of GME in the polymer backbone and D_p is the degree of polymerization of the sample. For the Lipid-PEG, only the D_p is given. All samples carried the lipid group in α -position and a hydroxy group at the ω -position. The polymerization reaction and some exemplary analytical data are given in **Figure 1**.

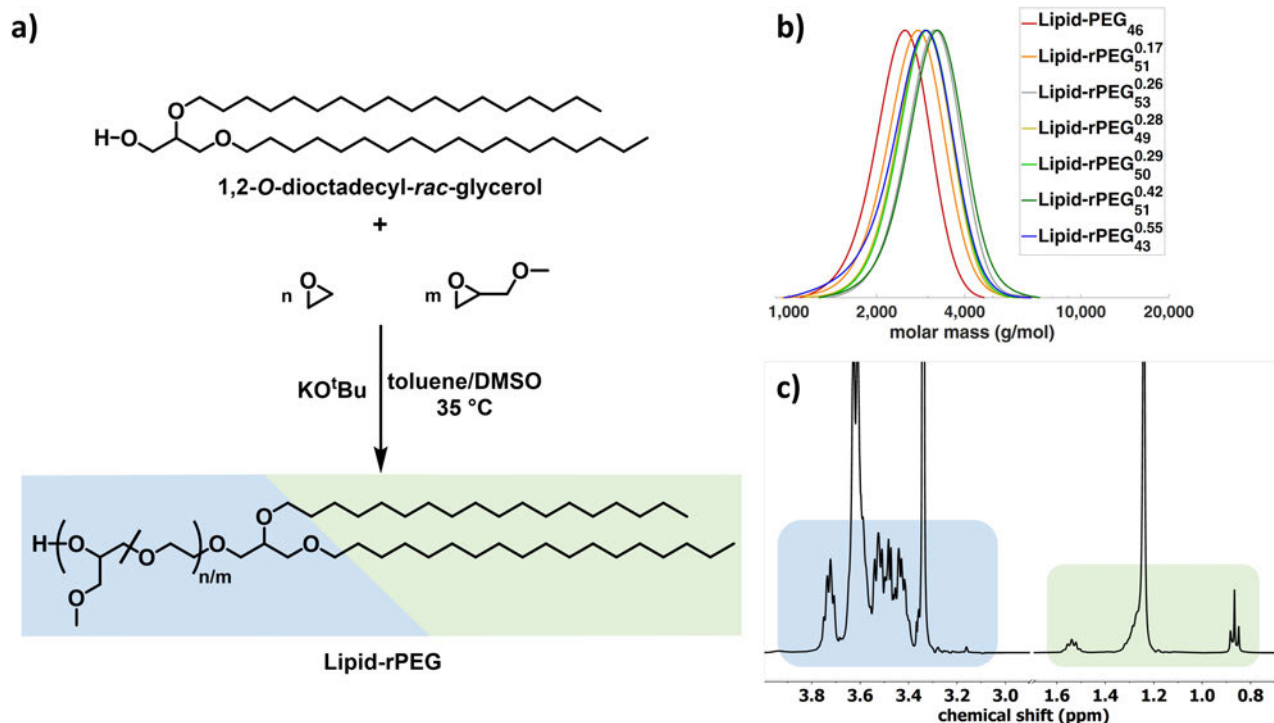


Figure 1. a) polymerization reaction of the synthesized initiator with EO and GME; b) SEC curves of the synthesized polymers (detector: RI, calibration: PEG); c) exemplary ^1H NMR spectrum of Lipid-rPEG₄₃^{0.55} with marked signals of the polyether backbone (blue) and the lipid chains (green).

During the polymerization procedure, both monomers were transferred to the reaction flask at -90°C to ensure that they could be added in their entirety before polymerization was initiated. Initially, it was hypothesized that the slow dissolution of the initiator while heating the mixture to 45°C would lead to partially delayed initiation of the polymerization and therefore broadening of the chain length distribution and increase of the polydispersity, but no such effect was observed during analysis of the copolymers. For Lipid-rPEG₄₉^{0.28} and Lipid-rPEG₅₁^{0.42}, small traces of allyl initiation were observed in the MALDI TOF MS, but since no signals were visible in the NMR spectra these were considered negligible.

For analytics of the polymers, a combination of ^1H NMR spectroscopy, SEC and MALDI TOF mass spectrometry was applied. By calculating the ratio of the integrals of the methyl signal produced by the GME units in the backbone and the signal of the methylene groups of EO and GME, the ratio of both monomers could be determined from the ^1H NMR spectrum. Furthermore,

using the signals of the terminal lipid group introduced through the initiator as a reference for integration, the molecular weight of the polymers could also be determined by ^1H NMR spectroscopy. Since signal integration in NMR spectroscopy can be prone to errors up to 5 %, MALDI TOF MS was performed to receive absolute values for the molar masses of the polymers. Because of the isomeric nature of EO and GME, mass signals of both monomers overlap so that only a repeating unit of 44 g mol^{-1} was visible in the mass spectra. Nevertheless, molar masses of the polymers and end group mass could both be determined by MALDI TOF MS. Accurate values for the polydispersities $\bar{D}$ of the samples were obtained by SEC. Analytical data are summarized in **Table 2**. More details about the analysis are given in the **Supporting Information**.

Table 2. Analytical data of the synthesized polymers.

<i>Polymer</i>	mol% <i>GME</i> (<i>target</i>)	mol% <i>GME</i> (<i>NMR</i>)	$\bar{D}_p$ (<i>MALDI</i>)	M_n (<i>MALDI</i>) / g mol^{-1}	M_n (<i>SEC</i>) / g mol^{-1}	$\bar{D}$ (<i>SEC</i>)
<i>Lipid-PEG</i> ₄₆	0	0	46	2,700	2,360	1.05
<i>Lipid-rPEG</i> ₅₁ ^{0.17}	15	17	51	3,250	2,600	1.06
<i>Lipid-rPEG</i> ₅₃ ^{0.26}	25	26	53	3,570	3,020	1.05
<i>Lipid-rPEG</i> ₄₉ ^{0.28}	25	28	49	3,410	2,780	1.05
<i>Lipid-rPEG</i> ₅₀ ^{0.29}	25	29	50	3,460	2,830	1.05
<i>Lipid-rPEG</i> ₅₁ ^{0.42}	40	42	51	3,800	3,060	1.06
<i>Lipid-rPEG</i> ₄₃ ^{0.55}	50	55	43	3,550	2,680	1.07

Molar masses found in SEC were systematically lower than the ones found in MALDI TOF MS. This suggests that the hydrodynamic radius of the copolymers did not increase linearly with the GME content. At the same time, the molar GME content was slightly higher than expected in all samples which could be attributed to the use of a toluene/DMSO mixture as solvent for the polymerization. As mentioned before, the reactivity ratios for copolymerization in toluene show a

slightly higher probability of propagation of GME, leading to moderately higher GME content in the copolymers.

Differential scanning calorimetry (DSC) was performed to see if crystalline, EO-rich segments could be found in the copolymers. The measurements showed typical glass transition temperatures (T_g) found for rPEGs at $T_g \approx -62$ °C and melting temperatures (T_m) between 43 °C and 24 °C, suggesting a mixed phase of lipid and rPEG which cannot be found for rPEGs without lipid end groups (see **Supporting Information, Figure S30** and **Table S2**). These melting temperatures showed a clear distinction from Lipid-PEG₄₆ with $T_m = 47$ °C and regular mPEG with $T_m > 50$ °C, demonstrating that no EO-rich epitopes are present in the copolymers.

Protein interactions

Analysis of the rPEGylated silica nanocapsules

For the investigation of the protein corona, the polymers were coated onto SiO₂ nanocapsules (NCs). These were synthesized by an oil-in-water miniemulsion process, employing tetraethyl orthosilicate for a condensation reaction at the oil-water interface and cetyltrimethylammonium chloride (CTMA-Cl) as stabilizing surfactant. Subsequently, the templating surfactant CTMA-Cl was replaced with Lutensol AT50 (Lut AT50) or the respective Lipid-PEG/Lipid-rPEG via dialysis (see **Supporting Information**).

The data showed consistent values for the hydrodynamic radii (R_h) between 111 and 166 nm with polydispersity indices of $PDI \leq 0.20$. ζ -potentials showed values between -6 mV and -11 mV. Together with the hydrodynamic radii derived from dynamic light scattering (DLS) and transmission electron microscopy (TEM), stability of the PEGylated/rPEGylated particles could be confirmed. An exception was found for Lipid-rPEG₅₃^{0.26}, for which a R_h of 236 nm and PDI of 0.50 were determined. It was concluded that the particles aggregated in this case. To ascertain if this behavior was related to the specific rPEG composition, two more polymer samples were synthesized (Lipid-rPEG₄₉^{0.28} and Lipid-rPEG₅₀^{0.29}) and NCs were produced again using CTMA-Cl. No aggregation was found for these polymers (see **Table 3**), indicating that the aggregation behavior of Lipid-rPEG₅₃^{0.26} was an outlier. No impurities could be detected via NMR or MALDI TOF and the reason for this behavior remained unclear. Comparability of the protein data required

an approximately equal total particle surface of the samples (here 0.05 m^2) which was controlled by the particle concentration during the incubation with the plasma samples. In the case of aggregation, the total surface area deviated from this value and therefore did not provide comparable data for this investigation. Consequently, Lipid-rPEG₅₃^{0.26} will be omitted for discussion of the protein data.

Table 3. Analytical Data of the synthesized nanocapsules (orange = observed aggregation during NC synthesis).

<i>Polymer</i>	<i>M_n</i> (SEC) / g mol ⁻¹	<i>R_h</i> / nm	<i>PDI</i>	<i>ζ-potential</i> / mV
<i>CTMA-Cl-a</i>	--	258 ± 26	0.40	- 2 ± 0.2
<i>Lut AT50</i>	--	108 ± 11	0.18	- 14 ± 1.4
<i>Lipid-PEG₄₆</i>	2,360	124 ± 12	0.16	- 7 ± 0.7
<i>Lipid-rPEG₅₁^{0.17}</i>	2,600	111 ± 11	0.12	- 7 ± 0.7
<i>Lipid-rPEG₅₃^{0.26}</i>	3,020	236 ± 24	0.50	- 12 ± 1.2
<i>Lipid-rPEG₅₁^{0.42}</i>	3,060	130 ± 13	0.15	- 6 ± 0.6
<i>Lipid-rPEG₄₃^{0.55}</i>	2,680	126 ± 13	0.20	- 8 ± 0.8
<i>CTMA-Cl-b</i>	--	203 ± 20	0.21	- 14 ± 1.4
<i>Lipid-rPEG₄₉^{0.28}</i>	2,780	158 ± 16	0.14	- 9 ± 0.9
<i>Lipid-rPEG₅₀^{0.29}</i>	2,830	166 ± 17	0.13	- 11 ± 1.1

Composition of the protein corona

For analysis of the protein corona, the NCs were incubated in undiluted human citrate plasma for 1 h. Total protein concentration was determined by Pierce Assay and total IgG concentration in the corona by Pierce Assay and liquid chromatography – mass spectrometry (LC-MS). Finally, the fraction of anti-PEG IgG was determined via enzyme-linked immunosorbent assay (ELISA). **Table 4** shows the results of these investigations. As can be seen, the total protein concentration for the particles functionalized with the surfactant CTMA-Cl was significantly higher than for the PEGylated/rPEGylated particles. Since CTMA-Cl is a cationic surfactant and cationic surfaces

have shown to adsorb a higher number of proteins than neutral or anionic ones^{33,41,42} because the majority of proteins has a net negative surface charge⁴³, this observation was expected and was also found in previous analogue experiments.⁴⁰

Total protein concentration and total concentration of IgG in the corona for the NCs functionalized with Lut AT50 were in a similar range found in previous experiments. Unexpectedly, the fraction of anti-PEG IgG was found to be significantly lower than previously determined (4 % vs 15 %).

The particles functionalized with the synthesized Lipid-PEG₄₆ and Lipid-rPEG samples showed similar total protein concentrations which were considerably lower than the concentration found for the NCs functionalized with Lut AT50. Similarly, the total IgG concentration is also lower for the Lipid-PEG₄₆ and Lipid-rPEG samples with a slight increase in concentration for increasing molar GME amounts between Lipid-PEG₄₆, Lipid-rPEG₅₁^{0.17} and Lipid-rPEG₅₁^{0.42}. For Lipid-rPEG₄₉^{0.28}, Lipid-rPEG₅₀^{0.29} and Lipid-rPEG₄₃^{0.55}, a noticeably lower total IgG concentration was observed. However, while Lipid-rPEG₄₃^{0.55} also showed a relatively low concentration of anti-PEG IgG, the anti-PEG IgG concentration of Lipid-rPEG₄₉^{0.28} and Lipid-rPEG₅₀^{0.29} was found to be comparatively high, leading to anti-PEG-IgG fractions of > 50 %. For the other samples functionalized with Lipid-PEG₄₆ and the Lipid-rPEGs, the fraction of anti-PEG IgG in the corona remained constant between 10 and 15 %.

A similar trend can be seen in **Figure 2**, where the relative abundance of 4 immunoglobulin components is illustrated. Leaving Lipid-rPEG₅₃^{0.26} out of the consideration, we saw a constant increase in Ig components up to Lipid-rPEG₅₁^{0.42} with a stark drop in abundance for Lipid-rPEG₄₃^{0.55}. Overall, Lipid-rPEG₄₃^{0.55} as well as Lipid-rPEG₄₉^{0.28} and Lipid-rPEG₅₀^{0.29} showed the lowest abundances of Ig components between all synthesized samples in the same range that was found for the Lut AT50 functionalized NCs.

Table 4. Protein corona analysis of the synthesized nanocapsules (orange = unusually high anti-PEG IgG concentration).

Polymer	Protein conc.^a / $\mu\text{g mL}^{-1}$	anti-PEG IgG^b / $\mu\text{g mL}^{-1}$	Total IgG^c / $\mu\text{g mL}^{-1}$	Fraction anti-PEG IgG/Total IgG / %
<i>CTMA-Cl</i>	2076 ± 148	1.8 ± 0.4	47.3	3
<i>Lut AT50</i>	996 ± 156	2.6 ± 1.3	56.3	4
<i>Lipid-PEG₄₆</i>	227 ± 24	2.5 ± 0.1	20.1	15
<i>Lipid-rPEG₅₁^{0.17}</i>	200 ± 18	2.6 ± 0.7	25.7	10
<i>Lipid-rPEG₅₃^{0.26}</i>	268 ± 8	6.8 ± 0.5	30.1	23
<i>Lipid-rPEG₄₉^{0.28}</i>	166 ± 42	4.6 ± 0.5	8.2	56
<i>Lipid-rPEG₅₀^{0.29}</i>	171 ± 43	4.6 ± 0.2	8.5	54
<i>Lipid-rPEG₅₁^{0.42}</i>	173 ± 8	3.0 ± 0.2	29.5	10
<i>Lipid-rPEG₄₃^{0.55}</i>	200 ± 29	1.5 ± 0.3	11.1	13

^a concentration determined via Pierce Assay^b concentration determined via ELISA^c concentration determined via Pierce Assay and LC-MS

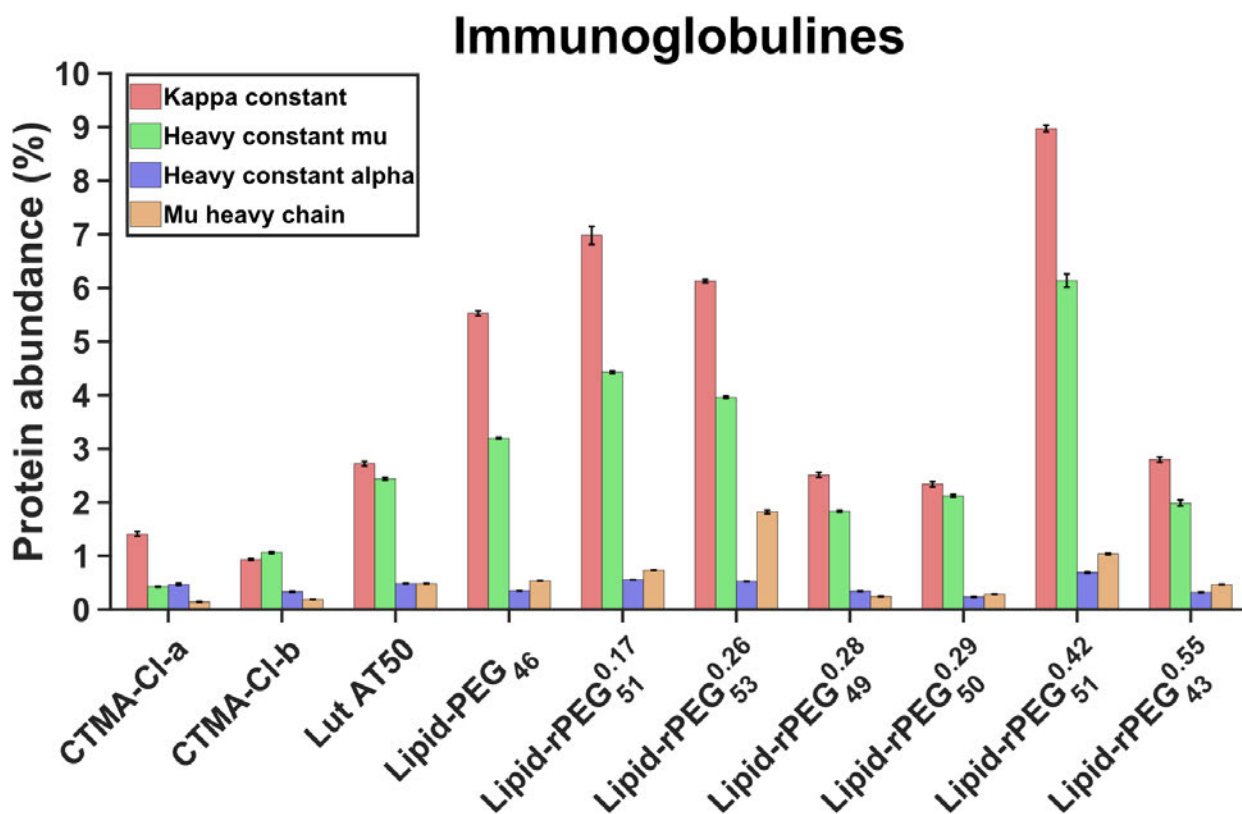


Figure 2. Relative abundance of Immunoglobulin components in the protein corona of all investigated samples analyzed *via* liquid chromatography – mass spectrometry (LC-MS).

Generally, multiple factors can influence the amount of adsorbed proteins on nanoparticles as well as the composition of the protein corona. These include holistic properties like particle size⁴⁴ and shape^{45,46} as well as surface properties like hydrophobicity⁴⁷, steric shielding through surface functionalization^{48,49}, roughness^{35,50} and surface charge³⁵. Additionally, the properties of the biological environment like protein concentration^{51,52}, incubation time⁵³, temperature⁵² and pH^{32,54} of the medium will also determine the composition of the protein corona.

Many of these factors can be excluded from discussion in this case. Since all NCs were synthesized from the same material under identical conditions and have been shown through DLS and TEM to possess similar size and shape, differences in the protein corona composition are not expected to be influenced by these factors. With biological parameters like incubation time, temperature and plasma concentration being kept constant for all incubation procedures, two relevant distinctions in the properties of the NCs remain: different lipid anchors between Lut AT50 and the synthesized

Lipid-PEG/Lipid-rPEG polymers and varying molar amount of GME units in the backbone between the Lipid-rPEG samples.

The stark difference in the total protein concentration between the Lut AT50 functionalized NCs and the NCs functionalized with the synthesized polymer samples was most likely caused by the difference in the lipid anchors of the samples. An anchor with two lipid chains – as used for the Lipid-PEG/Lipid-rPEG samples – requires more surface area when attached to the NCs, thereby reducing the packing density of the polyether chains compared to Lut AT50 which possesses only a single lipid chain. A differing lipid packing parameter would furthermore change the solubility of the polymers. Naturally, variations in the packing density would also influence the hydrophobicity of the NCs. Additionally, the rPEGs could be more sterically demanding than simple PEG chains thanks to their side chains, enhancing this effect. At high packing density, the polyether chains form brush conformations which prevent coiling due to steric hindrance and form an effective hydrophilic shield between the plasma proteins and the hydrophobic particle surface. It has been shown that hydrophilic particles adsorb smaller amounts of proteins than hydrophobic ones^{33,55} since hydrophobic (van der Waals) interactions contribute strongly to protein adsorption.⁵⁶ At low packing density, hydrophilic shielding of the particle surface will be less efficient (**Figure 3**).

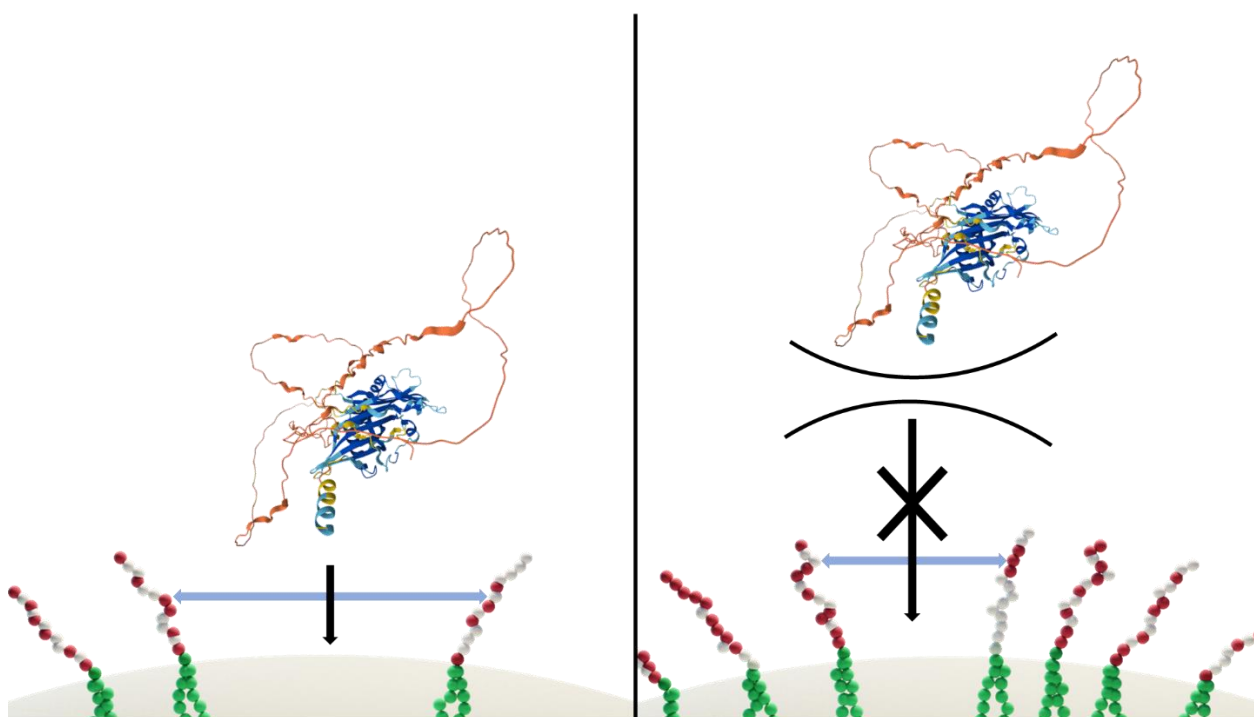


Figure 3. Illustration of rPEGylation for large particles of different packing density. At low packing density (left), interaction of proteins (here: histidine-rich glycoprotein) with the particle surface is likely. At high packing density (right), interaction is limited by steric hydrophilic shielding of the polyether chains.

The difference in IgG concentrations for of Lipid-rPEG₄₉^{0.28} and Lipid-rPEG₅₀^{0.29} could stem from a difference in packing density on these particles caused by this specific polymer composition. Data from a previous study (see **Chapter 2** of this Dissertation) showed a dependence of hydrophobicity and the formation of the hydration shell on the molar GME content in the polyether chains, which could have affected the packing density during the functionalization procedure. Conclusive deductions about why this behavior is only observed for Lipid-rPEGs of this exact composition could not be made yet.

Additionally, differences in hydrophobicity caused by the lipid anchor used for the Lipid-PEG and Lipid-rPEG samples as well as the varying amount of GME in the polyether chains could have altered the diffusion behavior during the dialysis applied for the PEGylation/rPEGylation procedures. Deviations in the polymer concentration during the functionalization process could have led to the proposed difference in packing density of polymers on the surface of the NCs.

One could reasonably expect to find higher amounts of adsorbed proteins on the Lipid-PEG/Lipid-rPEG samples because interaction with the lipid anchor or the particle surface should be favoring hydrophobic binding to the proteins. Instead, we found the total concentration of adsorbed proteins to be 4 to 5-fold higher for the NCs functionalized with more hydrophilic Lut AT50. The exact reason for this behavior could not be determined yet. Previous studies have shown that certain proteins can bind strongly to hydrophilic i.e. PEGylated nanoparticles⁵⁷ and that hydrogen bonds could be another significant factor in protein adsorption.⁵⁶ Others were found to bind in larger quantities to particles of moderate hydrophobicity but not to particles of strong hydrophobicity.⁵⁸ These results demonstrated that hydrophobic interactions alone do not determine the composition of the protein corona even though they are among the driving forces of adsorption. Considering this could also explain the unusually low concentration of IgG in the protein corona of Lipid-rPEG₄₉^{0.28} and Lipid-rPEG₅₀^{0.29}, which simultaneously showed higher concentrations in anti-PEG IgG than the other samples. The specific hydrophobicity exhibited by Lipid-rPEG₄₉^{0.28} and Lipid-rPEG₅₀^{0.29} might have favored the binding of anti-PEG IgG while the binding of other IgGs was disfavored.

Furthermore, conformational changes in the proteins could also have contributed to the concentration of the proteins and the composition of the corona. These changes are induced by the hydrophobic interactions of the proteins with the particles and can affect adsorption of certain proteins.⁵⁹ It is not yet completely understood if PEG chains themselves contribute to these changes as well, although differences in protein conformations have been described for gold nanoparticles which were functionalized with PEGs of varying chain lengths.^{31,60} It is however possible that hydrophobic interactions of proteins with the lipid anchor of the polymers or the surface of the NCs functionalized with Lipid-PEG/Lipid-rPEGs caused a conformational change which reduced binding to certain proteins and reduced overall protein concentration. Moreover, if the conformational change caused by Lipid-rPEG₄₉^{0.28} and Lipid-rPEG₅₀^{0.29} differed from polymers with different hydrophobicities, this could also explain the distinct behavior of these polymers compared to the other samples. At this point, the reason for these observations remains a hypothesis. Further investigations will be necessary to assess the exact cause of the behavior of the proteins regarding the PEGylated/rPEGylated NCs.

Previous *in vitro* investigations³¹ showed a reduced PEG-antigenicity with increasing GME content of fluorescein functionalized rPEGs. Furthermore, competitive ELISA also showed reduced affinity to backbone-selective and end group-selective anti-PEG IgG for unfunctionalized rPEGs of varying molar GME content.³⁰ Contrarily in this study, concentration of anti-PEG IgG was found to increase slightly with increasing GME content in the polyethers. For Lipid-rPEG₄₃^{0.55} however, the anti-PEG IgG concentration was significantly reduced. But since the total IgG concentration decreased as well for this sample, the fraction of anti-PEG IgG remained approximately equal for all samples except the ones with GME contents between 25 – 30 mol%.

Nevertheless, the overall significantly reduced protein concentration found for the NCs functionalized with Lipid-PEG and the Lipid-rPEGs suggested that the variation of the lipid anchor had a larger effect on the composition of the protein corona than the varying molar content of GME in the backbone of the polyether chains. Therefore, influence of the GME content on the composition of the protein corona cannot be precisely quantified for now.

Figure 4 shows the heatmap of all proteins with an abundance > 1 % identified in the protein corona of the NCs. Some proteins found in the corona showed only small differences in abundance between the NCs functionalized with Lut AT50, Lipid-PEG₄₆ and the Lipid-rPEGs. The relative concentration of Albumin showed only minimal changes between the different samples. The same can be said about the Coagulation factors XI and XII and the Fibrinogen chains alpha, beta and gamma as well as Plasma kallikrein, Protein Red and Septin-7. For Immunoglobulin heavy constant mu and Immunoglobulin kappa constant, a slight increase in relative concentration with increasing molar GME amount in the polymer backbone was observed with the exception of Lipid-rPEG₄₃^{0.55}, where a notable decrease in abundance was seen as previously illustrated in **Figure 2**. Meanwhile, the relative abundance of Immunoglobulin mu heavy chain remained constant for all samples.

The heatmap shows the dependency of the opsonin and dysopsonin concentrations on the GME content in the polymers. A stark difference in the abundance between the Lut AT50 functionalized NCs and the Lipid-PEG₄₆ and Lipid-rPEGs could be seen for the Apolipoproteins A-I (ApoA1) and A-IV (ApoA4) as well as the Histidine-rich glycoprotein (HRG). The Apolipoproteins showed a much higher concentration in the corona of the Lipid-PEG₄₆ NCs compared to the Lut AT50 NCs but the concentration decreased with increasing molar GME amount in the backbone. Especially the concentration of ApoA4 dropped to a very low value for a GME amount of 55 mol%. These

concentrations were inversely proportional to the concentration of HRG in the corona, which was observed to be very high for the Lut AT50 NCs and low for Lipid-PEG₄₆ NCs, whereas concentration increased again for higher molar amounts of GME in the polymer backbone.

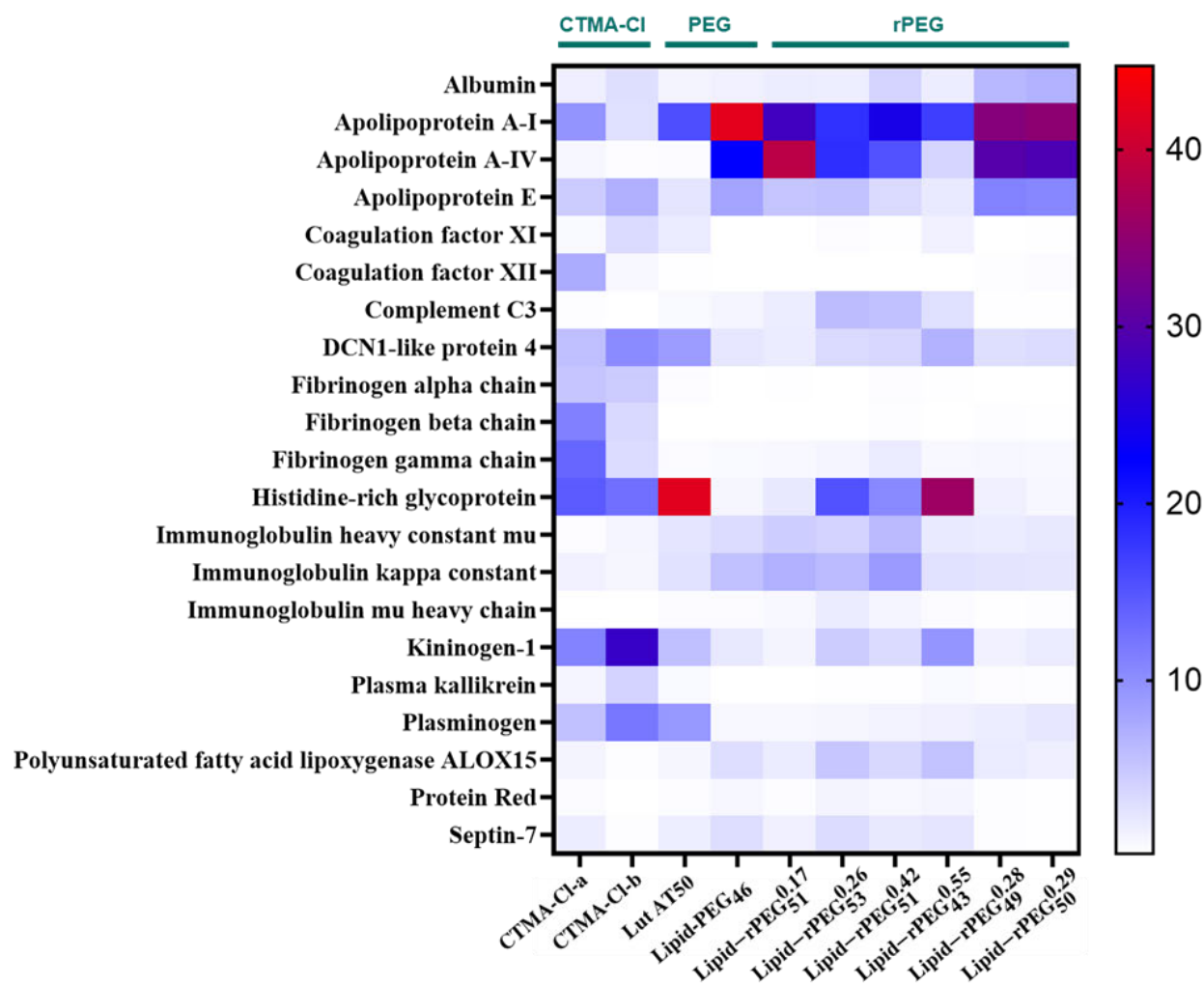


Figure 4. Heatmap of the most abundant proteins found in the protein corona of the Lipid-rPEG functionalized SiO₂ nanocapsules. All proteins with an abundance of at least 1 % in one of the samples are collected. Relative values were calculated based on the absolute amount of each protein (fmol).

A direct comparison of the relative abundance of the Apolipoproteins and HRG for all samples can be seen in **Figure 5**.

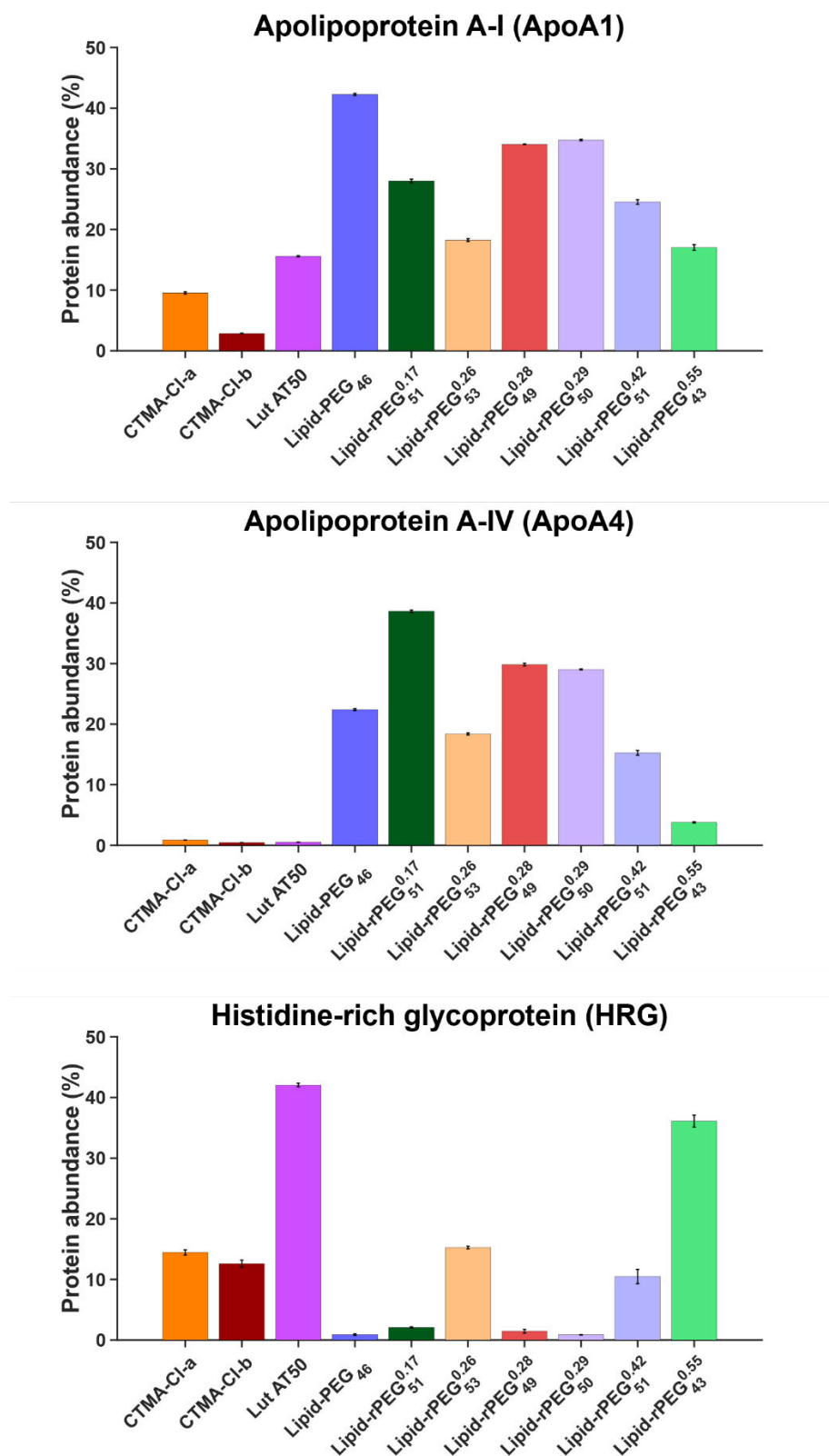


Figure 5. Direct comparison of the relative abundance of Apolipoproteins A-I and A-IV and Histidine-rich glycoprotein in the protein corona of the samples investigated via LC-MS.

These results were unexpected compared to previous investigations, where ApoA1 and Clusterin (Apolipoprotein J) were found to be the most abundant proteins in the corona of Lut AT50 functionalized NCs while HRG showed a relative concentration below 1 % for these NCs.⁴⁰

The results indicated the binding of HRG and the Apolipoproteins to be mutually exclusive for the NCs functionalized with Lipid-PEG and the Lipid-rPEGs, showing a clear decrease in the fraction of ApoA1 and an increase in the fraction of HRG for increasing molar GME content. A similar behavior was recently found for non-PEGylated silica nanoparticles of varying sizes.⁴⁴ This similarity could indicate that the corona composition for the NCs functionalized with Lipid-PEG and the Lipid-rPEGs was mainly driven by protein-particle interactions because of low packing density of the polyether chains on the particle surface.

Specifically for PEGylated silica nanocapsules, previous studies found an abundance of ApoA1 in the protein corona. Cai *et al*⁶⁰ found that the diameter of PEGylated gold nanoparticles does not change after adsorption of lysozyme and suggested that the protein enters the PEG layer when adsorbed. Adsorption of ApoA1 could work similarly since both are relatively small proteins (129 amino acids / 14.3 kDa for lysozyme; 243 amino acids / 28.3 kDa for Apo A1). The side chains in the rPEG chains could impede the proteins from entering the PEG layer and therefore explain why the concentration of ApoA1 decreased with higher contents of GME in the polymers.

Conclusion

In this work we have investigated the composition of the protein corona formed around nanocapsules coated with lipid-functionalized rPEGs. The results showed that dysopsonins like the Apolipoproteins A-I and A-IV were found in highest concentrations for samples functionalized with polymers that possessed low amounts of GME in the polyether backbone (Lipid-PEG₄₆ and Lipid-rPEG₅₁^{0.17}). Lipid-rPEG₅₁^{0.42} showed relatively high concentrations for these dysopsonins and a low concentration of HRG, which exhibits opsonic properties. For Lipid-rPEG₄₃^{0.55}, the concentration of Apolipoproteins dropped drastically while the HRG concentration was found to be very high. Other dysopsonins like the Fibrinogen chains and the Coagulation factors remained at a nearly constant low concentration for all samples. The dysopsonin Clusterin, which was previously found to be the second most abundant protein in the corona of PEGylated silica NCs, showed a relative abundance of less than 1 % for all samples.

Together with the overall concentrations in the protein corona, it is difficult to make predictions about the behavior of these nanomaterials in an *in vivo* environment. The samples with low GME content showed high concentrations of dysopsonins in the protein corona which could reduce interaction with phagocytes and prolong the circulation time of these particles in the blood stream. Although the concentration of IgG in the protein corona was found to be lower for the Lipid-PEG/Lipid-rPEG functionalized samples compared to the Lut AT50 sample, the concentration of anti-PEG IgG for all PEGylated/rPEGylated samples remained approximately the same up to a molar GME content of 42 mol% and only declined for Lipid-rPEG₄₃^{0.55}. The fraction of anti-PEG IgG in relation to the total IgG concentration remained constant and independent of the GME content in all synthesized polymers. While the dysopsonin concentration was low and the opsonin concentration was high for Lipid-rPEG₄₃^{0.55}, this sample showed the lowest total IgG concentration in the protein corona, except for Lipid-rPEG₄₉^{0.28} and Lipid-rPEG₅₀^{0.29}, which showed deviating behavior compared to all other samples.

As mentioned before, it is not yet clear to which degree the low protein concentration found for the NCs functionalized with Lipid-PEG and Lipid-rPEG and the therefore assumed low packing density of polyether chains dictated the formation of the protein corona and what influence the molar content of GME had. The influence of the lipid anchor was found to be greater than expected, which did not allow for confirmation of the previously found results regarding the effect of the

GME content of the polymers. Therefore, further investigations should include rPEGs functionalized with a single chained lipid anchor. Additionally, higher concentrations of antibodies could be utilized to recreate the biological environment of severe immune reactions to PEG. Changing these parameters could more conclusively determine the potency of rPEG to reduce the antigenicity of functionalized nanomaterials and help make predictions about their immunogenicity under *in vivo* conditions.

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Supporting Information

Effect of surface functionalization with lipidated random poly(ethylene glycol – glycidyl methyl ether) copolymers (Lipid-rPEGs) on the formation of the protein corona of silica nanocapsules

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1. Materials and Methods

Calculation of monomer fractions in the copolymers from ^1H NMR spectra

For calculation of the monomer fractions f_{EO} and f_{GME} , the integral of the methoxy group signal of the GME units I_{MeO} (3.36 – 3.15 ppm) has to be put in relation to the integral of the signal of the backbone methylene and methine groups $I_{backbone}$ (4.00 – 3.37 ppm). The signal of the methyl groups of the initiator (0.90 – 0.85 ppm ; 6H) is hereby used as a reference point.

The number of GME units in the backbone can be calculated by:

$$D_{p,GME} = \frac{I_{MeO}}{3} \quad (\text{eq S1})$$

The number of EO units in the backbone can then be derived by subtracting the integral of the remaining GME hydrogens ($I_{GME} = D_{p,GME} \cdot 5$) and the integral of the overlapping initiator hydrogens ($I_{Init} = 9$) from $I_{backbone}$ and dividing the result by the number of hydrogens in an EO unit:

$$D_{p,EO} = \frac{I_{backbone} - I_{GME} - I_{Init}}{4} \quad (\text{eq S2})$$

The molar fractions of EO and GME in the backbone can then be calculated by:

$$f_{EO} = \frac{D_{p,EO}}{D_{p,EO} + D_{p,GME}} \quad (\text{eq S3})$$

$$\text{and } f_{GME} = \frac{D_{p,GME}}{D_{p,EO} + D_{p,GME}} \quad (\text{eq S4})$$

Using Lipid-rPEG₄₃^{0.55} (**Figure S21**) as an example:

$$D_{p,GME} \approx \frac{81}{3} = 27$$

$$D_{p,EO} \approx \frac{234 - 27 \cdot 5 - 9}{4} = 22.5 \approx 23$$

Which leads to:

$$f_{EO} = \frac{22.5}{22.5 + 27} \approx 0.45$$

$$\text{and } f_{GME} = \frac{27}{22.5 + 27} \approx 0.55$$

1.1. Materials

Ethylene oxide was purchased from *Air Liquide Deutschland*. Epichlorohydrin (99 %), benzyl glycidyl ether (99 %) and octadecylbromide (99 %) as well as all solvents for the monomer-, initiator- and polymer syntheses were purchased from *Thermo Fisher Scientific*. Other substances were purchased from *TCI Chemicals*, *Sigma-Aldrich*, and *Thermo Fisher Scientific*. All substances were used as received.

Tetraethoxysilane (TEOS, *Alfa Aesar*, 98 %), 3-aminopropyltrimethoxysilane (APTES, *Alfa Aesar*, > 98 %), hexadecane (*Sigma Aldrich*, 99 %), chloroform (*Sigma Aldrich*, highly refined, low acidity), cetyltrimethylammonium chloride (CTMA-Cl, *Acros Organics*, 99 %) and Lutensol AT50 (*BASF*) were used as received.

To obtain pooled plasma, blood was taken from 10 healthy donors after obtaining informed consent and pooled subsequently. All experiments containing human blood plasma from these donors were approved by the ethics committee of the Landesärztekammer Rheinland-Pfalz, Mainz, Approval No. 837.439.12 (8540-F). After collection, the citrate plasma was centrifuged at 20,000 g for 1 h at room temperature to remove residual protein precipitates and stored at - 80 °C until further use.

1.2. ^1H NMR (nuclear magnetic resonance)

^1H and ^{13}C NMR spectra were recorded on a *Bruker Avance III HD 400* spectrometer with 400 and 100 MHz, respectively, and referenced internally to residual proton signals of the deuterated solvent. All spectra were acquired at 23 °C. Spectra were processed and analyzed utilizing the *MestReNova 14.3.3-33362* software. Deuterated chloroform was purchased from *Deutero GmbH*.

1.3. Matrix-assisted laser desorption ionization using time-of-flight detection (MALDI TOF) mass spectrometry

MALDI TOF MS measurements were carried out at a *Bruker autoflex maX MALDI TOF/TOF* using a Smartbeam-II solid-state laser with a wavelength of 337 nm. Spectra were recorded using the software *Bruker flexControl 3.4* and analyzed using *Bruker flexAnalysis 3.4* and *Bruker polytools 1.31*. The potassium salt of trifluoroacetic acid (KTFA) and trans-2-[3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) were utilized as ionization salt and matrix, respectively. For sample preparation, the polymers were dissolved in chloroform at 10 mg mL⁻¹. 20 µL of this solution was combined with 20 µL of a 10 mg mL⁻¹ solution of the matrix in chloroform. 5 µL of a 0.1 M solution of the salt in methanol were added and 1 µL of the resulting mixture was spotted onto a *MTP 384 ground steel target plate*. The solvents were allowed to evaporate completely before the measurement. All spectra were recorded in linear mode.

1.4. Size exclusion chromatography (SEC)

Measurements were conducted using an *Agilent 1100 series HPLC* system, which included a degasser, isocratic pump (G1310A), autosampler (G1313A), column oven (G1316A), and detectors for refractive index (RI) (G1310A) and variable wavelength (VWD) (G1314A). Separations were carried out employing a four-column set-up (*MZ-Analysentechnik GmbH*) connected sequentially:

- i) HEMA-40 guard column (40 Å pore size, 10 µm particle size, 50 x 8.0 mm)
- ii) HEMA-40 analytical column (40 Å pore size, 10 µm particle size, 300 x 8.0 mm)
- iii) HEMA-100 analytical column (100 Å pore size, 10 µm particle size, 300 x 8.0 mm)
- iv) HEMA-300 analytical column (300 Å pore size, 10 µm particle size, 300 x 8.0 mm)

The eluent consisted of DMF (Fisher Chemical) with 1 mg mL⁻¹ anhydrous LiBr (Acros Organics), delivered at a flow rate of 1 mL min⁻¹. Both the column oven and RI detector cell were maintained at 50 °C. Calibration was performed using well-defined poly(ethylene glycol)s from PSS (PSS Standards Kit) with molar mass values (M_p) ranging from 106 to 42700 g mol⁻¹. Samples were dissolved in DMF (with 1 mg mL⁻¹ anhydrous LiBr) at a concentration of 1 mg mL⁻¹ with the addition of 1 drop of toluene. The injection of 100 µL of the stock solutions was carried out via the autosampler, with a measurement duration of 45 min. Elution times were referenced using toluene as an internal standard. RI traces were analyzed using the *PSS WinGPC Unichrom V8.31 software*.

1.5. Differential scanning calorimetry (DSC)

DSC measurements were performed on a *DSC250* device from *TA Instruments* using indium and *n*-octane as calibrations standards. 3 – 5 mg of the polymer samples were weighed in *Tzero* aluminum pans and measured under nitrogen atmosphere against an empty pan as a reference point. The following steps were performed for all samples:

- i) Heating from room temperature to 100 °C at a heating rate of 10 °C min⁻¹
- ii) Cooling from 100 °C to – 90 °C at a cooling rate of 10 °C min⁻¹
- iii) Temperature kept isothermally at – 90 °C for 10 min
- iv) Heating from – 90 °C to 180 °C at a heating rate of 10 °C min⁻¹
- v) Cooling from 180 °C to – 90 °C at a cooling rate of 10 °C min⁻¹
- vi) Heating from – 90 °C to 180 °C at a heating rate of 10 °C min⁻¹

Melting temperatures (T_g) and glass transition temperatures (T_g) were calculated from the last heating cycle to ensure prior removal of solvent residues as well as thermal history of the sample.

1.6. Nanocapsule and protein corona analysis

1.6.1. Characterization of the nanocapsules (NCs)

- a) **Dynamic light scattering (DLS) measurements** of SiNCs were performed using an instrument from *ALV* (Langen, Germany) consisting of an electronically controlled goniometer and an ALV-5000 multiple τ full-digital correlator with 320 channels having a measurement range between 10⁻⁷ s and 10³ s. A helium-neon laser (Type 1145 P) from *JDS Uniphase* (Milpitas, USA) of 632.8 nm wavelength and 25 mW output power was used as a source of light. Before measurements, samples were filtered into quartz cuvettes for light scattering from *Hellma* (Müllheim, Germany) with an inner radius of 9 mm. Millex-SV filters (*Merck Millipore*, Billerica, USA) with 5 μ m pore size were used for filtration. Prior to use, the quartz cuvettes were cleaned with acetone using a Thurmond apparatus.

- b) **Zeta potential measurements** were performed using a *Nano Z Zetasizer* (Malvern Instruments GmbH, Herrenberg, Germany). 20 μL of the sample were diluted with 1 mL of a 1 mM KCl solution and measured at 25 °C after two minutes of equilibration. Each measurement was repeated in triplicate and mean values as well as standard deviations were calculated.
- c) **Transmission electron microscopy (TEM) micrographs** were taken on an *FEI Tecnai F20 transmission electron microscope* operated at 200 kV. Micrographs were taken using a 2k charge-coupled device camera from *Gatan* (Type: Ultrascan1000).

1.6.2. Analysis of the protein corona

- a) **Protein quantification** was performed via Pierce 660 nm protein Assay (*Thermo Fisher Scientific*). Absorption was measured at 660 nm with a Tecan infinite M1000 plate reader and Bovine serum albumin (*Serva*, Germany) was used as a standard.
- b) **Liquid chromatography-mass spectrometry (LC-MS)** proteomic analysis was carried out as previously described.^{2,3} Briefly, SDS was removed from the protein sample via *Pierce Detergent Removal Spin Columns* (*Thermo Fisher Scientific*). Further, 20 μg of proteins were precipitated using *ProteoExtract protein precipitation kit* (*CalBioChem*) overnight. Afterwards, the protein pellets were isolated via centrifugation (10 000g, 10 min), washed three times with wash solution at 10 000 g, 2 min. After drying the pellet for 2 min, the pellet was resuspended with *RapiGest SF* (*Waters*) in ammonium bicarbonate (50 mM) and incubated at 80 °C for 15 min. The protein solution was reduced with dithiothreitol (*Sigma*) at a concentration of 5 mM for 45 min at 56 °C and alkylated with 15 mM iodoacetamide (*Sigma*) for 1 h in the dark. Tryptic digestion (protein : trypsin ratio 50 : 1) was carried out for approx. 18 h at 37 °C. Afterwards, the reaction was quenched with 2 μL hydrochloric acid (*Sigma*). Tryptic peptides were diluted with 0.1 % formic acid spiked with 50 fmol μL^{-1} Hi3 *E. coli* (*Waters*) for absolute protein quantification. The peptide solution was injected into a *nanoACQUITY UPLC* system coupled to a *Synapt G2-Si* mass spectrometer. Glu-fibrinopeptide (150 fmol μL^{-1}) was also injected as reference. The system was operated in resolution mode, with a *NanoLockSpray* source in positive ion

mode. Data-independent acquisition (MSE) experiments were performed and data was analyzed with *MassLynx 4.1*. Proteins were identified with *Progenesis GI (2.0)* using a reviewed human database downloaded from *Uniprot*. For analysis, the following criteria were chosen: max. protein mass 600 kDa, one missed cleavage, fixed modifications for carbamidomethyl and cysteine, variable oxidation for methionine, and a false discovery rate of 4 %. Peptide identification required three identified fragments and for protein identification, five identified fragments and two peptides were needed. Based on the TOP3/Hi3 quantification, the amount of each protein in fmol is provided.

The **ELISA experiments** were performed based on a previously published procedure from Chen *et al.*⁴

Maxisorp 96-well microplates (*Thermo Fisher Scientific*, Waltham, USA) were coated with 0.5 µg NH₂-PEG10000-NH₂ in 50 µL 0.1 M NaHCO₃/Na₂CO₃ buffer (adjusted to pH 9.5) per well overnight at 4 °C. Afterwards, the content of the wells was discarded and the well blocked with 200 µL of 5 % (w/v) skim milk powder in Dulbecco's phosphate-buffered saline (PBS, *Thermo Fisher Scientific*) per well at room temperature for 2 h. Plates were washed once with 100 µL PBS per well immediately before use. In wells where no competition took place, 50 µL of 2 % (w/v) skim milk in PBS was added. The wells were incubated for 30 min at room temperature. Human plasma samples were pre-diluted to 20 %, 50 %, 80 %, or no dilution in 2 % (w/v) skim milk in PBS. Serial dilutions of chimeric anti-PEG antibodies c3.3-IgG in 2 % (w/v) skim milk powder in PBS were prepared starting from 8 µg mL⁻¹. For each sample (including standards), 60 µL of the sample were diluted with 120 µL of 2 % (w/v) skim milk in PBS (33 %-dilution). 50 µL of these 33 %-diluted samples were added to the respective well and incubated for 1 h at room temperature. Unbound antibodies were removed by washing the plates twice with 0.1 % 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in PBS and once with pure PBS. Afterwards, 50 µL of anti-human IgG-peroxidase antibody (0.25 mg L⁻¹ in 2 % (w/v) skim milk in PBS, Fc-specific) was added to each well and incubated for 1 h at room temperature. The content of the wells was discarded, and each well was washed four times with 100 µL 0.1 % (w/v) CHAPS in PBS and once with 100 µL pure PBS. Then, 100 µL ABTS substrate was added per well and incubated for 30 min in the dark. The absorbance was measured at $\lambda = 405$ nm in a microplate reader (*Tecan infinite M1000 plate reader*, Switzerland).

2. Preparative procedures

2.1. Monomer synthesis

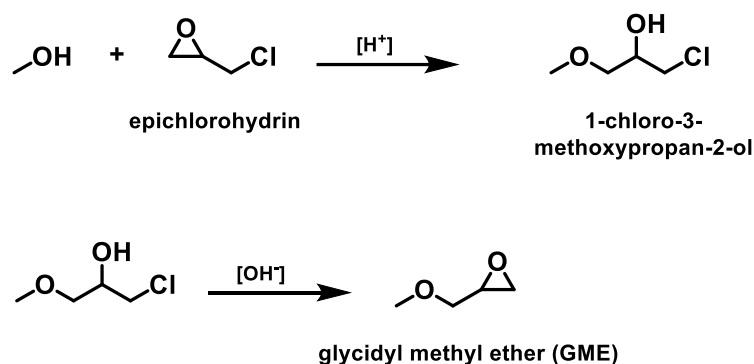


Figure S1. Synthesis of glycidyl methyl ether (GME) from epichlorohydrin in a 2-step reaction.

1-Chloro-3-methoxypropan-2-ol In a flame-dried 1000 mL three neck round bottom flask equipped with a magnetic stirring bar, a dropping funnel and a Dimroth condenser, 233 mL (183.9 g, 5.74 mol, 3.00 eq) of methanol were combined with 4 mL (7.5 g, 0.08 mol, 0.04 eq) conc. sulfuric acid (98 %). Under rigorous stirring and argon atmosphere 150 mL (177.0 g, 1.91 mol, 1.00 eq) of epichlorohydrin were added dropwise to the mixture. After the addition was finished, the mixture was stirred under reflux overnight. After cooling to room temperature, 22.65 g (0.11 mol, 0.06 eq) of barium carbonate were added and the suspension was stirred for 1 h. Finally, methanol is removed at normal pressure before the product is purified by distillation at reduced pressure (Bp.: 100 – 102 °C, 60 mbar) in a fractional distillation. 191 g (80 %) of product were received as a clear, colorless liquid.

^1H NMR (CDCl_3 , 400 MHz) δ [ppm]: 3.99 – 3.95 (dd; $J = 5.32$ Hz, 5.26 Hz; 1H), 3.65 – 3.55 (qd; $J = 11.13$ Hz, 5.56 Hz; 2H), 3.50 – 3.48 (d; $J = 5.01$ Hz; 2H), 3.39 (s; 3H), 2.65 – 2.64 (d; $J = 5.50$ Hz; 1H).

Glycidyl methyl ether In a 500 mL three neck round bottom flask equipped with an electric overhead stirrer, a Dimroth condenser and a thermometer, 172 mL (200.0 g, 1.61 mol, 1.00 eq) of 1-chloro-3-methoxypropan-2-ol were combined with 400 mL of diethyl ether. The mixture was cooled to 0 °C in an ice bath and 77.07 g (1.93 mol, 1.20 eq) of sodium hydroxide were added in small portions (over a timespan of appr. 2 h) while the temperature was kept under 15 °C. Under constant stirring, the mixture was slowly allowed to reach room temperature over the span of 5 h. The residue was filtered off and flask and filter were washed 4 times with diethyl ether. The combined organic phases were dried over magnesium sulfate and diethyl ether was removed by distillation at normal pressure. Afterwards, the product was purified by fractional distillation at reduced pressure (Bp.: 70 – 72 °C, 150 mbar) to receive 95.6 g (68 %) of a clear, colorless liquid.

^1H NMR (CDCl_3 , 400 MHz) δ [ppm]: 3.70 – 3.66 (dd; $J = 11.37$ Hz, 5.26 Hz; 1H), 3.39 (s; 3H), 3.34 – 3.28 (dd; $J = 11.32$ Hz, 5.82 Hz; 1H), 3.15 – 3.10 (m; 2H), 2.80 – 2.77 (dd; $J = 4.62$ Hz, 4.57 Hz; 1H), 2.61 – 2.58 (dd; $J = 5.00$ Hz, 2.71 Hz; 1H).

^{13}C NMR (CDCl_3 , 101 MHz) δ [ppm]: 73.09, 59.10, 50.63, 44.05.

2.2. Initiator synthesis

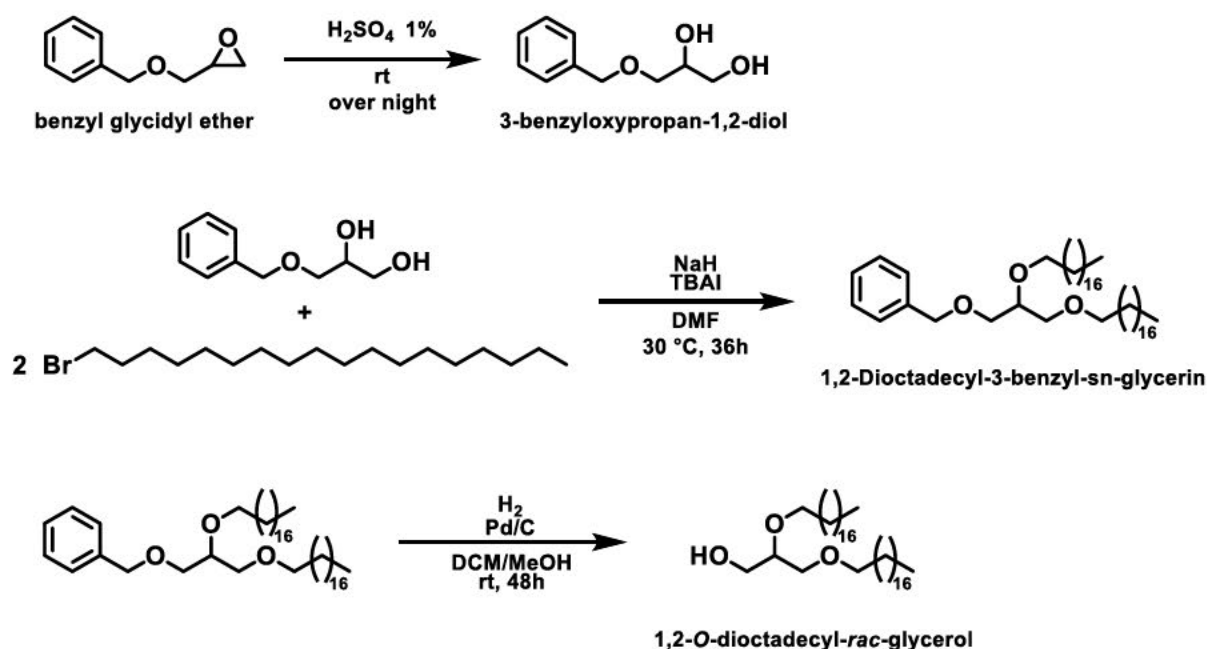


Figure S2. Synthesis of the initiator 1,2-O-di-octadecyl-*rac*-glycerol from benzyl glycidyl ether in a 3-step reaction.

3-Benzyloxypropan-1,2-diol Under rigorous stirring 5 g (4.64 mL, 30 mmol, 1 eq) of benzyl glycidyl ether were added to 150 mL of 1 % aqueous sulfuric acid. The mixture was stirred overnight at room temperature and extracted with 5 x 100 mL ethyl acetate. The combined organic phases were dried over magnesium sulfate, filtered and the solvent was removed by distillation to receive 4.46 g (80 %) of the product as a colorless oil.

^1H NMR (CDCl_3 , 400 MHz) δ [ppm]: 7.37 – 7.29 (m; 5H), 4.55 (s; 2H), 3.91 – 3.86 (m; 1H), 3.71 – 3.51 (m; 4H).

1,2-Distearylbenzyl-glycerolether A 250 mL three neck round bottom flask equipped with a magnetic stirring bar and Dimroth condenser was flame dried under vacuum two times before 1.32 g (55 mmol, 5 eq) of sodium hydride were suspended in a mixture of 25 mL water free DMF and 25 mL water free THF. The mixture was cooled to 0 °C in an ethanol cooling bath and 2g (11 mmol, 1 eq) of 3-benzyloxypropan-1,2-diol were slowly added in portions *via* syringe. After complete addition, the mixture was stirred for 60 min at room temperature. 15 g (45 mmol, 4.1 eq) of octadecyl bromide and 0.81 g (2.2 mmol, 0.2 eq) of tetra-*n*-butylammonium iodide were added and the mixture was stirred for 72 h at room temperature. The reaction was quenched with 50 mL of saturated aqueous ammonium chloride solution and stirred for 30 minutes. The mixture was extracted with 4 x 50 mL diethyl ether. The combined organic phases were washed with 2 x 20 mL of deionized water, dried over magnesium sulfate, filtered and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica (cyclohexane:EtOAc 20:1 ; R_f = 0.40) to receive 4.14 g (55 %) of the product as a colorless solid.

^1H NMR (CDCl_3 , 400 MHz) δ [ppm]: 7.34 – 7.27 (m; 5H), 4.55 (s; 2H), 3.63 – 3.41 (m; 9H), 1.60 – 1.51 (m; 2H), 1.25 (s; 60H), 0.90 – 0.86 (t; J = 6.92 Hz; 6H).

1,2-*O*-dioctadecyl-*rac*-glycerol 900 mg (1.3 mmol, 1 eq) of 1,2-distearylbenzyl-glycerolether were dissolved in DCM in a 50 mL Schlenk flask. 90 mg (0.1 mmol; 0.1 eq (wt)) of Pd/C 10% were suspended in 5 mL EtOH and carefully added under stirring and Argon atmosphere. The flask was sealed and vacuum was applied until the solvent just started to evaporate. The flask was then flooded with Argon and this process was repeated 2 more times. Subsequently, a hydrogen balloon was connected to the flask and vacuum was applied again until the solvent just started to evaporate. This time, the flask was flooded with hydrogen. This process was also repeated 2 more times to

receive a saturated hydrogen atmosphere in the flask. After stirring for 48 h under hydrogen atmosphere, the balloon was removed and the mixture was filtered to remove the catalyst. The solvent was removed under reduced pressure to receive 720 mg (92 %) of the product as a colorless solid.

^1H NMR (CDCl_3 , 400 MHz) δ [ppm]: 3.75 – 3.42 (m; 9H), 1.59 – 1.54 (m; 4H), 1.25 (s; 60H), 0.90 – 0.86 (t; $J = 6.85$ Hz; 6H).

^{13}C NMR (CDCl_3 , 101 MHz) δ [ppm]: 78.33, 72.01, 71.07, 70.55, 63.26, 32.08, 30.25, 29.86, 29.78, 29.63, 29.52, 26.26, 22.85, 14.28.

2.3. Polymerization procedure

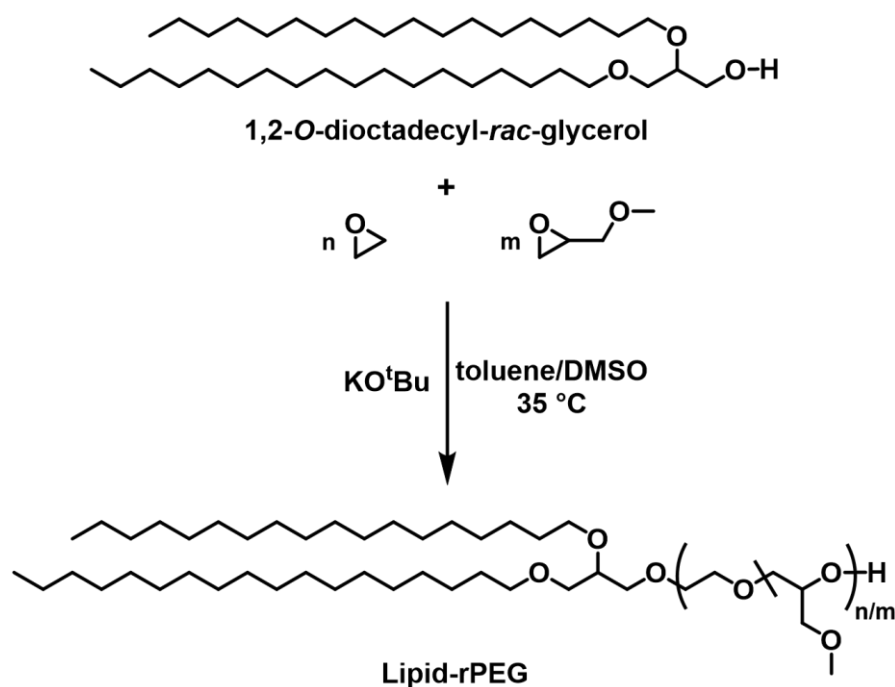


Figure S3. Anionic ring-opening copolymerization of EO and GME using 1,2-*O*-di-octadecyl-*rac*-glycerol as an initiator.

The composition of the copolymers in the Supporting Information is described with $\text{Lipid-rPEG}_{D_p}^{f_{\text{GME}}}$, where f_{GME} is the relative molar content of GME in the polymer backbone and D_p is the degree of polymerization.

Polymer synthesis was performed with identical procedures for most copolymers, varying only the ratio of monomers in the reaction mixture. It is exemplary described for the synthesis of Lipid-rPEG₄₃^{0.55} in the following paragraph. Generally, yields varied between 65 % and 80 %. For Lipid-PEG₄₆ only EO was used as a monomer.

GME was freshly dried before every polymerization by stirring over calcium hydride overnight in a flame-dried flask and subsequently cryo-transferred immediately before use. 135.3 mg of 1,2-*O*-dioctadecyl-*rac*-glycerol (227 μ mol, 1.00 eq) were dissolved in 5 mL of benzene and transferred into a flame-dried flask equipped with a stirrer bar, stop cock, and glass stopper. 24.9 mg of potassium *tert*-butoxide (222 μ mol, 0.98 eq) were dissolved in 3 mL freshly distilled THF and added to the solution in the flask. The mixture was stirred for 20 min before the solvents were slowly removed under vacuum at room temperature. The resulting initiator salt was dried at 60 °C under high vacuum overnight. After cooling to room temperature, the flask was flooded with Argon. Under Argon flow the glass stopper was removed and 3 mL of water free toluene and 1.5 mL of water free DMSO were added to the initiator salt. The flask was subsequently sealed again cooled to – 90 °C with an ethanol/nitrogen cooling bath. 509 μ L of GME (499 mg, 5.7 mmol, 25 eq) were added *via* syringe into the cooled flask. Vacuum was applied again to the flask and 240 μ L of ethylene oxide (5.7 mmol, 25 eq) were condensed into a graduated ampule at – 90 °C and then cryo-transferred to the reaction mixture. The flask was sealed and the coolingbath was removed. The mixture was warmed in a room temperature water bath while an oil bath was preheated to 45 °C. As soon as the solvents were thawed, the water bath was removed and the flask was brought to 45 °C with the preheated oil bath. After the initiator was completely dissolved and the solution was clear, the oil bath was slowly cooled down to 35 °C. At this temperature, the reaction mixture was stirred for 48 h. The polymerization was terminated by flushing the flask with argon and adding a mixture of 1M HCl and methanol (1:1, 1 mL). The reaction mixture was taken up in chloroform (20 mL) and extracted with 2 x 10 mL of deionized water and subsequently with 1 x 10 mL of brine. The organic phase was dried over MgSO₄, filtered, and the solvent was removed under reduced pressure. The polymer was dialyzed against methanol (regenerated cellulose, MWCO = 1000 g mol⁻¹) for 24 h, affording an amorphous pale-yellow solid (640 mg, 72 %).

¹H NMR (CDCl₃, 400 MHz) δ [ppm]: 3.75 – 3.37 (m; 234H), 3.36 – 3.16 (m; 81H), 1.56 – 1.52 (m; 4H), 1.24 (m; 60H), 0.88 – 0.85 (t; *J* = 6.87 Hz; 6H).

2.4. Synthesis of PEGylated and rPEGylated SiO₂ nanocapsules

The nanocapsules were synthesized in an oil water miniemulsion based on a previously published synthesis for the hydrolysis and condensation of alkoxysilanes.¹ 2.0 g (9.6 mmol) of tetraethoxysilane (TEOS) and 125 mg of hexadecane were dissolved in 1 g of chloroform. This dispersed phase was mixed under stirring with an aqueous solution of cetyltrimethylammonium chloride (CTMA-Cl, 30 mL at 0.77 mg mL⁻¹). Pre-emulsification was performed by stirring at 1,000 rpm for 1 h after which the obtained emulsion was sonicated by using a Branson 450 W sonifier with a ½'' tip at 70 % amplitude for 180 s (30 s of sonication, 10 s of pause) under ice cooling. Subsequently, the emulsion was stirred at 1,000 rpm for 12 h at room temperature to obtain an aqueous dispersion of SiNCs.

PEGylation and rPEGylation of the NCs was performed by replacement of the templating surfactant CTMA-Cl with Lutensol® AT50 or one of the synthesized polyether samples. For this, 70 mg of Lut AT50 or an equivalent molar amount of the synthesized polyether (see **Table S1**) were added to 2 mL of the SiNC dispersion and stirred at 1,000 rpm for 2 h before it was dialyzed against Millipore water in a dialysis tube with a MWCO of 1,000 g mol⁻¹ to remove CTMA-Cl ($M_w = 320 \text{ g mol}^{-1}$) while the polymers stayed inside. Afterwards, the dialyzed dispersion was centrifuged at 12,000 g to remove the excess of Lutensol® surfactant. The pellet was redispersed in water and again stirred at 1,000 rpm for 24 h. The samples were stored at room temperature and protected from light under constant agitation.

Table S1. Amount of polyether used for synthesis of the silica nanocapsules.

Polymer	amount used for NC synthesis / mg
Lipid-PEG₄₆	76.4
Lipid-rPEG₅₁^{0.17}	92.6
Lipid-rPEG₄₉^{0.28}	101.2
Lipid-rPEG₅₁^{0.42}	108.3
Lipid-rPEG₄₃^{0.55}	101.2

2.5. Protein corona preparation and analysis

For **preparation of the protein corona**, NCs with a total surface area of 0.05 m² were incubated in 1 mL of human citrate plasma for 1h at 37 °C under constant shaking. Afterwards, the protein coated NCs were isolated *via* centrifugation at 20 000 g (1 h at 4 °C) after which the supernatant was discarded. The pellet was resuspended in 1 mL PBS and the suspension was again centrifuged at 20 000 g for 1 h at 4 °C. The washing steps were repeated three times in total. After the last washing step, the pellet was suspended in 100 µL of a Tris*HCl solution (62.5 mM) containing 2 wt% of SDS for protein detachment. The suspension was incubated at 95 °C for 5 min and again centrifuged at 20,000 g for 1 h at 4 °C after which the supernatant was further used for analysis.

3. ^1H NMR (nuclear magnetic resonance) spectra

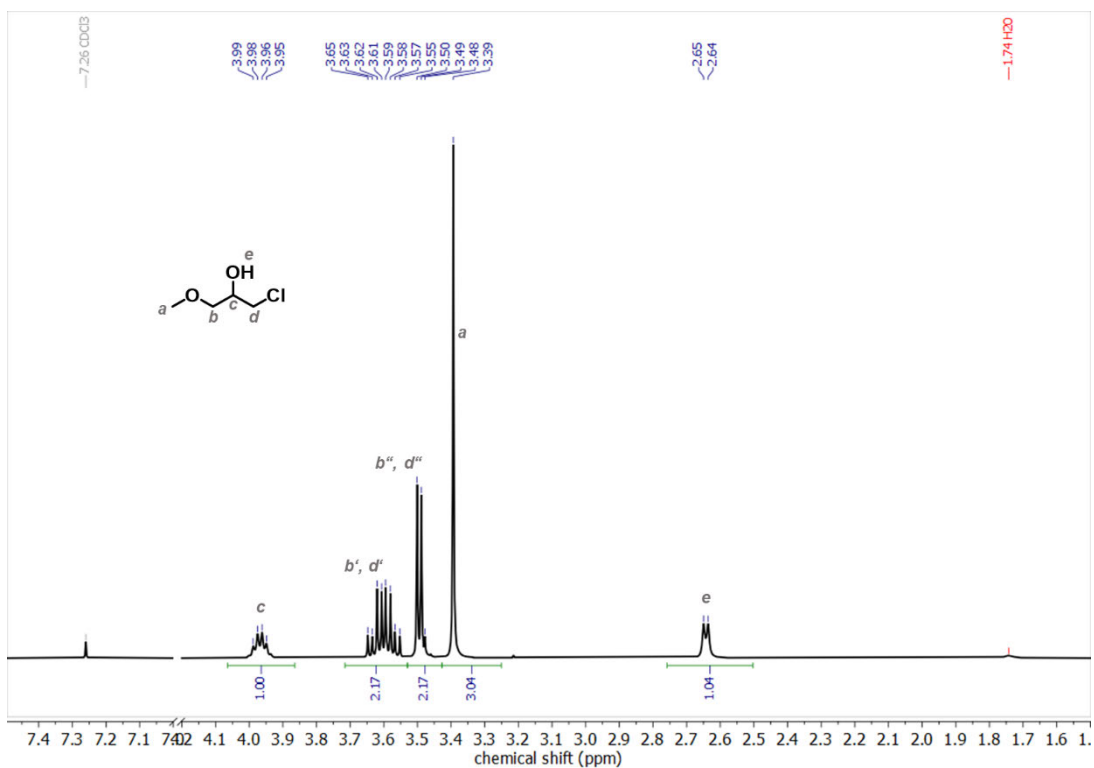


Figure S4. ^1H NMR spectrum (CDCl_3 , 400 MHz) of 1-chloro-3-methoxypropan-2-ol.

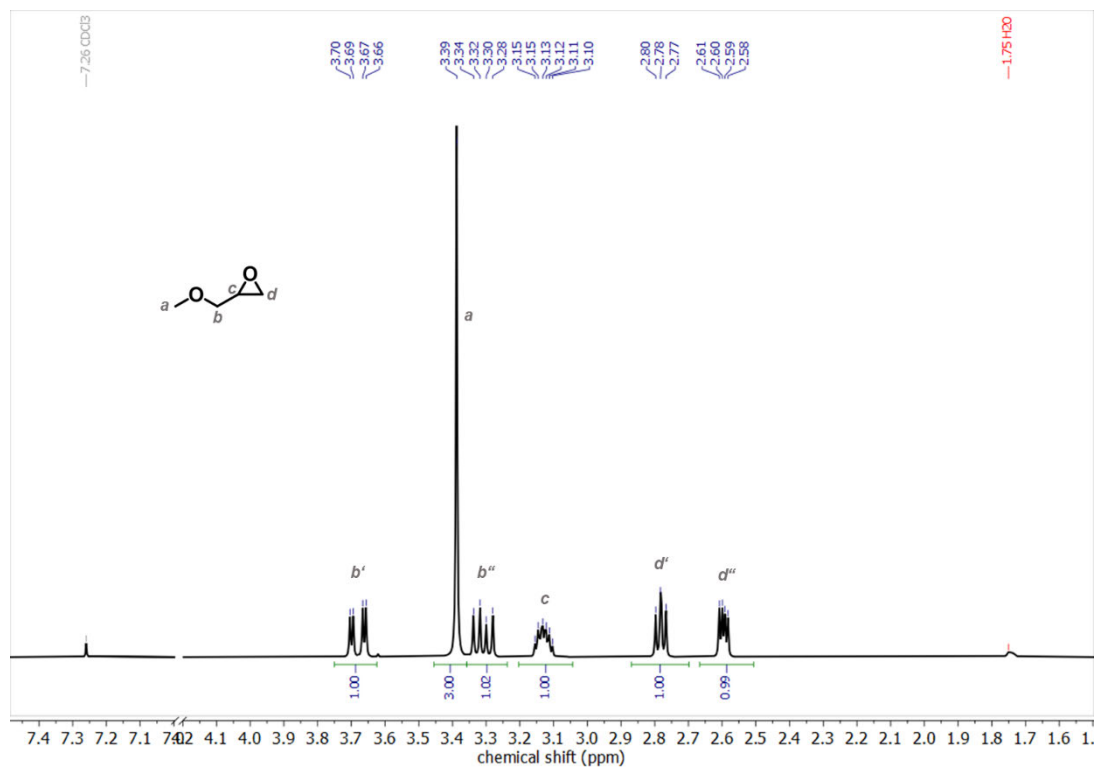


Figure S5. ¹H NMR spectrum (CDCl₃, 400 MHz) of glycidyl methyl ether.

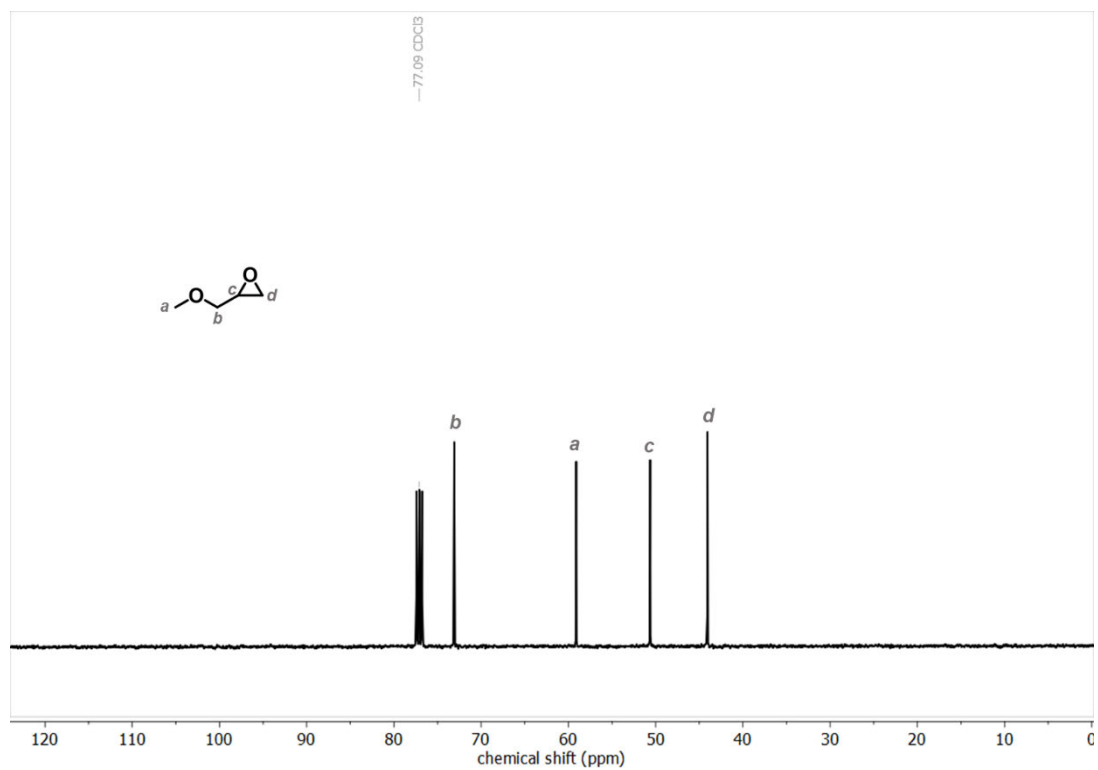


Figure S6. ¹³C NMR spectrum (CDCl₃, 101 MHz) of glycidyl methyl ether.

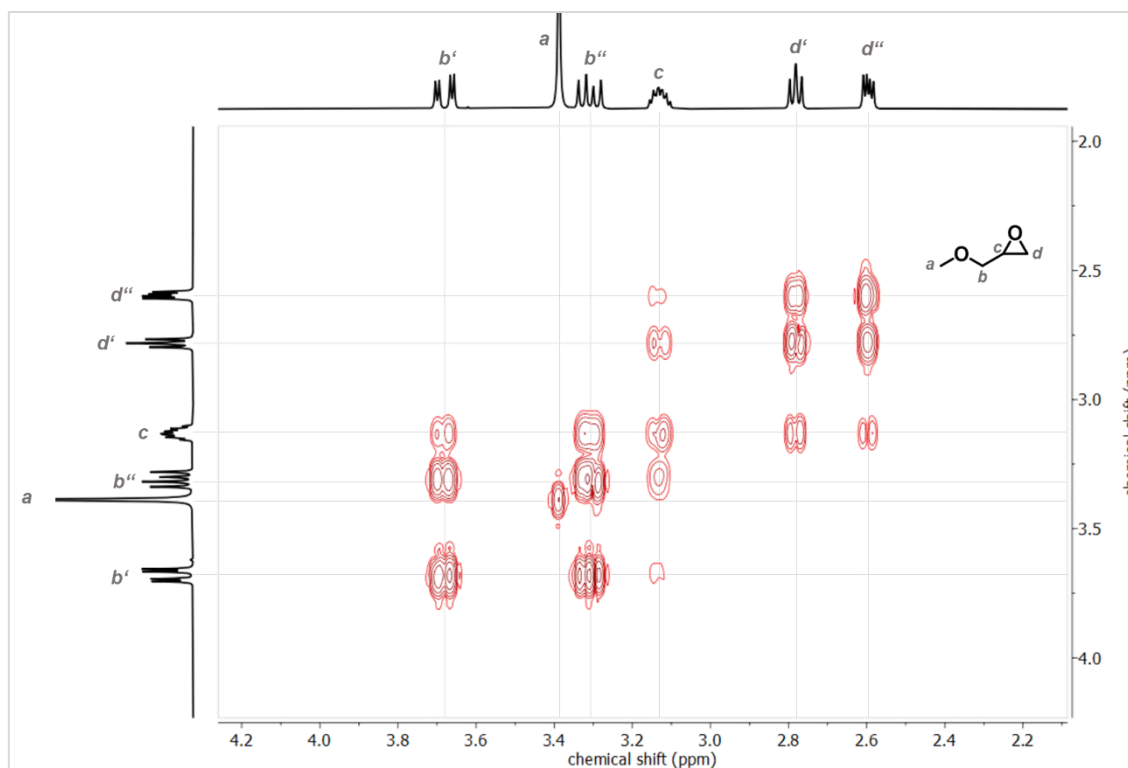


Figure S7. 2D [^1H , ^1H] COSY NMR spectrum of glycidyl methyl ether.

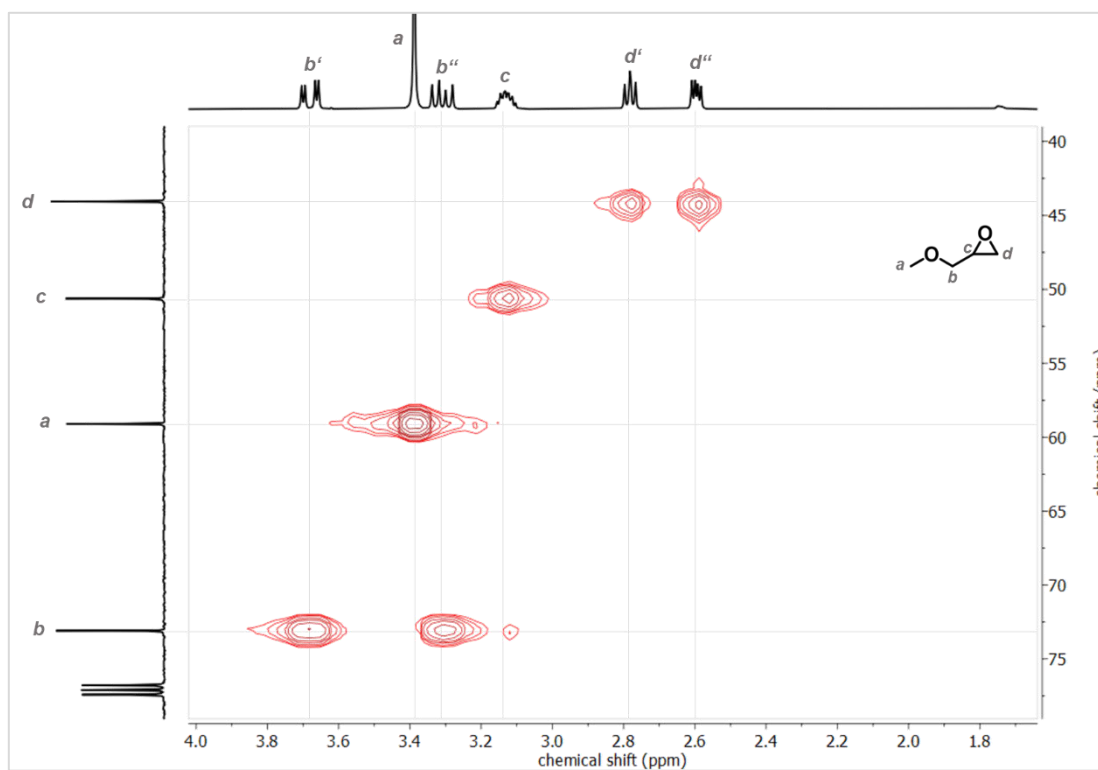


Figure S8. 2D [^1H , ^{13}C] HSQC NMR spectrum of glycidyl methyl ether.

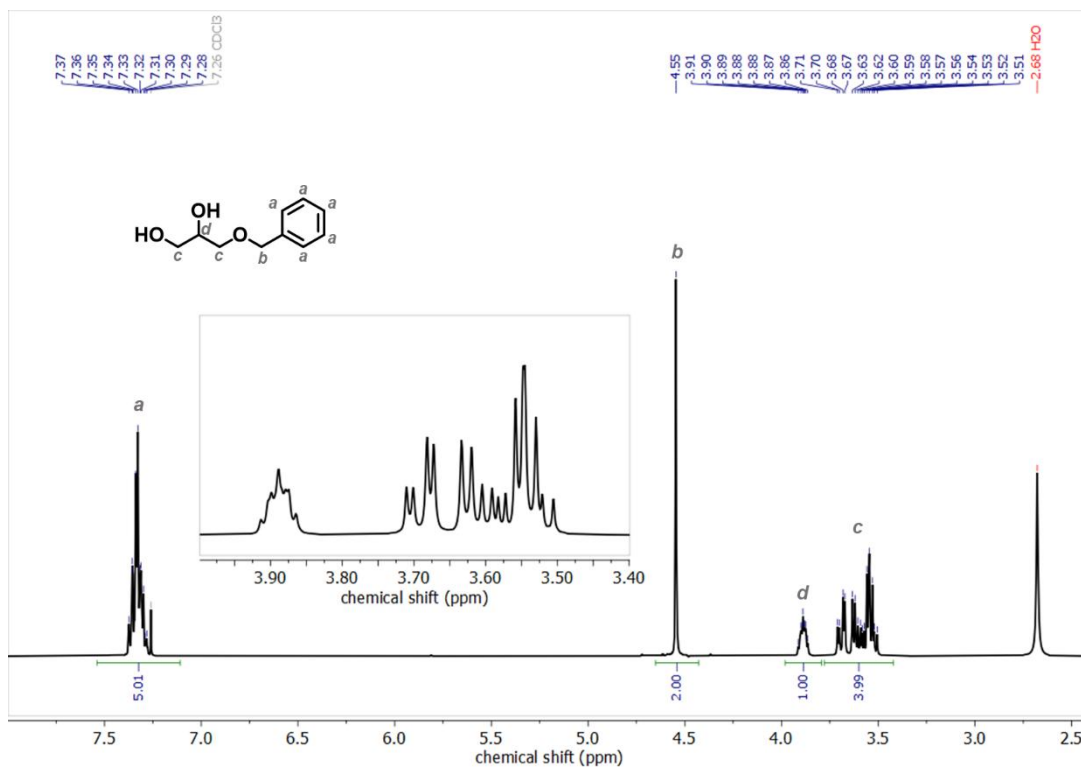


Figure S9. ^1H NMR spectrum (CDCl_3 , 400 MHz) of 3-benzyloxypropan-1,2-diol.

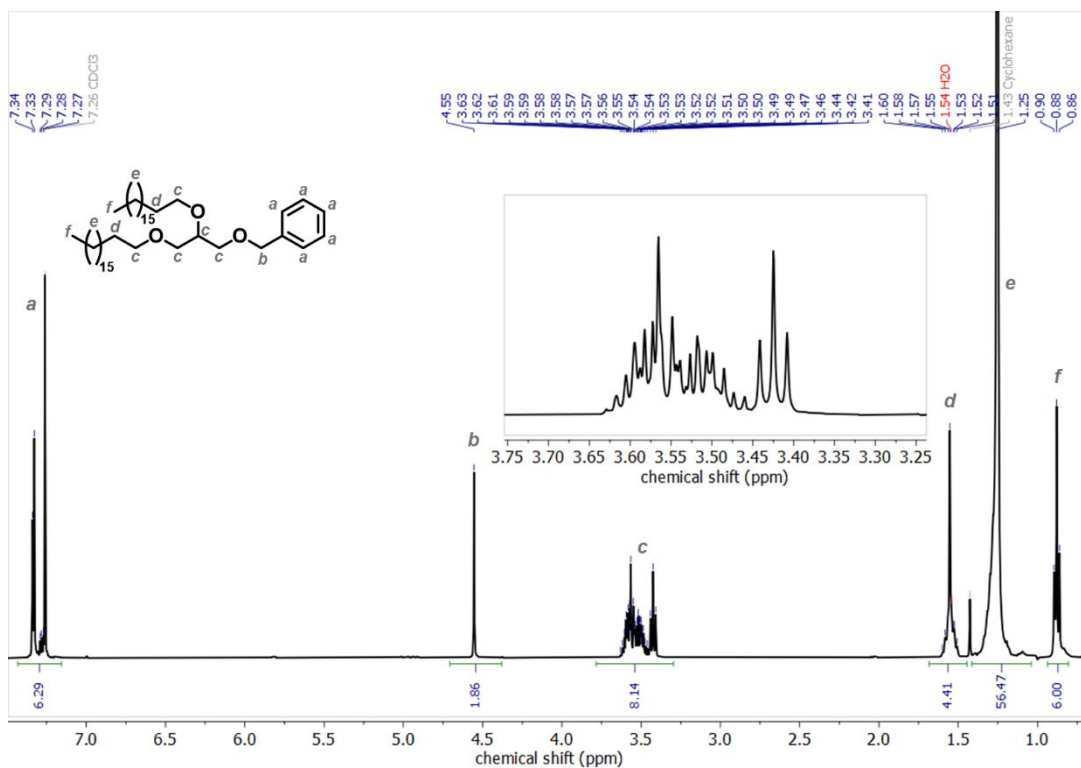


Figure S10. ^1H NMR spectrum (CDCl_3 , 400 MHz) of 1,2-dioctadecyl-3-benzyl-sn-glycerin.

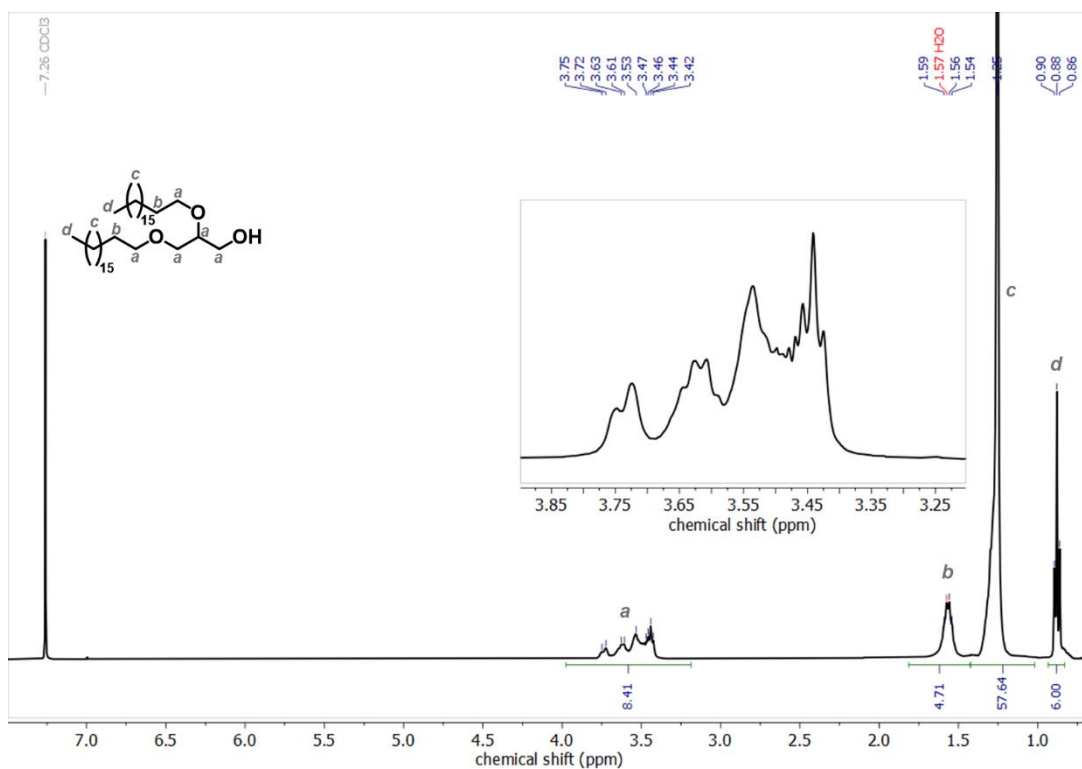


Figure S11. ¹H NMR spectrum (CDCl₃, 400 MHz) of 1,2-O-di-octadecyl-*rac*-glycerol.

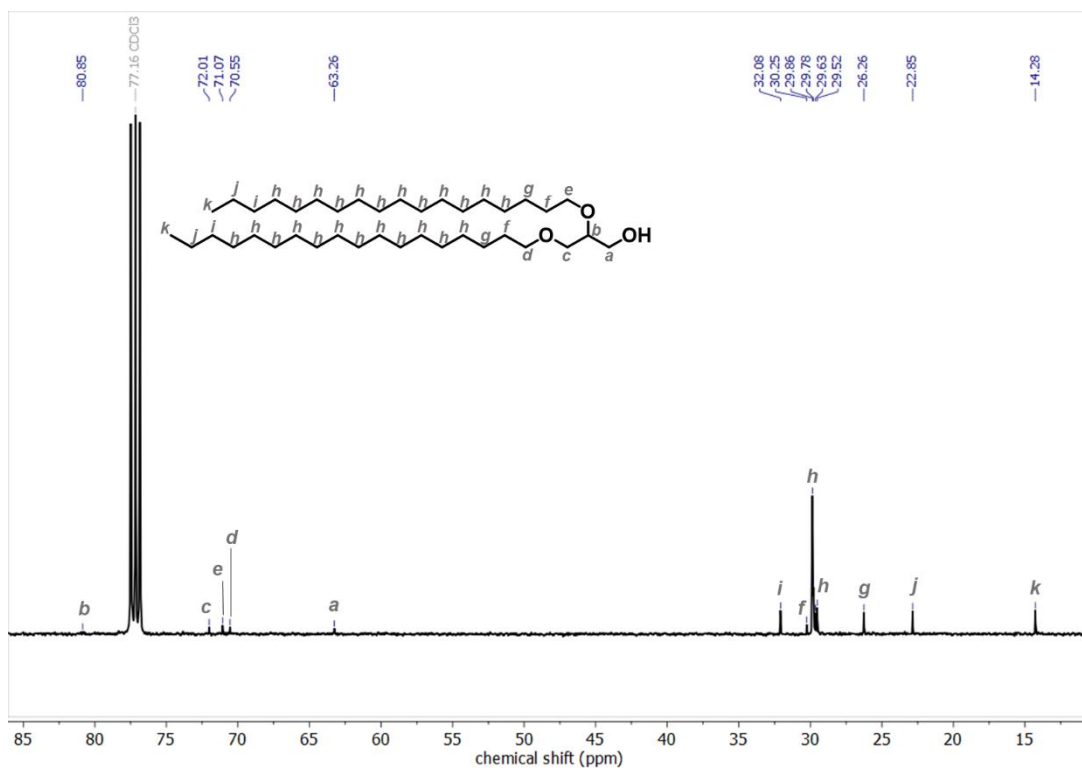


Figure S12. ¹³C NMR spectrum (CDCl₃, 101 MHz) of 1,2-O-di-octadecyl-*rac*-glycerol.

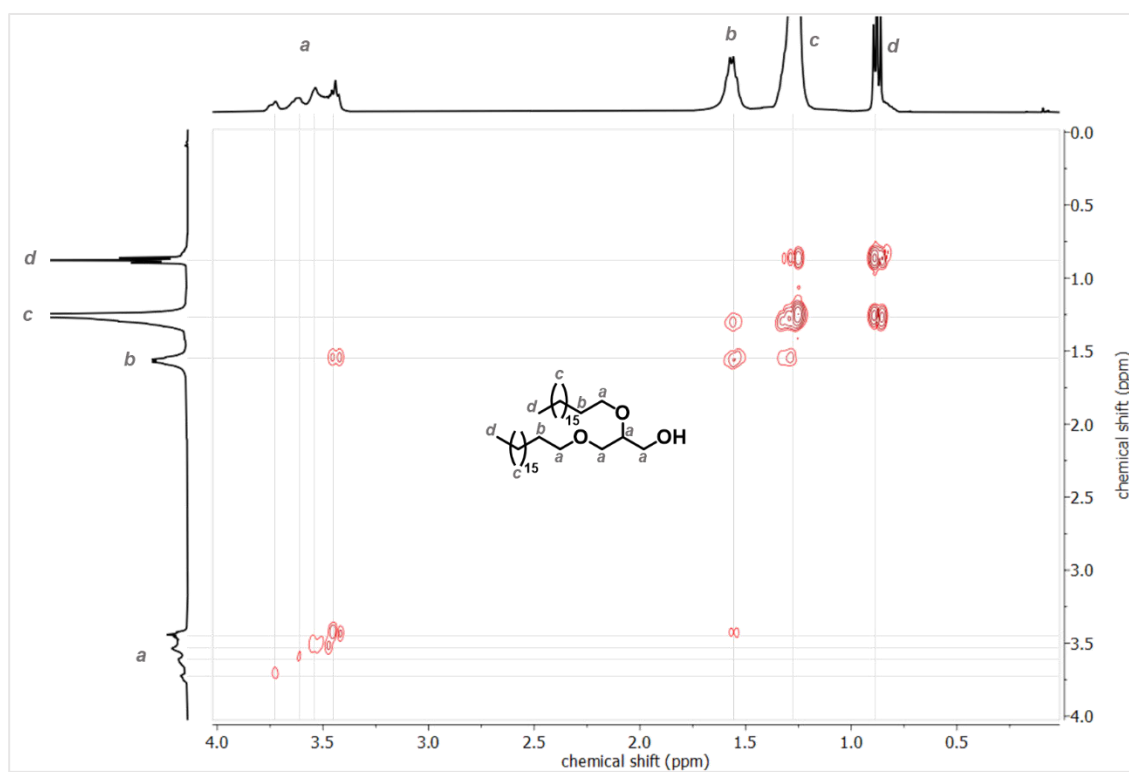


Figure S13. 2D [^1H , ^1H] COSY NMR spectrum of 1,2-*O*-di-octadecyl-*rac*-glycerol.

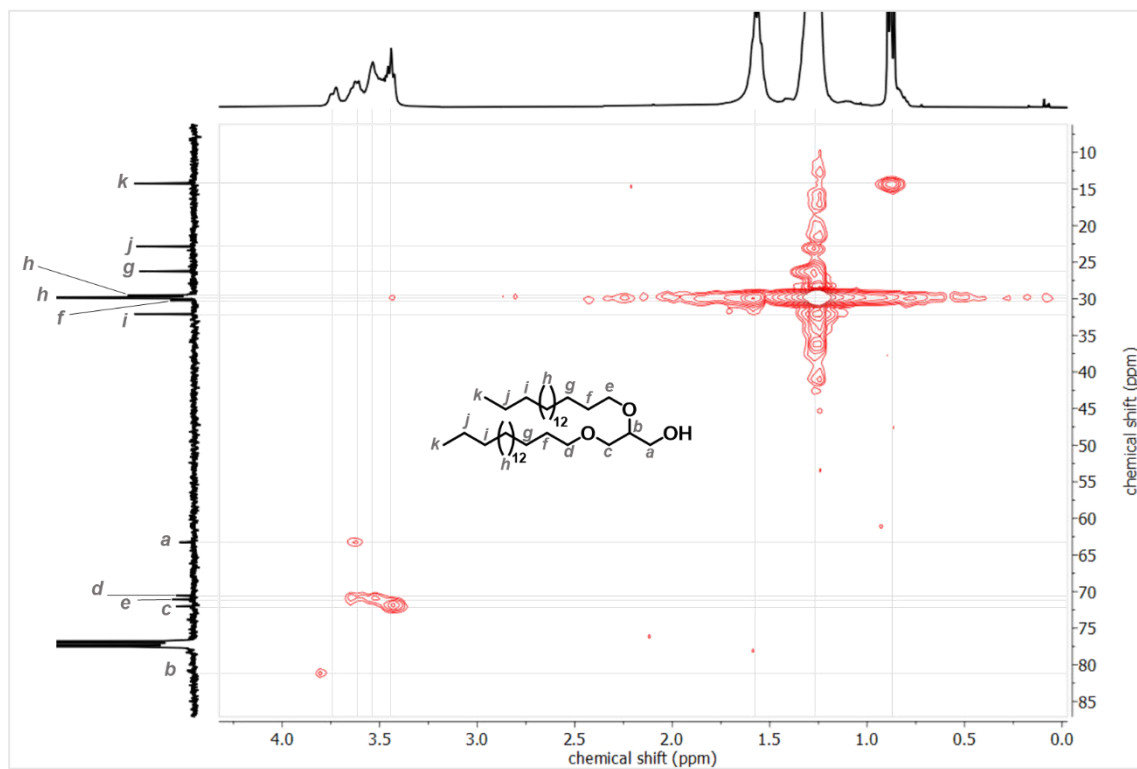


Figure S14. 2D [^1H , ^{13}C] HSQC NMR spectrum of 1,2-*O*-di-octadecyl-*rac*-glycerol.

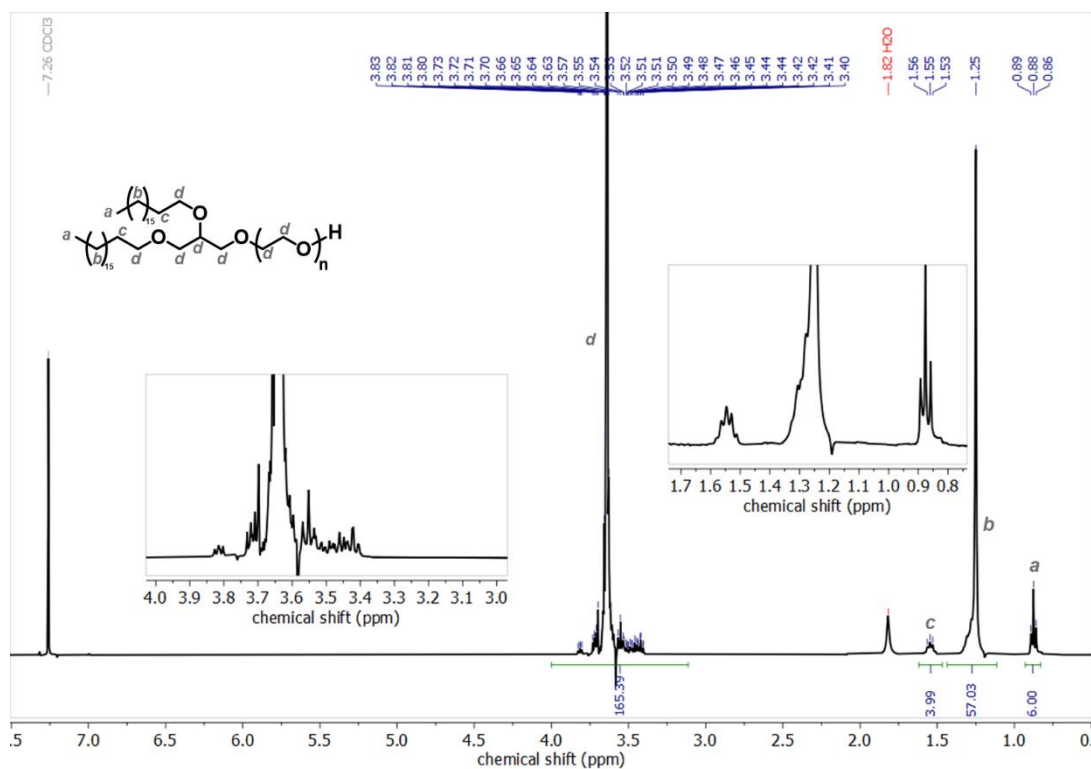


Figure S15. ^1H NMR spectrum (CDCl₃, 400 MHz) of Lipid-PEG₄₆.

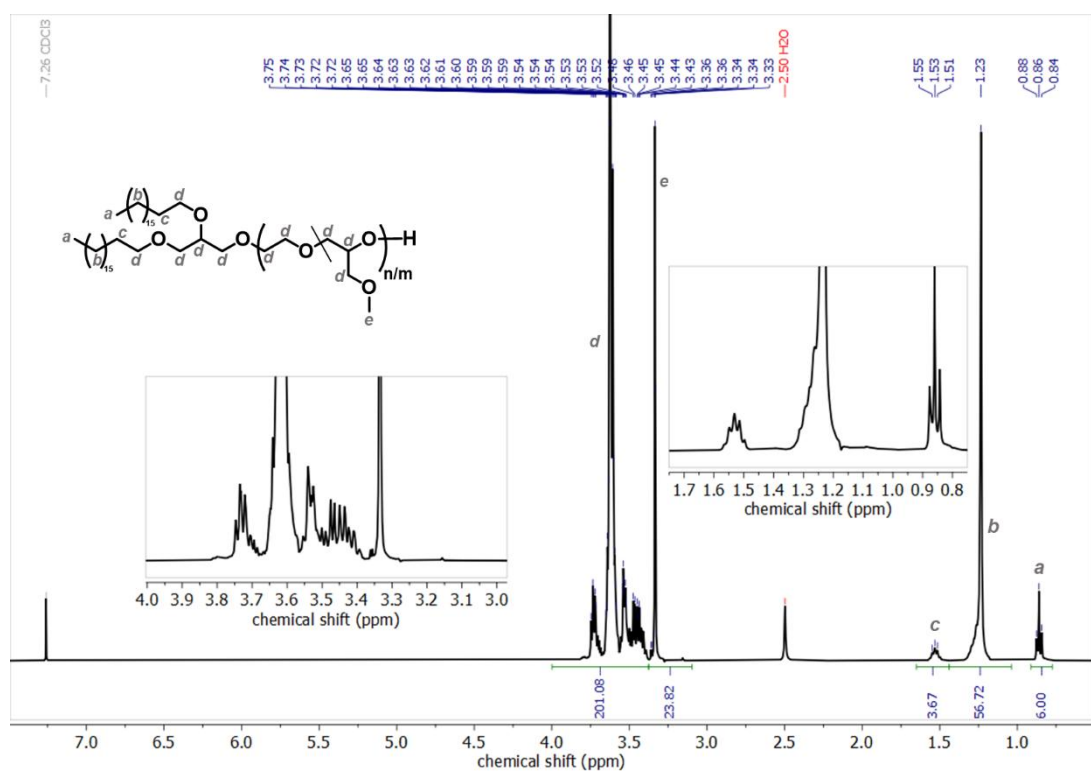


Figure S16. ^1H NMR spectrum (CDCl₃, 400 MHz) of Lipid-rPEG₅₁^{0.17}.

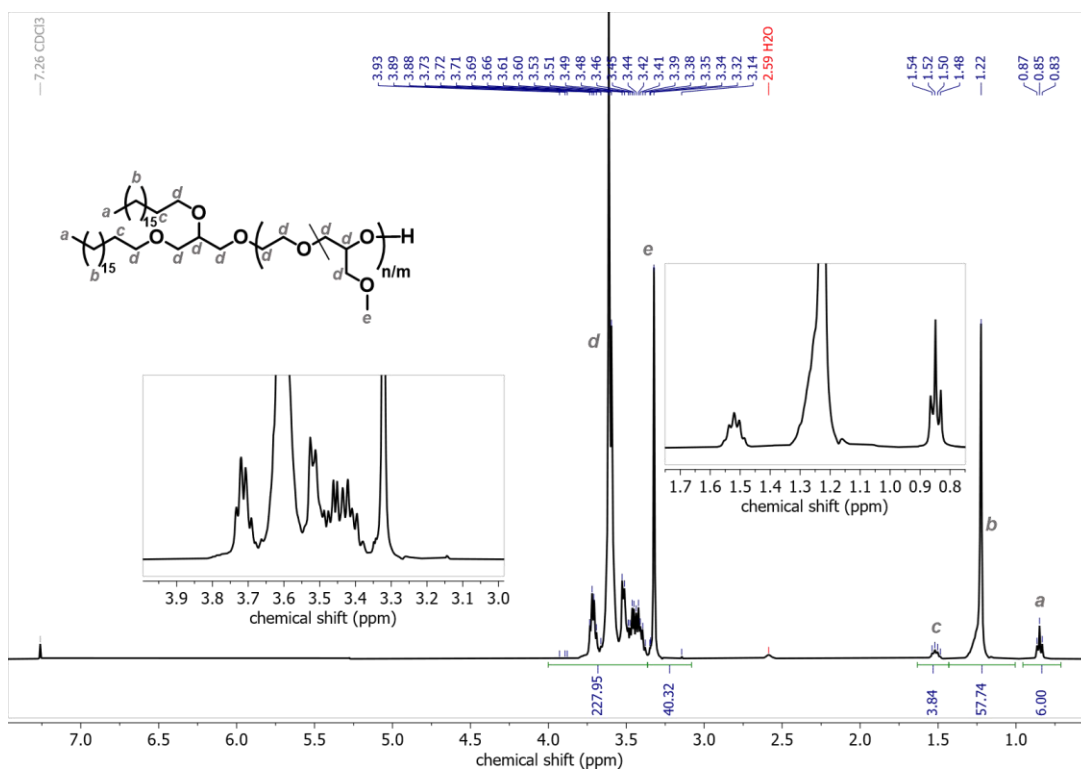


Figure S17. ^1H NMR spectrum (CDCl_3 , 400 MHz) of Lipid-rPEG₅₃^{0.26}.

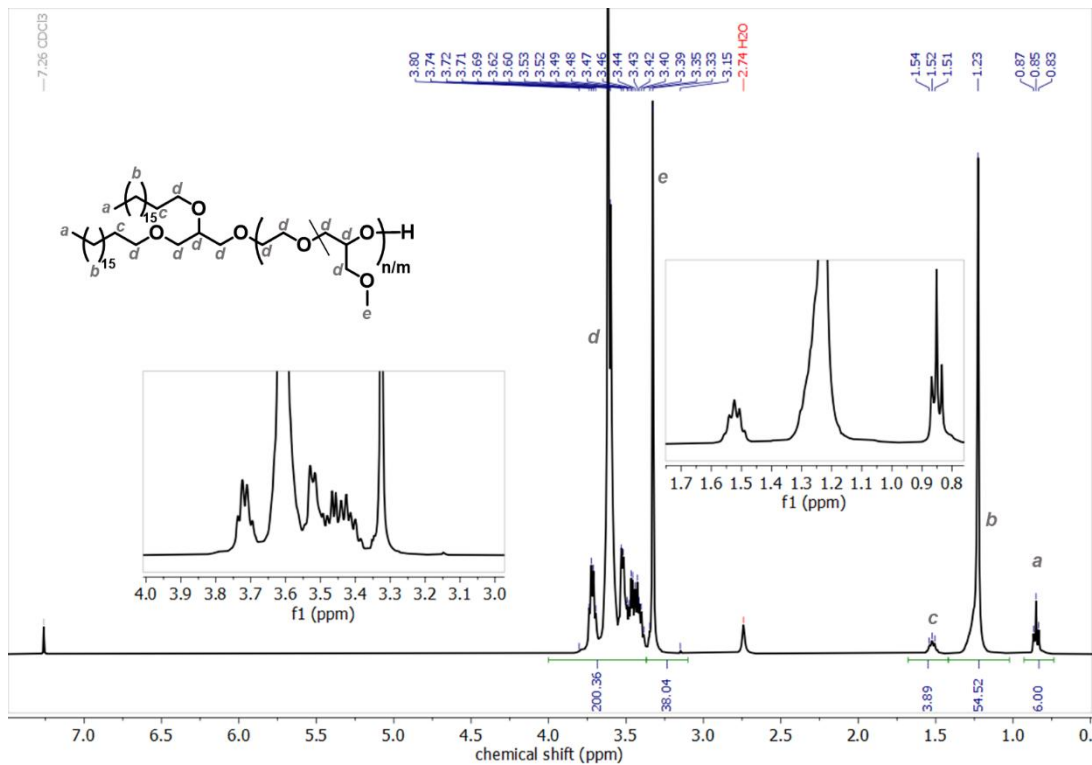


Figure S18. ^1H NMR spectrum (CDCl_3 , 400 MHz) of Lipid-rPEG₄₉^{0.28}.

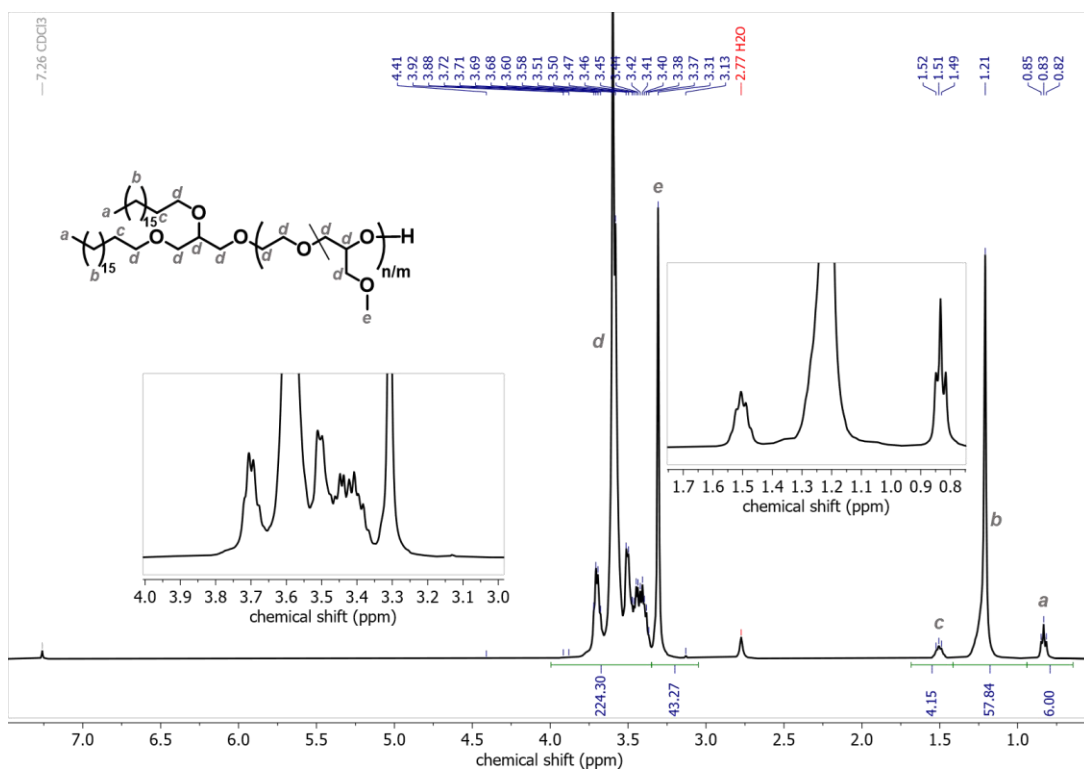


Figure S19. ¹H NMR spectrum (CDCl₃, 400 MHz) of Lipid-rPEG₅₀^{0.29}.

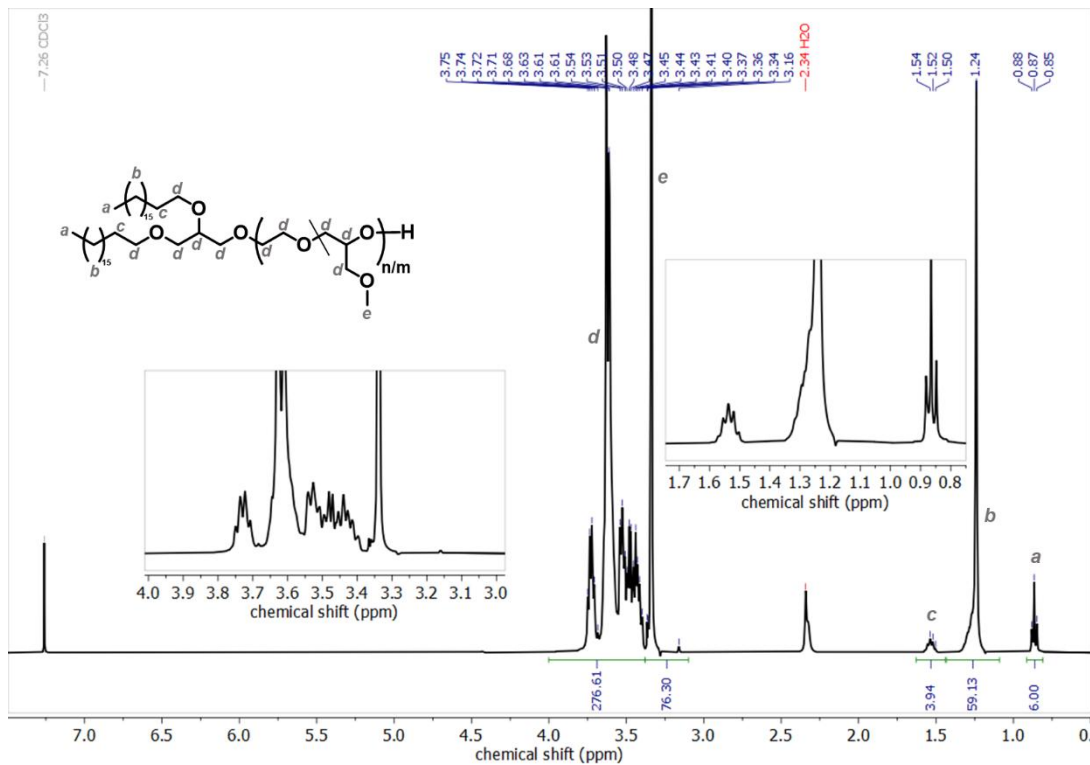


Figure S20. ¹H NMR spectrum (CDCl₃, 400 MHz) of Lipid-rPEG₅₁^{0.42}.

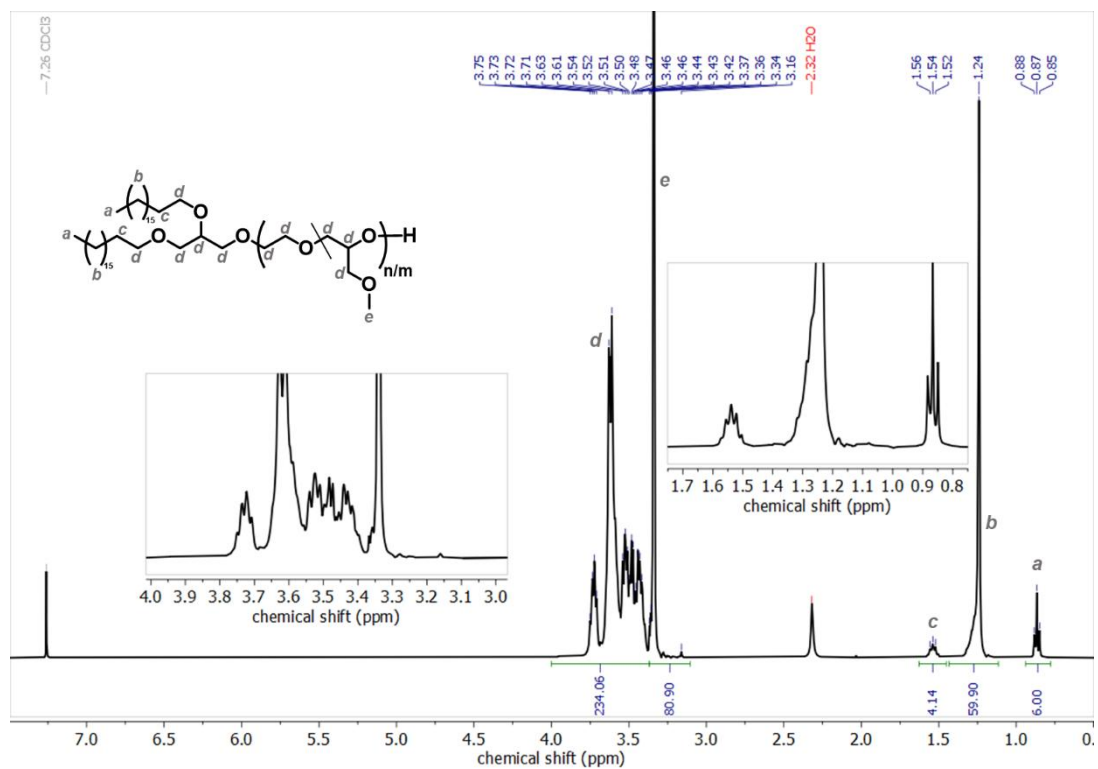


Figure S21. ^1H NMR spectrum (CDCl_3 , 400 MHz) of Lipid-rPEG $_{43}^{0.55}$.

4. Matrix-assisted laser desorption ionization using time-of-flight detection (MALDI TOF) mass spectra

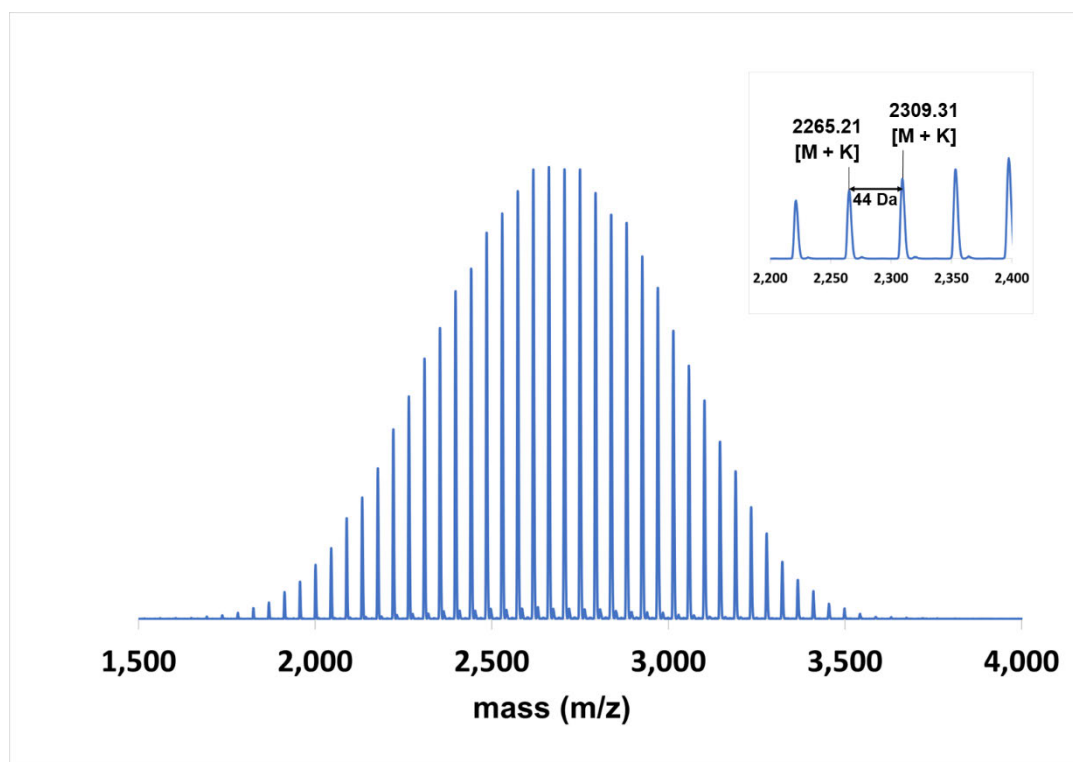


Figure S22. MALDI TOF mass spectrum of Lipid-PEG₄₆.

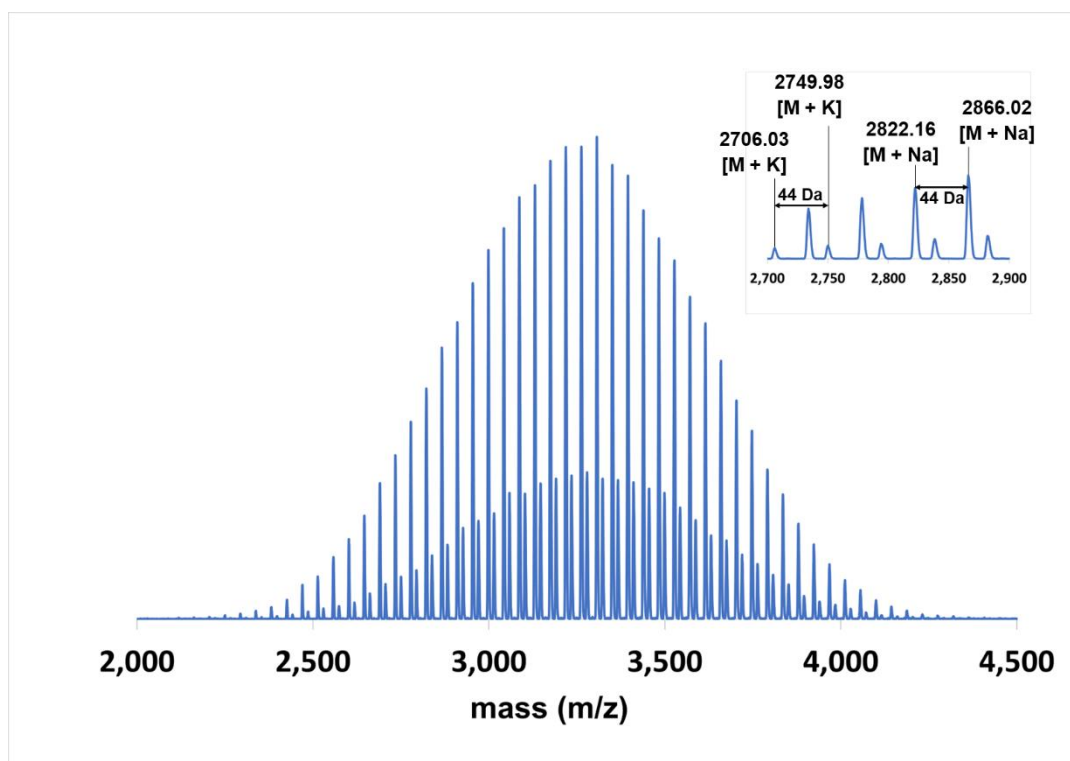


Figure S23. MALDI TOF mass spectrum of Lipid-rPEG₅₁^{0.17}.

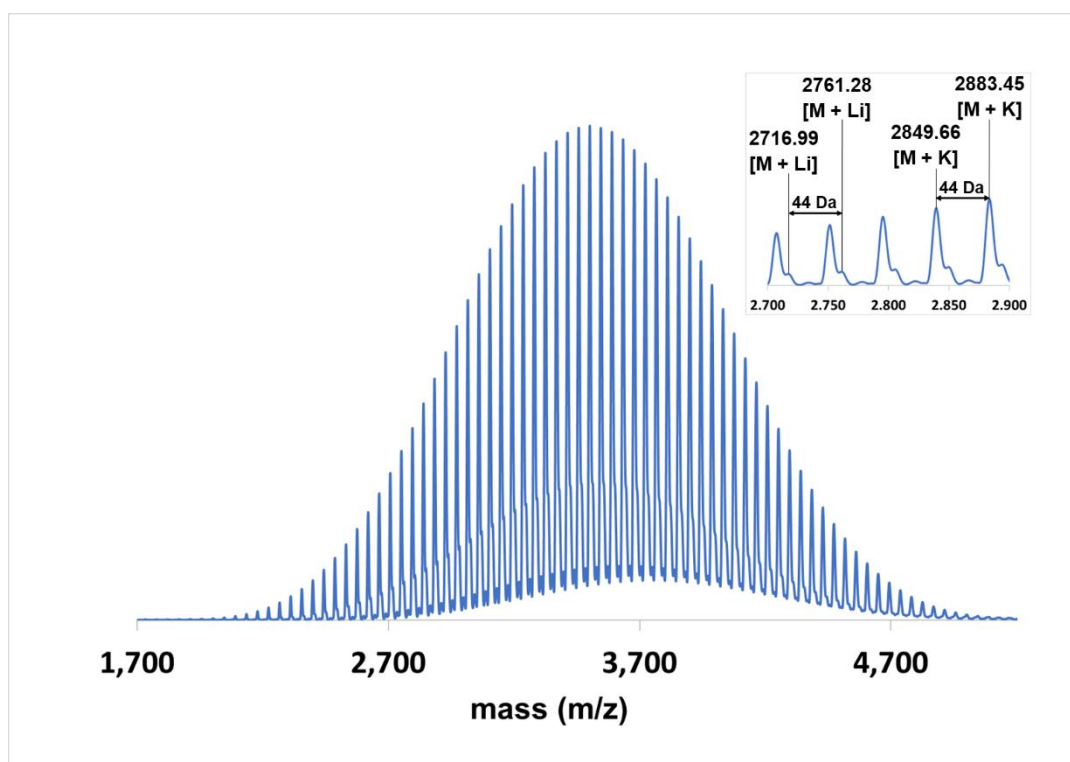


Figure S24. MALDI TOF mass spectrum of Lipid-rPEG₅₃^{0.26}.

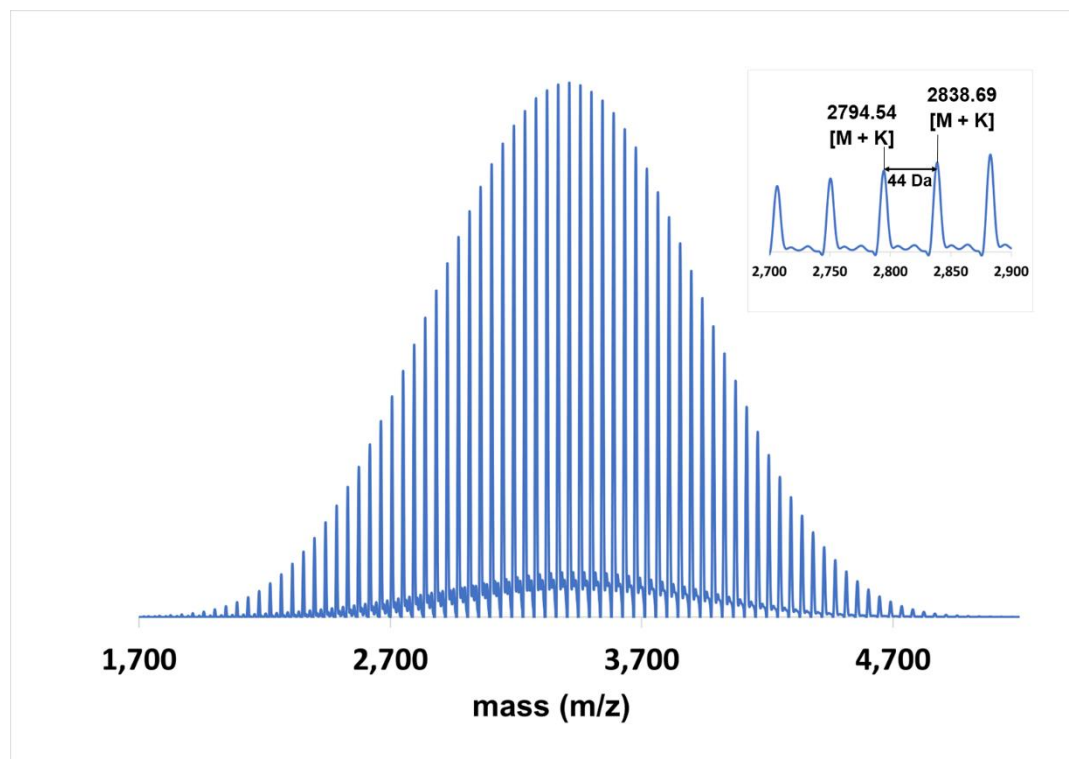


Figure S25. MALDI TOF mass spectrum of Lipid-rPEG₄₉^{0.28}.

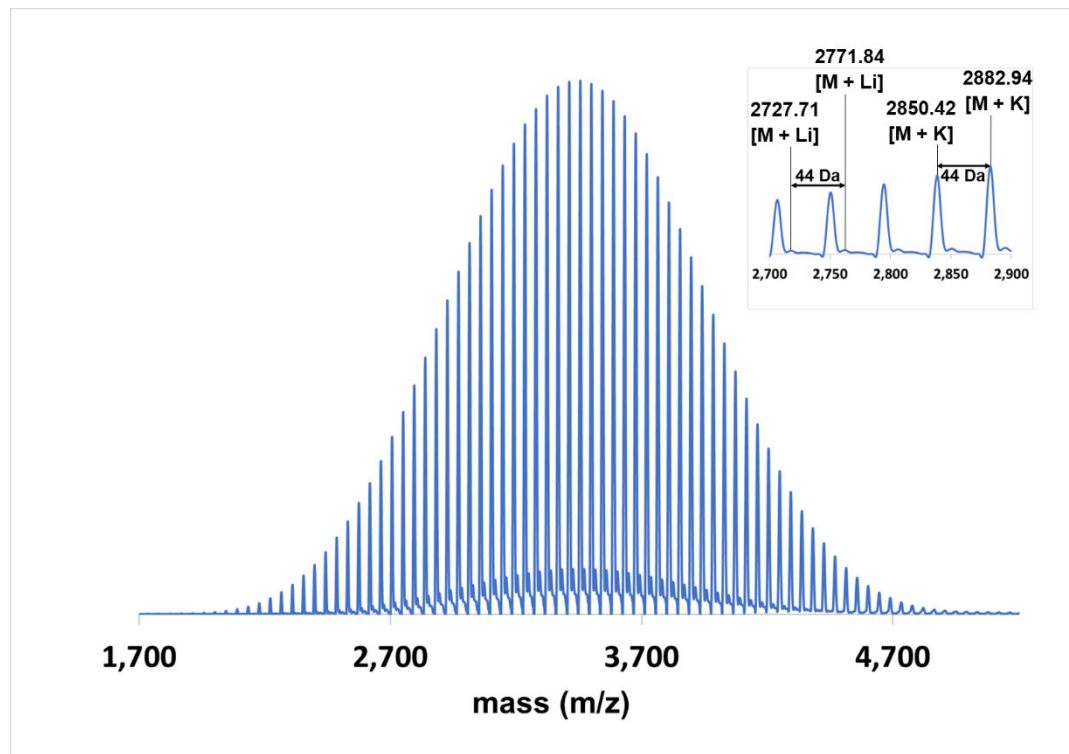


Figure S26. MALDI TOF mass spectrum of Lipid-rPEG₅₀^{0.29}.

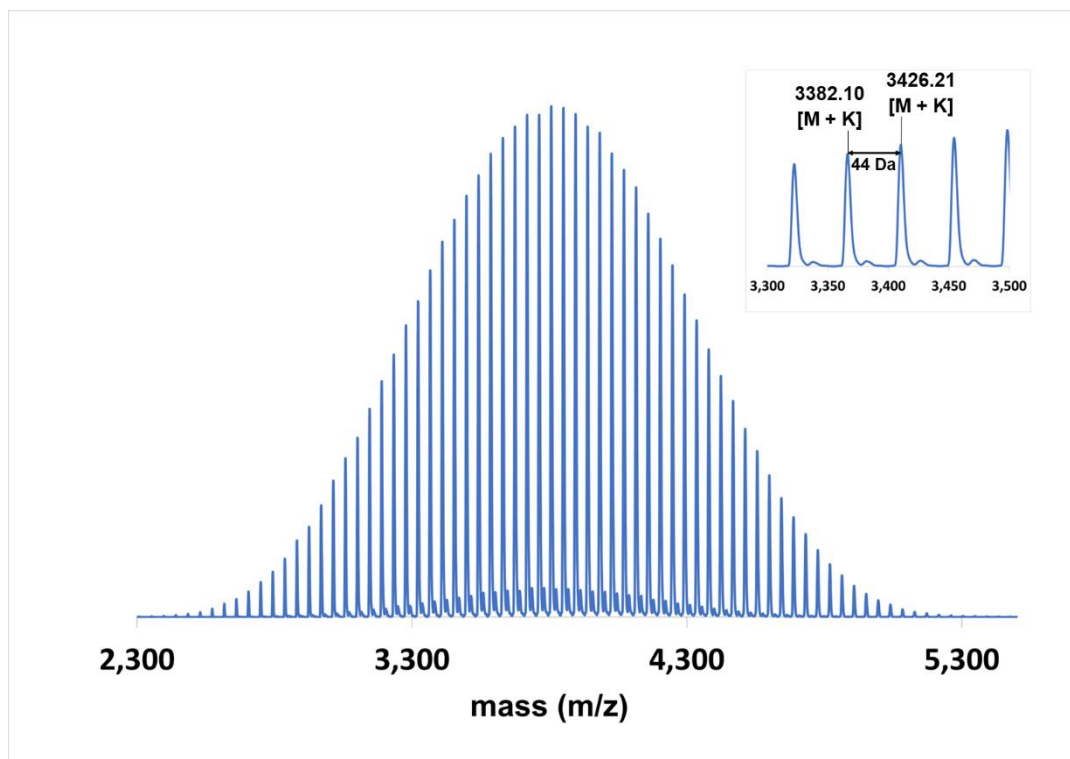


Figure S27. MALDI TOF mass spectrum of Lipid-rPEG₅₁^{0.42}.

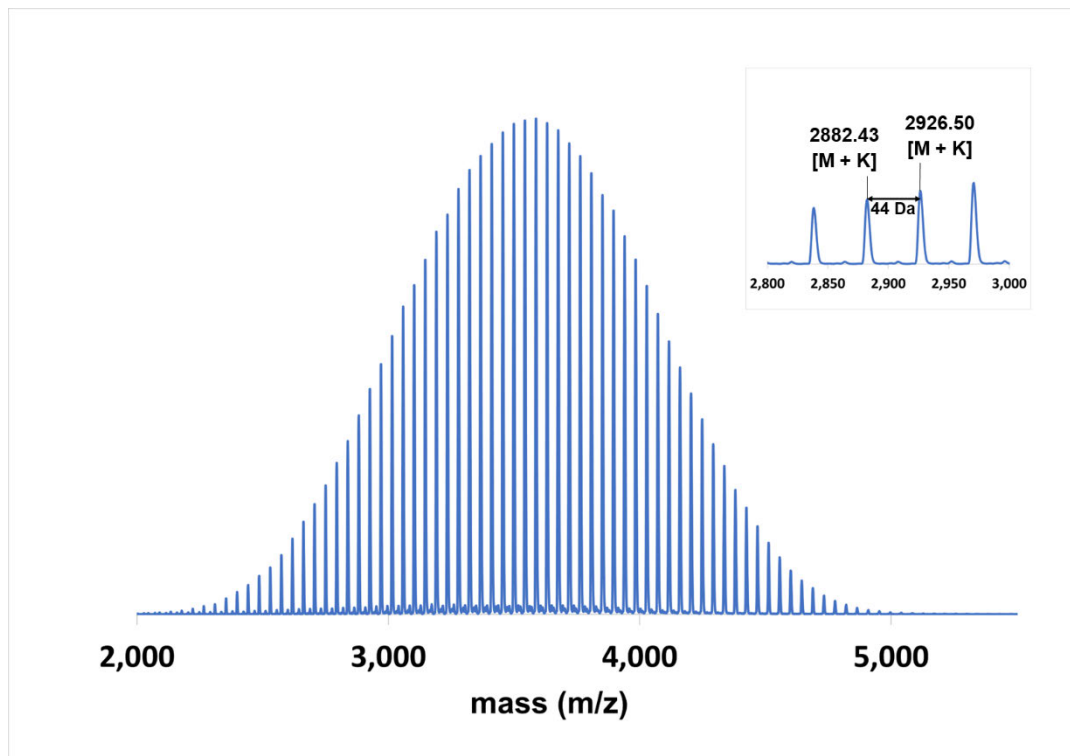


Figure S28. MALDI TOF mass spectrum of Lipid-rPEG₄₃^{0.55}.

5. Size exclusion chromatography (SEC) curves

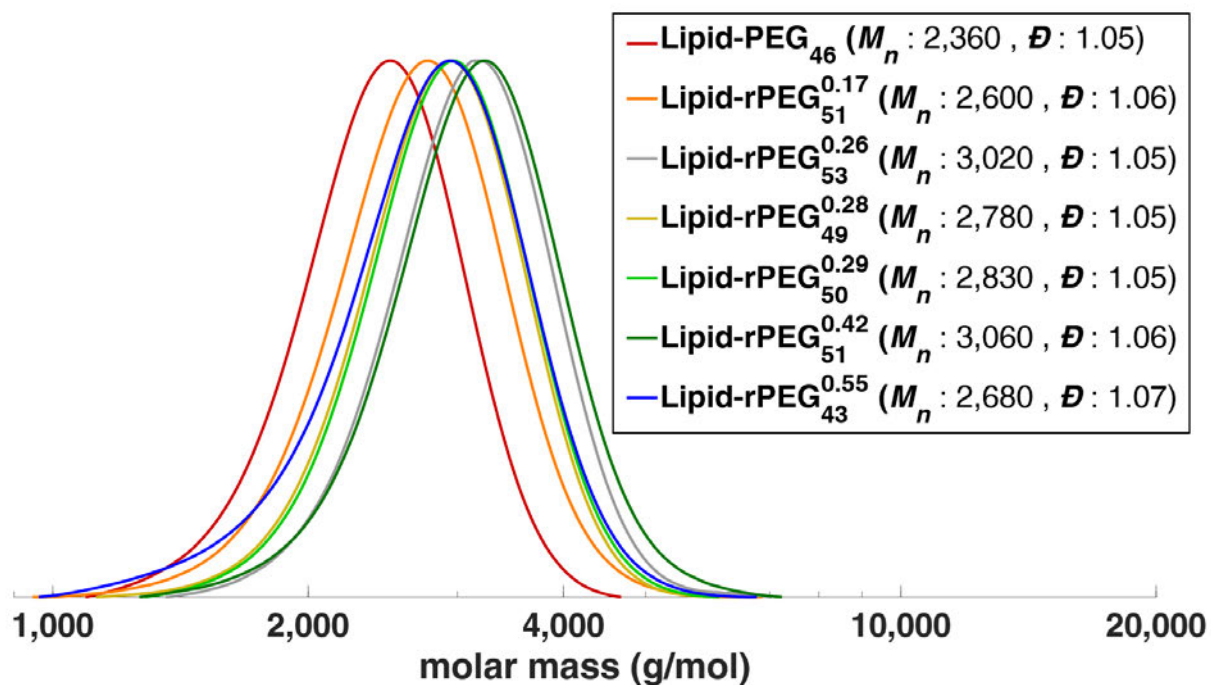


Figure S29. SEC curves (solvent: DMF; calibration: PEG) of the synthesized copolymers.

6. Differential scanning calorimetry (DSC)

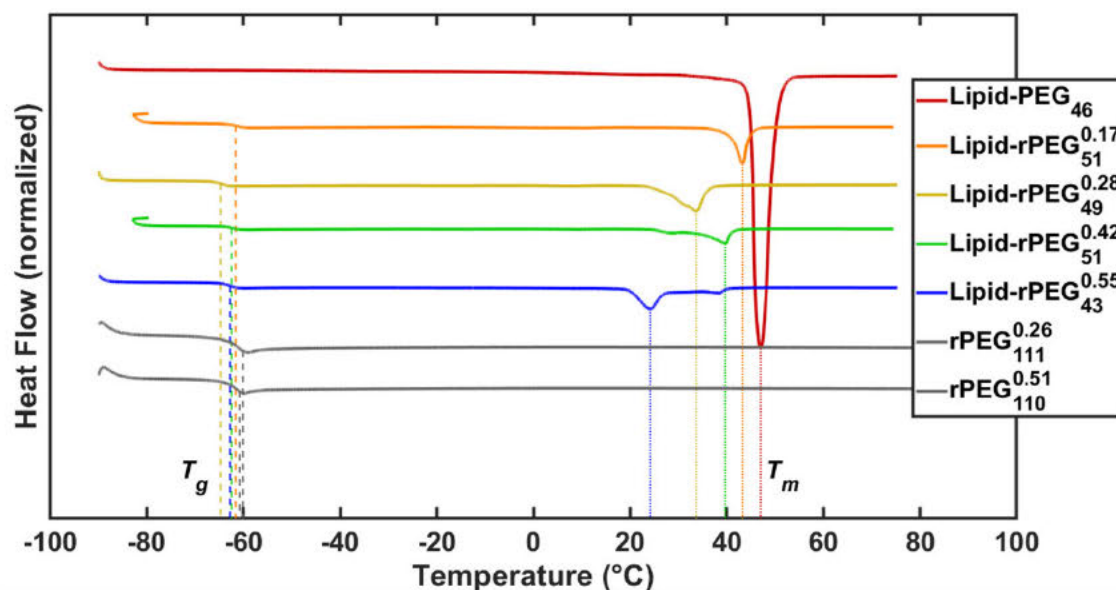


Figure S30. DSC curves of copolymers of different compositions. Glass transition temperatures T_g and melting temperatures T_m are marked in the graph. For comparison, two measurements of methoxy-rPEG are also depicted (gray curves) to show the absence of T_m if the copolymers do not possess a lipid group.

Table S2. DSC data of the synthesized polymers.

Polymer	T_g / °C	T_m / °C	ΔH_m / J·g ⁻¹
Lipid-PEG ₄₆	n.a.	47	154.94
Lipid-rPEG ₅₁ ^{0.17}	– 62	43	16.73
Lipid-rPEG ₄₉ ^{0.28}	– 65	34	23.43
Lipid-rPEG ₅₁ ^{0.42}	– 63	40	13.76
Lipid-rPEG ₄₃ ^{0.55}	– 63	24	21.76
rPEG ₁₁₁ ^{0.26}	– 60	n.a.	n.a.
rPEG ₁₁₀ ^{0.51}	– 61	n.a.	n.a.

7. Transmission electron microscopy (TEM) images

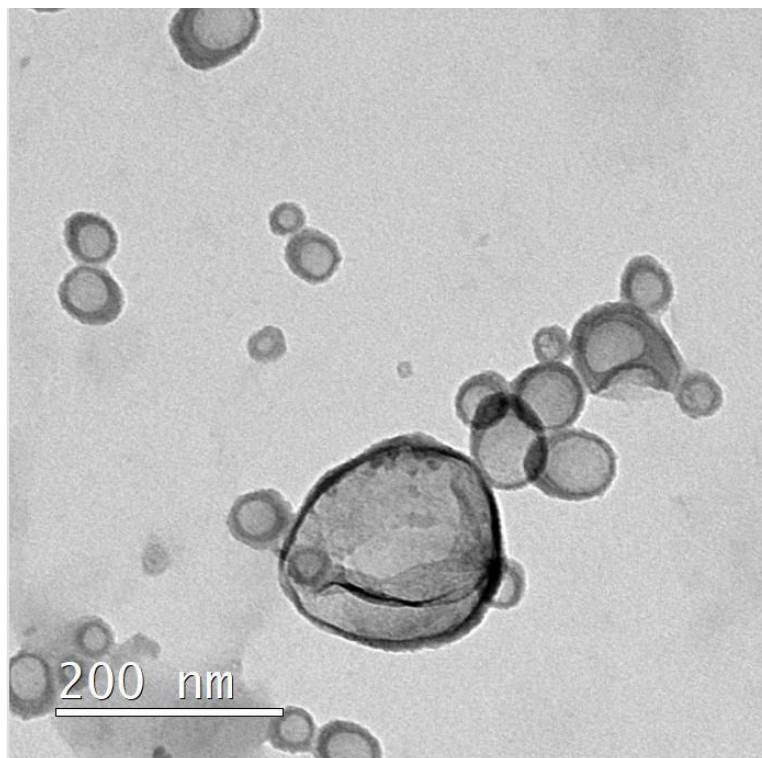


Figure S31. TEM image of nanocapsules functionalized with CTMA-Cl-a.

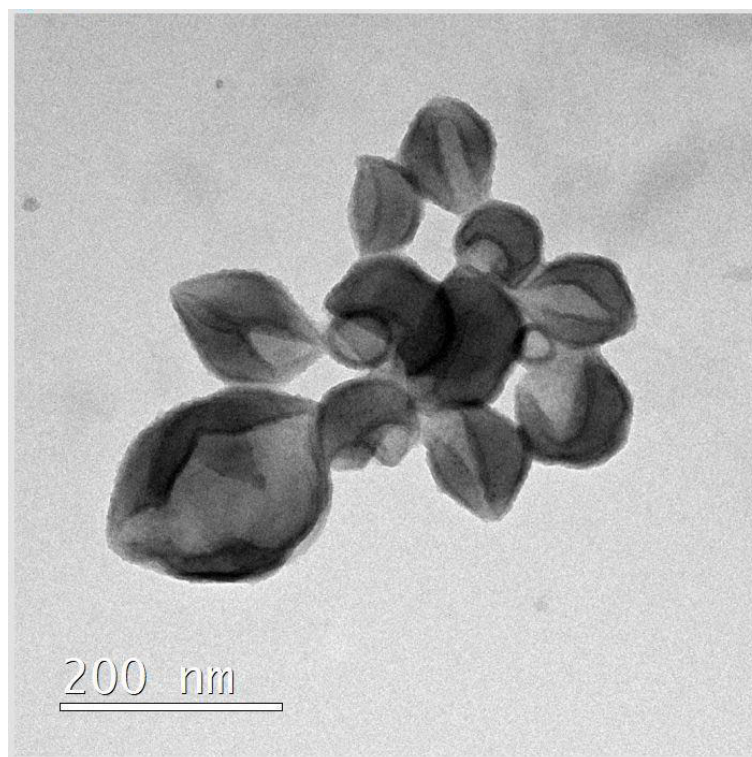


Figure S32. TEM image of nanocapsules functionalized with CTMA-Cl-b.

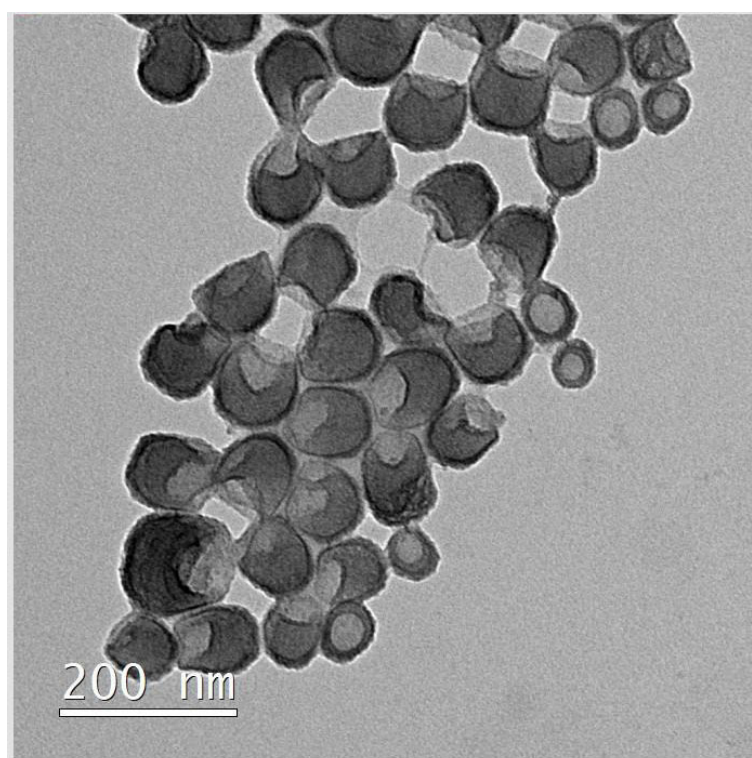


Figure S33. TEM image of nanocapsules functionalized with Lutensol AT50 (synthesized from CTMA-Cl-a NCs).

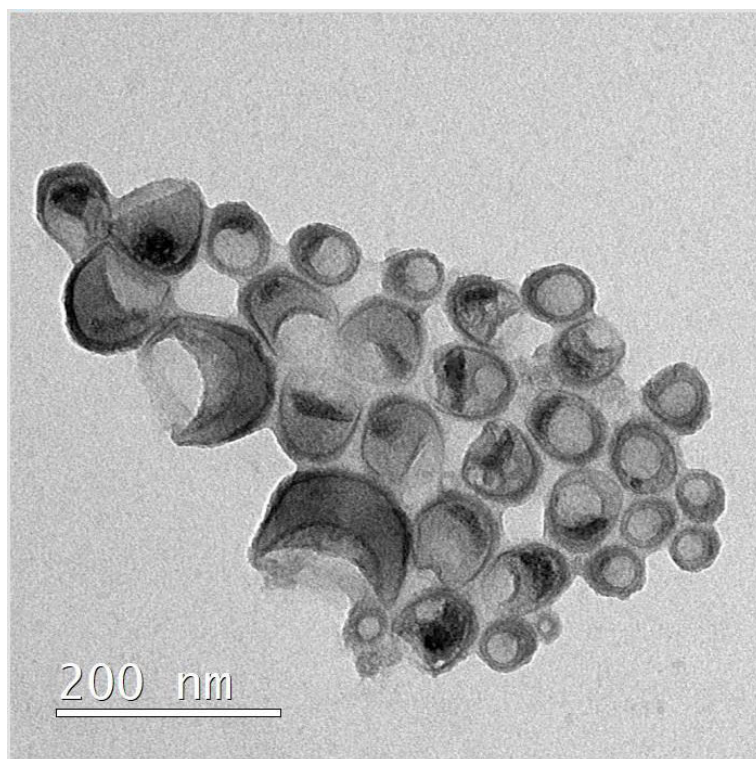


Figure S34. TEM image of nanocapsules functionalized with Lipid-PEG₄₆ (synthesized from CTMA-Cl-a NCs).

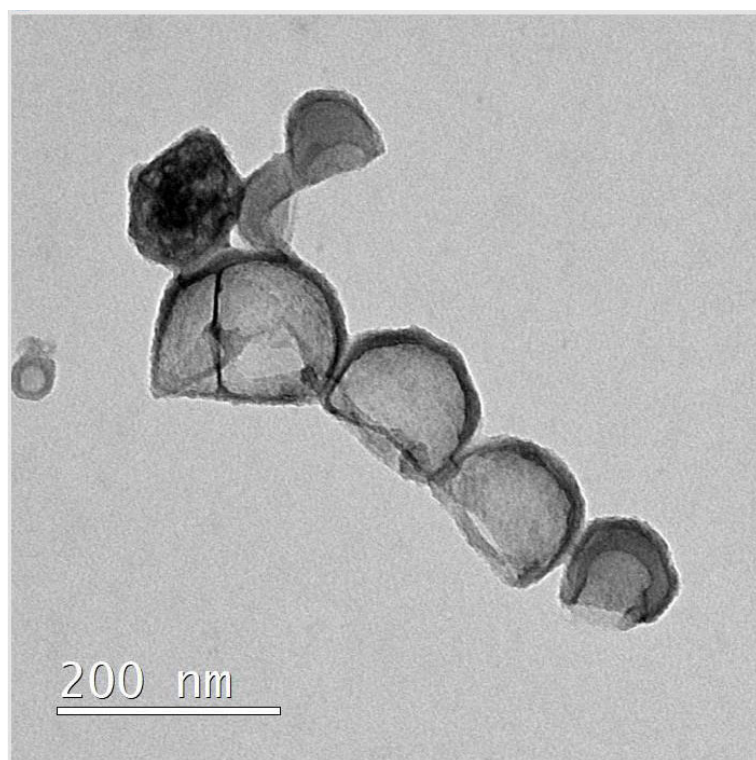


Figure S35. TEM image of nanocapsules functionalized with Lipid-rPEG₅₁^{0.17} (synthesized from CTMA-Cl-a NCs).

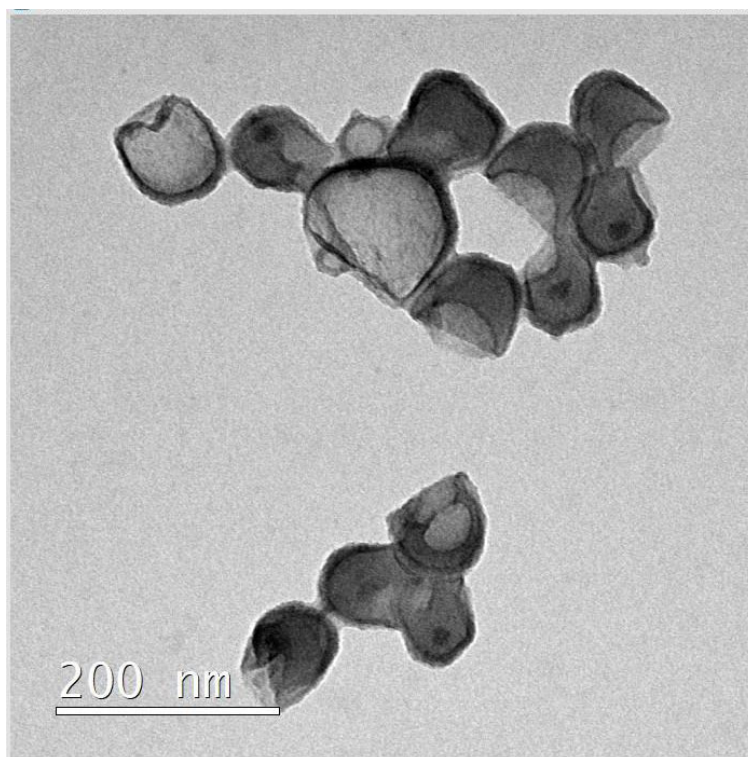


Figure S36. TEM image of nanocapsules functionalized with Lipid-rPEG₅₃^{0.26} (synthesized from CTMA-Cl-a NCs).

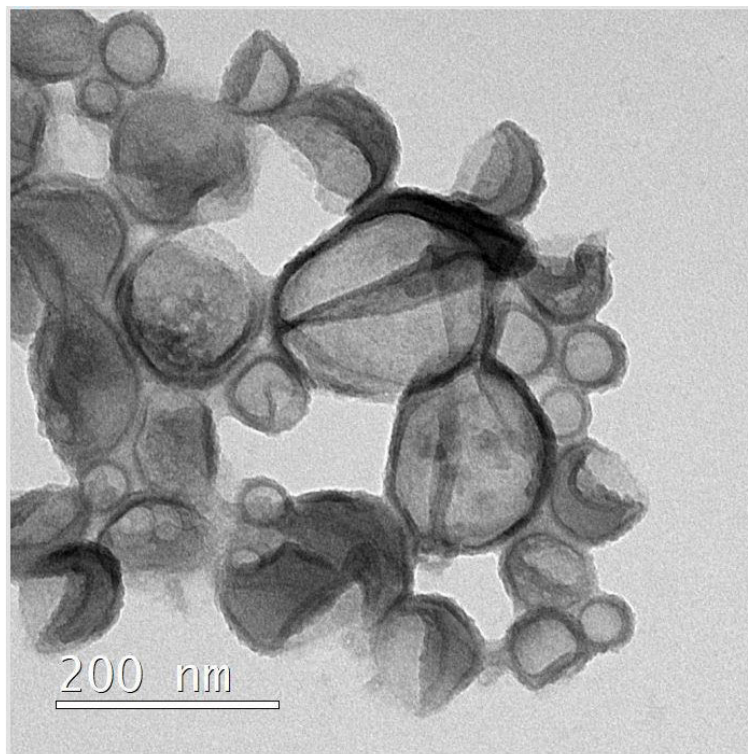


Figure S37. TEM image of nanocapsules functionalized with Lipid-rPEG₄₉^{0.28} (synthesized from CTMA-Cl-b NCs).

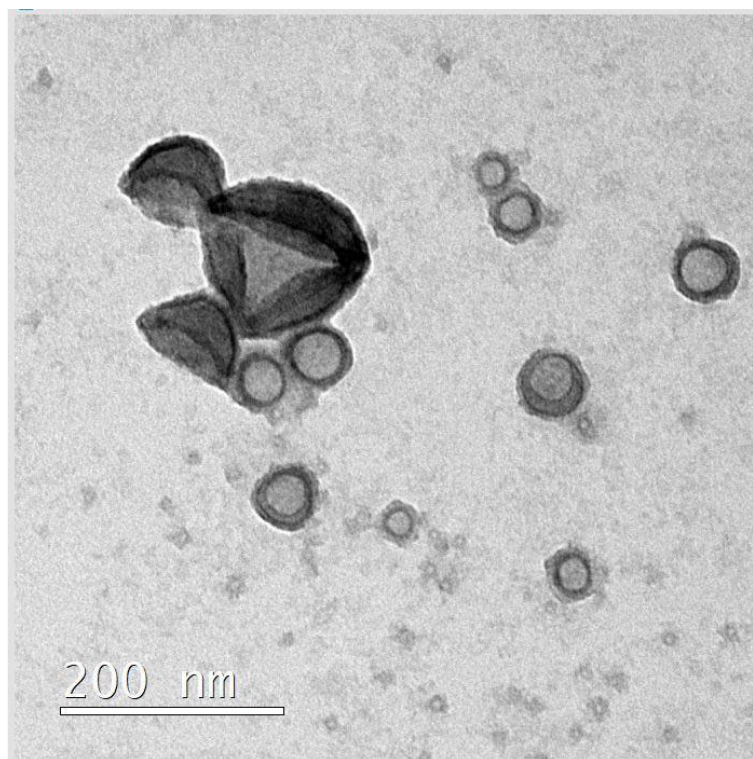


Figure S38. TEM image of nanocapsules functionalized with Lipid-rPEG₅₀^{0.29} (synthesized from CTMA-Cl-b NCs).

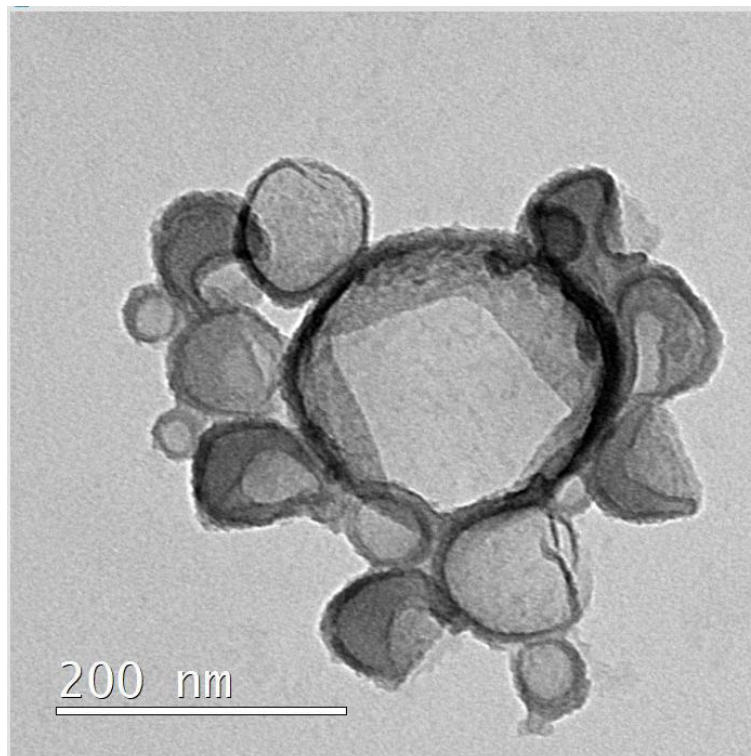


Figure S39. TEM image of nanocapsules functionalized with Lipid-rPEG₅₁^{0.42} (synthesized from CTMA-Cl-a NCs).

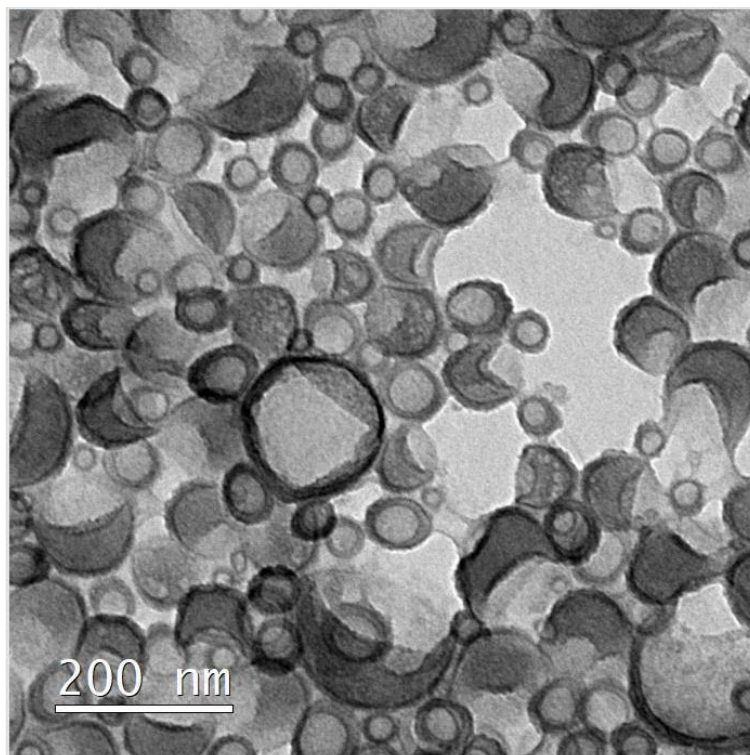


Figure S40. TEM image of nanocapsules functionalized with Lipid-rPEG₄₃^{0.55} (synthesized from CTMA-Cl-a NCs).

8. Summary of protein corona analysis

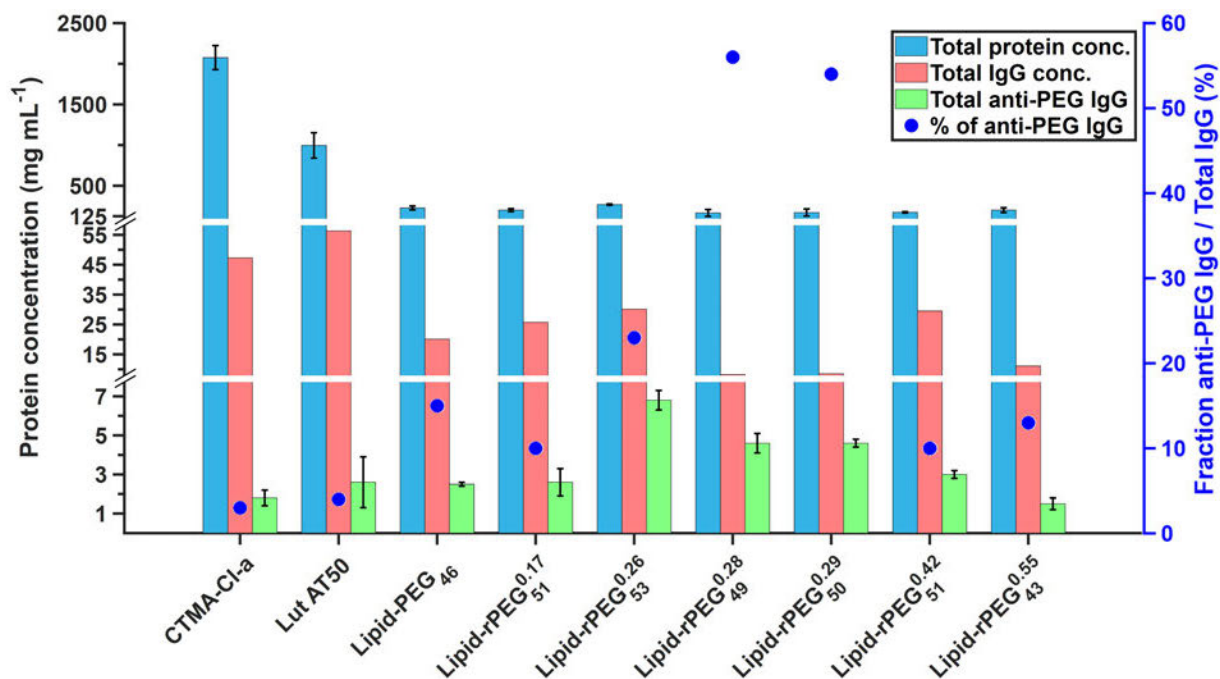


Figure S41. Total protein concentration (bars) and relative fraction of anti-PEG IgG compared to total IgG concentration (blue markers) in the hard protein corona (total protein concentration derived from Pierce assay, total IgG concentration derived from Pierce assay and LC-MS, anti-PEG IgG concentration derived from ELISA).

Supporting References

- (1) Jiang, S.; Prozeller, D.; Pereira, J.; Simon, J.; Han, S.; Wirsching, S.; Fichter, M.; Mottola, M.; Lieberwirth, I.; Morsbach, S.; Mailänder, V.; Gehring, S.; Crespy, D.; Landfester, K. Controlling protein interactions in blood for effective liver immunosuppressive therapy by silica nanocapsules. *Nanoscale* **2020**, *12* (4), 2626–2637. DOI: 10.1039/c9nr09879h.
- (2) Simon, J.; Wolf, T.; Klein, K.; Landfester, K.; Wurm, F. R.; Mailänder, V. Hydrophilicity Regulates the Stealth Properties of Polyphosphoester-Coated Nanocarriers. *Angew. Chem. Int. Ed Engl.* **2018**, *57* (19), 5548–5553. DOI: 10.1002/anie.201800272.
- (3) Dietz, L.; Simon, J.; Speth, K. R.; Landfester, K.; Mailänder, V. Plasma protein corona on silica nanoparticles enhances exocytosis. *Biomater. Sci.* **2025**, *13* (13), 3532–3543. DOI: 10.1039/d4bm01189a.
- (4) Chen, B.-M.; Su, Y.-C.; Chang, C.-J.; Burnouf, P.-A.; Chuang, K.-H.; Chen, C.-H.; Cheng, T.-L.; Chen, Y.-T.; Wu, J.-Y.; Roffler, S. R. Measurement of Pre-Existing IgG and IgM Antibodies against Polyethylene Glycol in Healthy Individuals. *Anal. Chem.* **2016**, *88* (21), 10661–10666. DOI: 10.1021/acs.analchem.6b03109.

Outlook

PEG has remained in a substantial role in nanomedicine for the last couple of decades. Since the first PEGylated API entered the market in 1990, PEG has been used to the circulation half time of dozens of other therapeutics that are FDA approved today. Yet, with the rising occurrence of pre-existing anti-PEG antibodies the research endeavor to find adequate alternatives for PEGylation is steadily increasing. Several promising alternatives have been explored so far, some of which are more hydrophilic than PEG, are comparably low in toxicity or possess biodegradability. The main disadvantages are high manufacturing costs and remainders of potentially toxic impurities, which make FDA approval challenging. Nevertheless, these problems could very well be solvable by process optimization in the future.

rPEGs are still in the very early stages of development. While certain PEG alternatives have already entered clinical trials, rPEGs have only shown their antigenicity and low toxicity via *in vitro* experiments. The next step towards application would therefore be a detailed *in vivo* investigation with focus on repeated administration to assess the potential immunogenicity of rPEGs. Contrary to many other PEG alternatives, which are homopolymers, rPEGs display a great variety in microstructures thanks to the random distribution of GME units in the backbone. Considering an ideal statistical sequence of monomer units in the backbone, an rPEG with a total chain length of 112 containing 28 GME units (25 mol%) could form approximately 2×10^{26} different chains. Additionally, since racemic GME is used for the synthesis, stereoisomeric variation is added as well, further increasing the chain variation. This property of rPEGs could render adaptation of the immune system much more difficult may impede or even suppress immunogenicity, although long-term studies will be necessary to further investigate this hypothesis. At the same time, industrial upscaling of the polymer synthesis faces less challenges than other PEG alternatives since rPEGs are produced *via* AROP under the same conditions as PEG. For comprehensive *in vivo* studies it will also be required to assess the reaction of the immune system towards rPEG conjugates, which can produce differing results than the free or immobilized polymers.

Regarding the synthesis of the copolymers, further optimization of reaction conditions will be necessary to obtain reliable and reproducible results. While the AROP yields satisfying results for copolymers with a molar content of up to 80 mol% GME and a chain length of approximately 113

units, the synthesis of the homopolymer PGME still proves to be a challenge. Variation of reaction temperature, reaction time, solvent or concentration of active chain ends (among other variables) has not yet given reproducible results. Instead, propagation is often times seemingly interrupted at a degree of polymerization of $D_p = 70 - 80$ ($6,000 - 7,000 \text{ g mol}^{-1}$) for reasons which remain unclear so far. Therefore, the homopolymerization of GME will need more in-depth investigation to determine the reason for this unexpected behavior so that the apparent limitation of accessible molar masses can be overcome.

On a longer timescale, copolymers of three different monomers would be achievable. Our group has already investigated copolymers of EO with other glycidyl ethers like isopropyl glycidyl ether or methoxy ethyl glycidyl ether. Hydrophilicity was found to be overall lower for these copolymers compared to PEG and rPEG, but less molar content of the comonomers was required to receive similar antigenicity in competitive ELISA tests. Terpolymers developed from monomers like this would increase the variety of the backbone sequence even more and could further enhance the antigenic effect of the distributed side chains.

Overall, rPEGs have so far shown potential of reducing antigenicity while maintaining the beneficial properties of PEG and therefore show potential for biomedical application as an alternative PEGylation agent which could provide relief for the rising concern about anti-PEG antibodies.

Appendix

Appendix Chapter 1

Isomerization of poly(ethylene glycol): A strategy for the evasion of anti-PEG antibody recognition

Isomerization of Poly(ethylene glycol): A Strategy for the Evasion of Anti-PEG Antibody Recognition

Philip Dreier,^{||} Rebecca Matthes,^{||} Fabian Fuß,^{||} Julian Schmidt,^{||} Dominik Schulz, Gregor M. Linden, Ramona D. Barent, Sandra Schüttner, Barry W. Neun, Edward Cedrone, Marina A. Dobrovolskaia, Matthias Bros, and Holger Frey*



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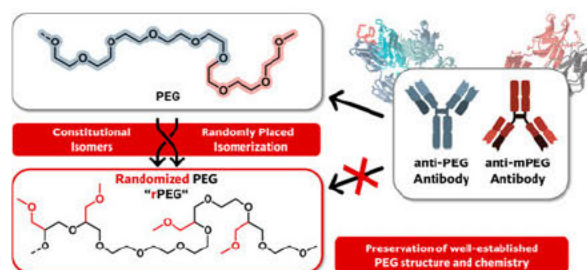
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ABSTRACT: PEGylation, the conjugation of poly(ethylene glycol) (PEG) to nanocarriers or protein-based active pharmaceutical ingredients (APIs), is a key strategy in nanomedicine to extend the circulation time of therapeutics in the bloodstream based on the stealth effect of PEG. However, the growing prevalence of anti-PEG antibodies in the population can lead to pronounced immune responses upon drug administration and accelerated blood clearance of PEGylated drugs, resulting in the loss of the stealth effect. We introduce the randomized PEG (rPEG) technology designed to strongly reduce the antigenicity of PEG while preserving its core benefits. This conceptually novel approach is based on an introduction of hydrophilic side chains along the PEG backbone. The synthesis is performed via anionic ring-opening copolymerization of ethylene oxide (EO) and glycidyl methyl ether (GME), resulting in constitutional isomers of PEG. By optimization of the reaction conditions, an ideally random distribution of the side chains in the polymer backbone could be achieved. Since previous studies show a relation between polymer chain regularity and immune system response, our approach specifically aims at introducing an irregular comonomer sequence via copolymerization, while translating the hydrophilicity and low toxicity of PEG to rPEG. Biocompatibility was evaluated using peripheral blood mononuclear cells (PBMC). Increasing the GME content in the copolymers did not decrease cell viability. Furthermore, all rPEG samples did not show complement activation in vitro at all tested concentrations. Enzyme-linked immunosorbent assays (ELISA) utilizing backbone- and end group-selective anti-PEG antibodies showed drastically reduced recognition and antibody binding for the constitutional isomers of PEG.



INTRODUCTION

PEGylation, the conjugation of poly(ethylene glycol) (PEG) to proteins, small molecules or lipids, is a crucial strategy for the delivery of peptide and protein drugs.^{1,2} It is frequently used in liposomal formulations^{3,4} and in PEGylated lipids as solubilizing and crucial stabilizing components of lipid nanoparticles, e.g. SARS-CoV-2 vaccines.^{5,6} Presently, over 40 PEGylated therapeutics are on the market or in clinical phase III, with market introduction pending.⁷ It is appropriate to state that PEGylation represents a key technology of current nanomedicine. The nonionic PEG provides a hydrophilic shield that protects conjugates from recognition by the patient's immune system, commonly referred to as "stealth effect". It effectively increases the size of the biomolecule, consequently reducing clearance from the bloodstream.⁸ The investigation of PEG-coated nanocarriers additionally revealed the impact of PEG on the composition of the protein corona formed at the particles, which is important to prevent nonspecific cellular uptake.⁹ However, whereas it was initially believed that PEG is immunologically inert, it has become evident in recent decades that an increasing number of

individuals have developed anti-PEG antibodies (APAs), extensively boosted by PEG lipid-containing SARS-CoV-2 lipid nanoparticles (LNPs) used for mRNA vaccinations.^{10–12} The induction of APAs in humans may not only result from PEGylated therapeutics but could also arise from exposure to PEG present in food, cosmetics and other common sources. The potential antigenicity of PEG has been confirmed by the existence of APAs in healthy individuals who have never received PEGylated therapeutics systemically.^{13,14} A recent study revealed that 83% of individuals in a typical western population are positive for either anti-PEG IgG or IgM.¹⁵

The presence of APAs results in the recognition and accelerated blood clearance of PEGylated therapeutics, premature drug release from PEGylated nanocarriers, and

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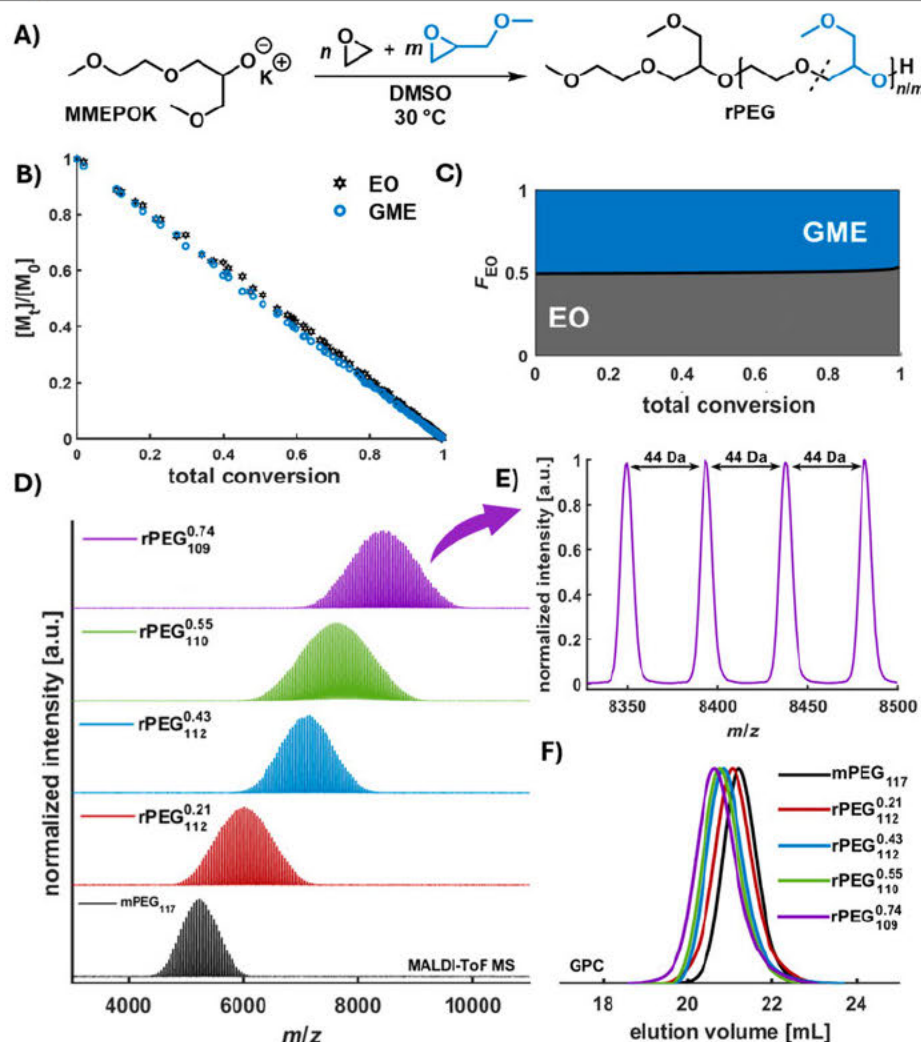


Figure 1. Synthesis and characterization of rPEGs. (A) Applied synthesis strategy for rPEG by copolymerization; (B) comonomer consumption ($[M]_t/[M]_0$) vs total conversion obtained from in situ ^1H NMR kinetics in $\text{DMSO}-d_6$; (C) molar-based composition diagram of rPEG (50 mol %GME) derived from the in situ ^1H NMR kinetics data in $\text{DMSO}-d_6$; (D) stacked MALDI ToF mass spectra of investigated rPEGs; (E) zoom-in of $\text{rPEG}_{109}^{0.74}$ MALDI ToF MS; (F) stacked GPC traces of investigated rPEGs.

hypersensitivity reactions, including complement activation-related pseudoallergy (CARPA) and, in severe cases, anaphylactic shock.¹⁶ A phase III clinical study regarding anticoagulation factor IXa RNA PEG-conjugated aptamer had to be interrupted since anaphylactic reactions in 0.6% of the patients were observed, most likely caused by the pre-existence of APAs in the bloodstream.¹⁷ Recent clinical works regarding Pegaspargase have shown that the presence of APAs permits the prediction of allergic reactions and failure of rechallenge, emphasizing the clinical relevance of APA-Fabs for the success of treating leukemia in this case.¹⁸ Based on these concerns, the search for PEG replacement structures has intensified in recent years. Several alternatives based on polymer classes other than polyethers, i.e. polysarcosine, polyoxazolines, and polymethacrylates have been discussed.^{7,8,19} These alternatives feature well-studied biocompatibility.^{20,21} However, it is important to note that antigenicity was also observed for some homopolymers of polymer classes other than PEG^{22,23} due to the flexibility of the adaptive immune system.

In contrast to the aforementioned approaches, the presented study targets the “PEG alternative dilemma” via a fundamentally different “PEG isomerization” approach. Our strategy aims to preserve the wide spectrum of beneficial properties of PEG, while diminishing its immunological drawbacks by the introduction of statistical heterogeneity, retaining precise control over the chain length. Additionally, we aim at a PEG-based alternative that could be seamlessly integrated into existing PEG manufacturing and PEGylation processes.

Recently, crystal-structures of two types of APAs that bind to the PEG backbone²⁴ or the methoxy end group of mPEG²⁵ were determined for the first time. In both cases, an open ring-like substructure of the APA Fab paratope recognizes ethylene glycol (EO) units of PEG via multiple van der Waals and polar interactions. While the backbone-selective APA binds 16 EO units, seven units and the methoxy residue at the chain end are recognized by the investigated end group-selective APA. Additionally, a third type of APA has been reported which interacts with a core PEG fragment in combination with a PEG

satellite fragment.²⁶ Inspired by these observations, we hypothesized that an interruption of the periodic and linear PEG structure by randomly distributed branching points should significantly alter the interaction of the APA with the PEG epitope. This random disruption of the regular PEG structure may be viewed as an introduction of ‘synthetic point mutations’ along the polyether backbone. In this context, we chose methoxy methylene side chains as branching points to preserve the carbon-hydrogen-oxygen ratio and properties of PEG while altering the underlying architecture of the polyether (Figure 1A).

■ RESULTS AND DISCUSSION

The repeating unit can be introduced via the epoxide monomer glycidyl methyl ether (GME). As GME is a formal dimer of EO ($C_{2n}H_{4n}O_n$; $n = 1$ (EO), 2 (GME)), all copolymers of EO and GME represent constitutional isomers of PEG, independent of their monomer composition. We assumed that these randomized PEG (rPEG) structures hold potential as a highly suitable nonantigenic alternative to PEG, albeit preserving the polyether backbone and the beneficial physicochemical properties of PEG. The random incorporation of EO and GME units into the polymer backbone is an essential parameter to decrease the occurrence of EO-rich epitope segments in the polyether chains. In principle, the incorporation of GME repeating units can be achieved via copolymerization of EO and GME. Noteworthy, anionic homo- and copolymerization of GME has rarely been investigated to date due to synthetic challenges and impurities (Scheme S2), resulting in ill-defined polymers that are not suitable for pharmaceutical applications. PGME homopolymers were either obtained as oligomers²⁷ or with high dispersity.²⁸ EO-GME copolymer structures are accessible using an activated monomer mechanism²⁸ albeit resulting in ill-defined materials with poor end group fidelity and nontolerable traces of the aluminum catalyst in the products. It is important to note that for a polymer to be regarded as a viable alternative to PEG, it must meet the standard specifications of pharmaceutical-grade PEG. Specifically, it must possess high purity and a dispersity (M_w/M_n) lower than 1.10. High end group fidelity (>99%) is critical for the subsequent conjugation of the polymers to proteins, nano-carriers or surfaces.

Via utilization of analytically pure GME (>99%) (Scheme S1, Figures S1, S4 and S5) and anionic ring-opening (co)polymerization (AROP) of EO and GME (Figure 1A), the first successful synthesis of well-defined rPEGs was accomplished, circumventing the abovementioned synthetic challenges (Scheme S2). AROP was selected as polymerization method of choice, as it represents the standard polymerization technique for pharmaceutical grade PEG, characterized by the absence of toxic side products or catalysts, high end-group fidelity and the ability to precisely control molar masses due to quantitative conversion of the epoxide monomers.²⁹ The method is also utilized in the industrially established synthesis of pharmaceutical-grade PEG. Therefore, it is the key for the potential synthesis of a polyether-based PEG alternative in existing PEG production facilities.

In the AROP of epoxides, the choice of solvent is a crucial reaction parameter because it has a direct influence on the copolymerization kinetics and the resulting microstructure of the copolymer.³⁰ Therefore, in a first optimization step, we elucidated the incorporation of EO and GME into the polymer

backbone by following the mean composition at all chain positions during the copolymerization. This was achieved by performing and evaluating *in situ* ¹H NMR kinetics measurements in various nonprotic solvents suitable for AROP of monosubstituted epoxides (Figures S8–S39 and Tables S1 and S2). In the case of DMSO-*d*₆, an almost linear decrease of comonomer concentration with progressing conversion (Figure 1B) for both comonomers is observed. This demonstrates that the incorporation of EO and GME units occurs in an ideally random manner ($r_{EO} \approx r_{GME} \approx 1$) (Figure 1C) independent of temperature and degree of deprotonation (Figures S15–S18, S24–S26). In the case of less polar solvents, a slightly preferred incorporation of GME over EO was observed, resulting in soft gradient microstructures (Figures S19–S23, S27–S31). As a random distribution of GME along the polymer backbone is preferred to statistically minimize the occurrence of EO-rich segments, we chose DMSO as a suitable solvent for the optimization of the rPEG synthesis on multigram scale.

To ensure systematic comparability of the side groups’ influence on the physicochemical properties and their behavior in the bioassays, copolymers in analogy to 5000 g mol^{−1} mPEG were synthesized. This translates to a constant degree of polymerization of 114, while varying the GME content, leading to higher molar masses that depend on the introduced GME content. In this context, we designed and utilized the potassium salt of 1-methoxy-3-(2-methoxyethoxy)propan-2-ol (MMEPOK) as initiator (Figure 1A) for the synthesis of the rPEG samples to lock a ‘synthetic point mutation’ at the second repeating unit of each polymer chain (Supporting Information). The design of the initiator structure was derived from preliminary enzyme-linked immunosorbent assay (ELISA) experiments of PGME samples with differing initiator molecules (Figure S40). We further designed and conducted an additional purification protocol via analytical (Figures S41 and S42) and semipreparative high-performance liquid chromatography (HPLC) (Supporting Information) to obtain rPEG samples in high purity which is an essential requirement for potential pharmaceutical applications (Figure S43, Table S5). Additionally, the purification protocol was applied using a commercial mPEG sample to allow for comparison between mPEG and rPEG in the following studies. Hence, rPEGs with varying molar compositions of 21–74 mol % GME and molar masses of 6.0–8.4 kg mol^{−1} were synthesized and purified (Tables S3 and S4). All isomeric rPEGs and mPEG were analyzed by ¹H NMR spectroscopy (Figure S44) and MALDI ToF MS. The latter reveals one distinct distribution which is assigned to the potassium-ionized and methoxy-initiated species ($[rPEG + K]^+$) carrying an alcohol group at the ω -chain end exclusively (Figures 1D,E, S45 and S46). In addition, primary and secondary hydroxyl end group functionalities were quantified with ³¹P NMR spectroscopy to verify the ideally random copolymerization (Figure S54). As an example, rPEG₁₁₀^{0.55} was investigated and the expected content of secondary hydroxyl end groups (54%) was confirmed.³¹ This supports the purity and an excellent end group fidelity of the synthesized rPEGs. It is important to note that due to the isomeric character of the rPEGs, only a single distribution with intervals of 44 *m/z* is observed, despite the copolymer structure. Additionally, all synthesized rPEGs show monomodal distributions and dispersity <1.10 (GPC) independent of their molar comonomer composition (Figures 1F, S47).

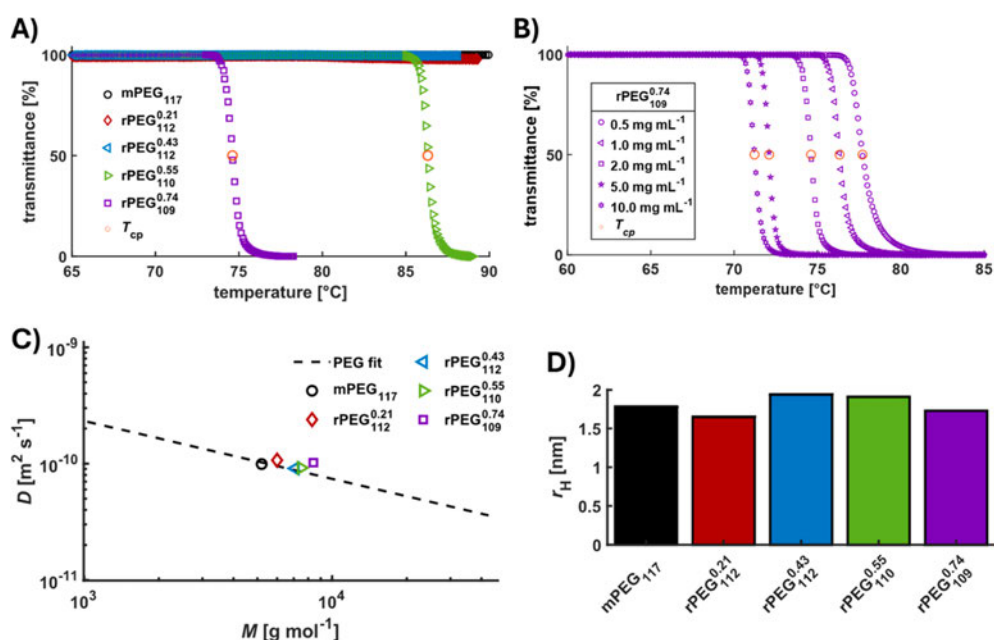


Figure 2. Physicochemical properties of rPEGs. (A) Stacked turbidimetry plots (heating curves) (2.0 mg mL⁻¹) of mPEG₁₁₇ and rPEG samples in PBS buffer; (B) stacked turbidimetry plots (heating curves) of rPEG_{0.74}₁₀₉ at different concentrations in PBS buffer; (C) diffusion coefficients in dependence of the molar mass for mPEG and rPEG samples in D₂O obtained via DOSY NMR; dashed line shows PEG fit determined from different well-defined PEG samples; (D) comparison of hydrodynamic radii of mPEG and rPEG samples based on the diffusion coefficients obtained from DOSY experiments.

Therefore, from a synthetic point of view, the requirements of rPEGs as a PEG alternative are fully met.

The application of PEG alternatives in polymer–protein conjugates and in lipid–nucleic acid formulations under physiological conditions necessitate sufficient solubility of the polymer in aqueous solutions significantly above body temperature. Since the formation of a hydration shell is crucial for the stealth effect and shielding of the active pharmaceutical ingredient (API) or nanoparticle, a collapse of the PEGylation alternative in aqueous solutions must be prevented.^{32,33}

Therefore, we investigated the thermoresponsive behavior of the synthesized rPEG samples in PBS buffer via turbidimetry. In the measurements, the cloud point temperatures (T_{cp}), marking phase separation of polymers and the solvent of the rPEGs with varying amounts of GME were determined at a concentration of 2 mg mL⁻¹ (Figure 2A, Table S6). The investigation shows that no cloud point is observed up to a molar GME content of 43% in the rPEG samples. The observed T_{cp} at a transmittance of 50% for rPEG_{0.55}₁₁₀ and rPEG_{0.74}₁₀₉ at 86 and 75 °C, respectively, demonstrate that even for high content of GME no coil-globule transition is to be expected at physiological temperatures. Additionally, cloud point temperatures for rPEG_{0.74}₁₀₉ at varying polymer concentrations (0.5–10.0 mg mL⁻¹) were investigated showing merely a slight decrease of the T_{cp} to 71 °C (10.0 mg mL⁻¹) with increasing polymer concentration (Figure 2B, Table S6). For comparison, the PGME homopolymer exhibits a T_{cp} of 66 °C (Figure S61) in PBS buffer at a concentration of 5 mg mL⁻¹. These findings underline the critical role of the EO/GME comonomer combination in modulating the overall hydrophilicity of the rPEGs.

Pursuing, we utilized diffusion-ordered NMR spectroscopy (DOSY NMR) in D₂O (Figures S48–S52) to evaluate the

influence of the methoxy methylene side chains on the properties of rPEGs in solution. The obtained data (Table S7) was referenced to a PEG fit (Figure S53) to elucidate differences of the rPEG samples to conventional PEG. In this context, the rPEG samples exhibit slight deviations from the expected PEG fit values, whereas the mPEG₁₁₇ sample aligns closely with the fit (Figure 2C). This confirms that the methoxy end group of the investigated samples has no significant influence on the diffusion of the polymers in the investigated molar mass range. Calculation of the hydrodynamic radii (r_H) from the diffusion data (Supporting Information) reveals unexpected behavior. Despite the significant increase in molar mass with higher content of GME, the r_H of the rPEG samples remains comparable to that of the mPEG reference (Figure 2D, Table S7). In comparison, a PEG sample with the same molar mass as rPEG_{0.74}₁₀₉ (r_H = 1.73 nm) possesses a r_H of ≈2.23 nm based on the determined PEG fit (Figure S53). From these observations, we conclude that the primary contribution to the hydrodynamic radius of the rPEG polymer coil derives from the hydration of the backbone, while the methoxy methylene side groups play a rather minor role.

In summary, the study of the thermoresponsive properties and hydrodynamic radii of rPEGs in buffer solution and D₂O, respectively, show similar results as for PEG, hinting at comparable shielding capability for drug- or protein-conjugates and nanoparticles. Naturally, the preservation of the stealth effect in a biological system can only be determined by in vivo studies, which are currently in progress and will be published in due course.

Further, we extensively assessed the in vitro biocompatibility of rPEG. Therefore, we analyzed cell viability, immunostimulatory effects as well as the hematological profile. It was shown

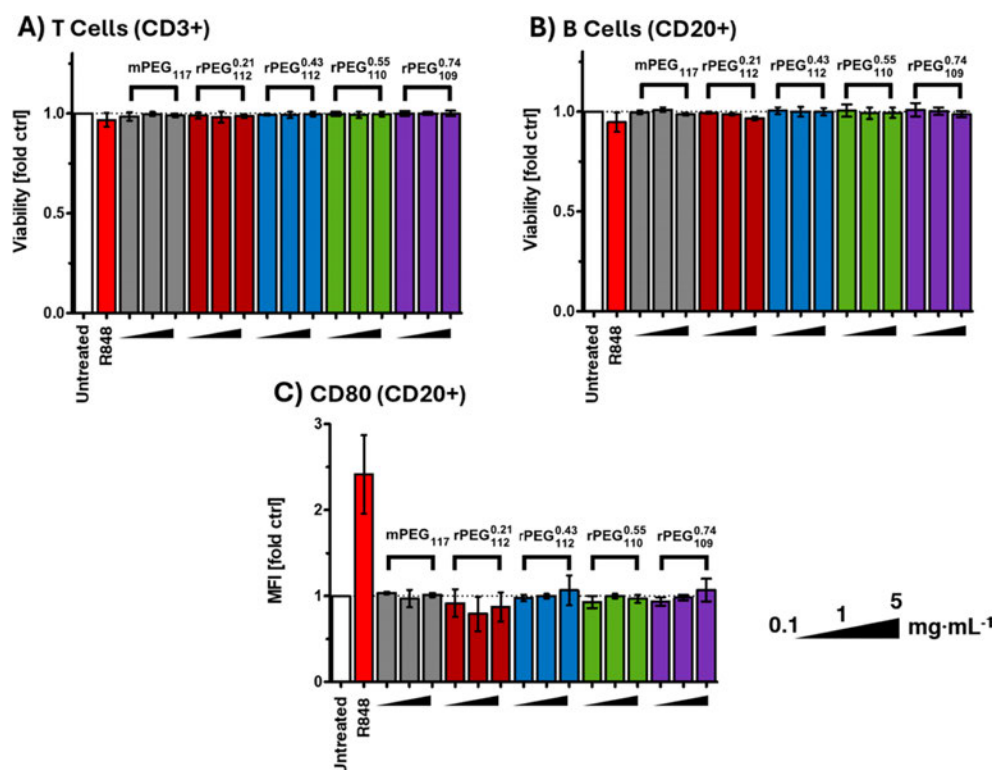


Figure 3. PBMC cell viability & B cell activation after exposure of mPEG₁₁₇ and rPEGs. (A,B) Cell viability of human leukocytes; (C) CD80 expression on B Cells (CD20 positive) as a biomarker for immunostimulatory effects; determined via flow cytometry at three different concentrations (0.1–5.0 mg mL⁻¹).

in earlier studies that the PGME homopolymer and other glycidyl ether-based polyethers show high biocompatibility.^{27,34} To establish a PEG-like safety profile for rPEG, two independent series were tested by the University Medical Centre of the Johannes Gutenberg University Mainz (Germany) and the National Cancer Institute's Nanotechnology Characterization Laboratory (NCL) (United States). In the first series peripheral blood mononuclear cells (PBMCs) of four healthy German donors were exposed to rPEGs and mPEG₁₁₇ with three concentrations (0.1–5.0 mg mL⁻¹) for 16 h to evaluate the cell viability and immunostimulation (Figures 3A, B, and S67–S69).

No influence on the viability of B cells, T cells, neutral killer cells (NK) as well as monocytes was observed for rPEGs, and no difference was noted between mPEG and rPEG samples. At the highest concentration of 5 mg mL⁻¹, which is equivalent to an extremely high in vivo human dose of 400 mg kg⁻¹, some rPEG samples showed a slight effect on dendritic cells (DC) and polymorphonuclear leukocytes. Nevertheless, their viability remains above 75% (fold ctrl) (Figures 3A,B, and S67). It can be assumed that this effect can be attributed to the high polymer concentration above any clinically relevant dose. A reduction of cell viability with increasing GME content is not observed. Additionally, the expression of surface activation markers, such as CD80 and CD86, remained at basal levels (Figures 3C, S68), providing the first indication that rPEG does not induce immunostimulation.

In the second series, slightly different samples were used for immunostimulatory and hematological testing, as these experiments were independently conducted by the NCL.

Specifically, rPEG_{0.45}⁴⁵ was used instead of rPEG_{0.43}⁴³, which also complied with the acceptance criteria as the other rPEG samples. rPEG_{0.55}⁵⁵ was excluded due to its compositional similarity to rPEG_{0.45}⁴⁵. The results of the in vitro studies suggest that the rPEG technology does not trigger innate immune responses under the tested conditions. When human PBMC from three healthy donors were exposed to various concentrations of rPEG, induction of cytokines responsible for the pyrogenic response and fever-like reactions (TNF, IL-1 β , and IL-6), produced by activated dendritic cells (IFN α , and IFN λ), and by activated T-cells (IL-2, IFN γ , and IP-10) was not observed at all tested concentrations (Figure 4). Therefore, it can be established that rPEG does not induce pyrogenic biomarkers, does not activate primary T-cells, resting B cells, and innate immune responses in agreement with the results from the series with the German donor population (Figures S67–69).

To investigate rPEG compatibility with human blood, we followed the ISO standard 10993-4 for medical devices, which is also generally adapted for hemocompatibility assessment of nanomedicines.^{35,36} None of the four samples (mPEG and rPEGs) with low (21 mol %), medium (45 mol %) and high GME content (74 mol %) lead to complement activation in vitro at all tested concentrations (Figure 5), and no difference was noted between the mPEG and rPEG samples. Similar results were obtained in plasma coagulation and platelet aggregation assays: all test samples did not induce platelet aggregation, did not affect collagen-induced platelet aggregation, and did not alter human plasma coagulation time in prothrombin, activated partial thromboplastin, and thrombin

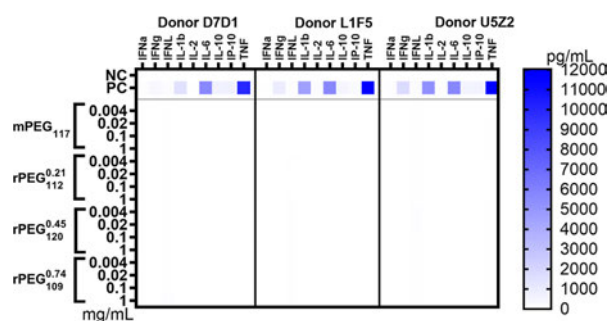


Figure 4. Cytokine biomarkers for activated myeloid and lymphoid cells in PBMC cultures exposed to mPEG₁₁₇, rPEG₁₁₂^{0.21}, rPEG₁₂₀^{0.45} and rPEG₁₀₉^{0.74}. PBS, and a combination of PHA-M, LPS, and ODN2216 were used as negative control (NC) and positive control (PC), respectively. The same data with masked positive control to highlight insignificant background cytokine levels is shown in the [Supporting Information](#) (Figure S75).

time assays (Figure 5). The data indicate rPEG would not induce complement activation when present in the blood at these concentrations. The highest tested concentration, 1 mg mL⁻¹, is equivalent to a rPEG in vivo human dose of 80 mg kg⁻¹. This suggests that the risk of complement activation-related pseudoallergy (CARPA) at rPEG doses up to 80 mg kg⁻¹ is marginal. In contrast, Cremophor-EL tested at a concentration equivalent to that of the clinical dose of Taxol (Cremophor-formulated paclitaxel) resulted in complement activation consistent with the current knowledge of CARPA in sensitive Taxol recipients. Likewise, the risk of thrombogenicity due to platelet activation, and hemorrhage due to the inhibition of platelet or coagulation factor functions is also low for rPEG at all tested concentrations equivalent to doses up to 80 mg kg⁻¹. To conclude, all investigated rPEGs are noncytotoxic, do not activate innate immune responses, are compatible with blood, and are not immunostimulatory at clinically relevant concentrations. Thus, rPEG appears to be suitable for utilization in in vivo experiments and additional preclinical studies.

Based on the reactivity ratios determined in DMSO-*d*₆ ($r_{EO} \approx r_{GME} \approx 1$) and considering an epitope of 16 or more consecutive EO repeating units, calculations and simulations with different molar compositions were performed ([Supporting Information](#)) to illustrate the random distribution of the ‘synthetic point mutations’ along the rPEG chains (Figures 6A,B, S62–S64). The targeted chemical heterogeneity within the polymer sample is evident from the simulation, as each distinct chain possesses a different sequence of repeating units (Figure 6B). The methoxy methylene groups as ‘synthetic point mutations’ are randomly distributed in the polyether backbone, and the statistical diversity within each chain within one sample is tremendously high. As an example, for a copolymer with 114 monomer units, among which 23 monomer units are GME (20 mol %), the number of possible chain isomers with different monomer sequences is 7.3×10^{23} .

This illustrates that the induction of polymer-selective antibodies targeting a regular chain segment should be highly hampered or even impossible. The calculations show that a decrease in the molar fractions of longer, merely EO containing segments with increasing GME amount occurs (Figure 6A). This is confirmed by the simulations (Figures 6B, S62–S64) where the probability of finding at least one

repeating pattern of 16 or more consecutive EO repeating units (highlighted in orange) in a rPEG chain decreases from 46% for rPEG₁₁₄^{0.20} to below 0.1% in the case of rPEG₁₁₄^{0.50} (Table S8). Besides the decrease in consecutive EO units, the increased spatial requirement of rPEGs and the random distribution of methoxy methylene side chains should additionally impede or even disable interaction with APAs according to the specific ‘lock and key principle’. For rPEGs, the GME monomer is utilized as a racemic mixture. In addition to employing two different comonomers and their sequence in the polymer chain, the stereochemistry of GME introduces another randomization parameter within one sample, rendering adaptation of the immune system and antibody formation even more improbable. This sets our system further apart from other approaches using hydrophilic homopolymers.

To investigate our hypothesis regarding APAs, we tested the effect of a varied concentration of synthetic point mutations (0–74 mol % GME) on the binding capability of backbone- and end group-selective APAs via competitive ELISA. The concentration-dependent interaction between the APA and polyether is detected by a decrease of absorbance intensity. In summary, the backbone-selective APA ELISA results confirm the effect of the incorporation of methoxy methylene groups along the PEG backbone on the APA recognition, in alignment with the simulations.

A major increase of the half maximal effective concentration (EC₅₀) value is observed with increasing GME content. Comparing the relative affinities (1/EC₅₀) of the APA toward mPEG₁₁₇ and rPEGs (Figure 6C,D, Table S9) underlines the significance of the diminished antigenicity. With incorporation of 21–74 mol % GME within the polyether, its affinity drops to 0.4 to 4.6×10^{-6} % relative to mPEG₁₁₇, respectively. Following the simulations, the random nature of ‘synthetic point mutation’ incorporation in the PEG chains reduces the length of undisrupted PEG segments, thereby minimizing the probability of forming a PEG epitope. Nevertheless, binding of the APA is still observed at very high rPEG concentrations. We consider two possible explanations for this behavior: (i) an interaction of a smaller epitope may already cause a weak recognition; (ii) the initial interaction between APA and PEG is based on van der Waals forces before trapping the polymer by a conformational change.²⁴ At polymer concentrations as high as tested, an unspecific aggregation or entanglement of polymer and APA could cause a decrease in binding affinity to the competitive PEG coated on the ELISA plate wells. Considering the overall polymer portion of PEGylated therapeutics, antigenic concentrations of rPEGs in the performed studies are magnitudes higher than those of PEG in current clinical applications.³⁷ While the introduction of pendant methoxy methylene groups strongly reduces the antigenicity of rPEGs toward backbone-selective APAs, this assay does not provide information regarding end group-specific APA interactions. In principle, each GME repeating unit contains a methoxy group which could theoretically be detected by the end group-selective APAs. To address this, a separate ELISA assay was conducted to evaluate interaction of the end group-selective APA with the rPEG polymers.

Compared to the backbone-selective APA, the EC₅₀ value of mPEG₁₁₇ in the end group-selective APA assay is shifted to higher concentrations, indicating an overall lower polymer affinity. In accordance with the backbone-specific APA ELISA, a notable shift of EC₅₀ values of all rPEGs in relation to mPEG₁₁₇ is observed (Figure 6E). The relative APA affinity

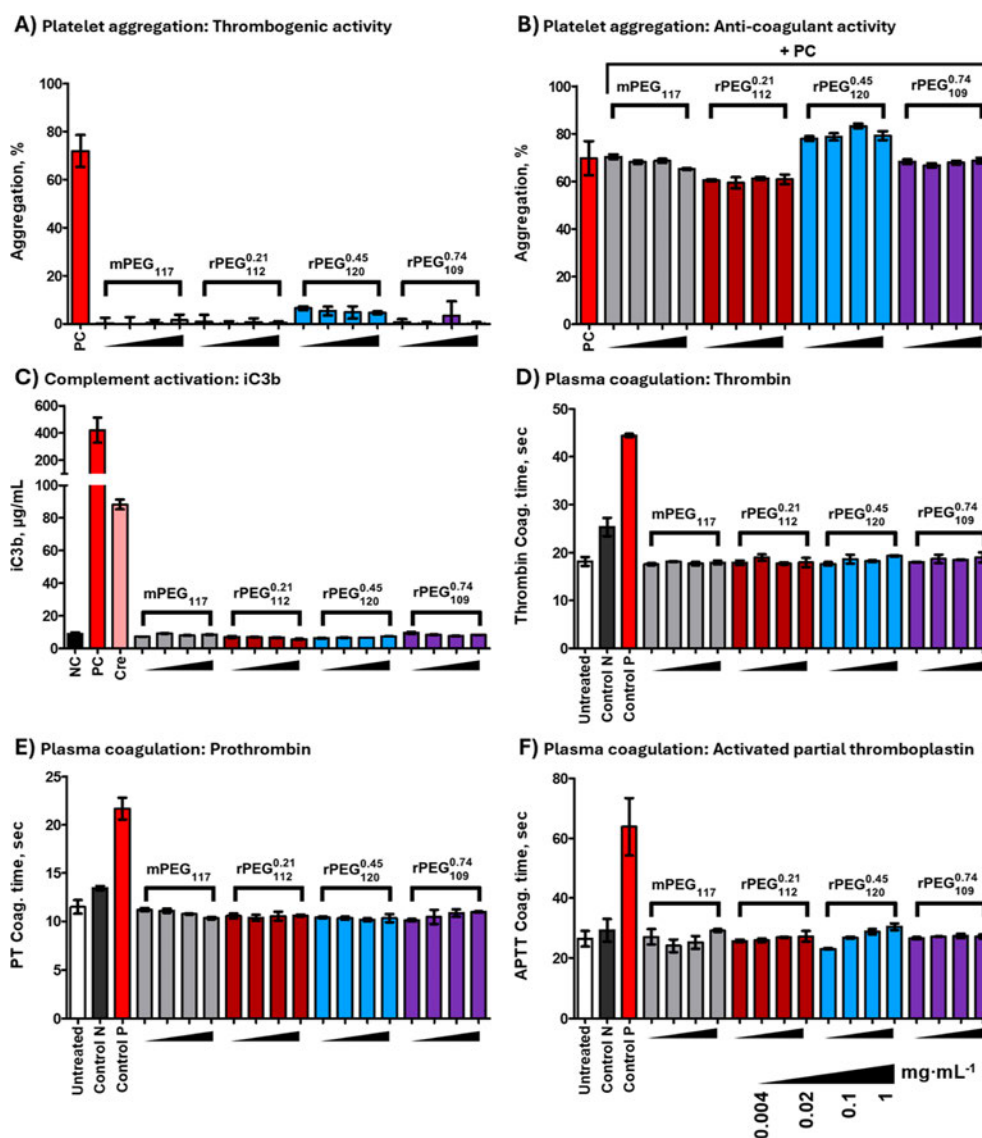


Figure 5. Assessing rPEG hemocompatibility in human blood in vitro. mPEG₁₁₇, rPEG₁₁₂^{0.21}, rPEG₁₂₀^{0.45} and rPEG₁₀₉^{0.74} were evaluated for their potential to induce/inhibit platelet aggregation, activate the complement system and affect normal plasma coagulation times. Each sample was tested at four concentrations (0.004–1 mg mL⁻¹). Two (complement assay) or three (all other tests) independent samples were prepared for each concentration and analyzed in duplicate (% CV < 20 for all assays and % CV < 5 for plasma coagulation assay). Shown is the mean response ±SD (A,B) Platelet aggregation: (A) particles alone were tested to verify their potential thrombogenic effect; (B) in combination with the assay positive control collagen (PC) to assess their anticoagulant activity. (C) Complement activation. PBS was used as the negative control (NC). Cobra venom factor (PC), and Cremophor-EL (Cre) were used as positive controls. (D–F) Plasma coagulation was assessed in thrombin, prothrombin (PT), and activated partial thromboplastin (APTT) time assays. WHO standard normal (Control N) and abnormal plasma (Control P) were used to qualify the instrument's performance.

drops to 1.6 to 6 × 10⁻²% (Figure 6F, Table S9). While the EC₅₀ value of rPEG₁₁₂^{0.21} significantly differs from mPEG₁₁₇, a further increase of the GME content has a minor impact on the APA binding relative to rPEG₁₁₂^{0.21}. This can be explained with the two different binding domains of the end group-selective APA. The APAs' end group binding domain cannot contribute to the overall binding, as all rPEGs are furnished with a blocked α-chain end (Figure 1A). Consequently, merely the domain that interacts with PEG₇ sequences along the polymer backbone can mediate binding. Since the change in affinity is significantly affected by blocking of the end group binding

domain, this appears to be the primary binding site for the end group-selective APA. The results further confirm that the methoxy groups of the side chains do not enable binding of the end group-selective APAs, in stark contrast to a methoxy end group.

CONCLUSION

The increasing abundance of anti-PEG antibodies (APA) in the population leads to undesired immune responses to PEGylated products, thereby altering their efficacy (e.g., via inducing premature drug release and causing accelerated blood

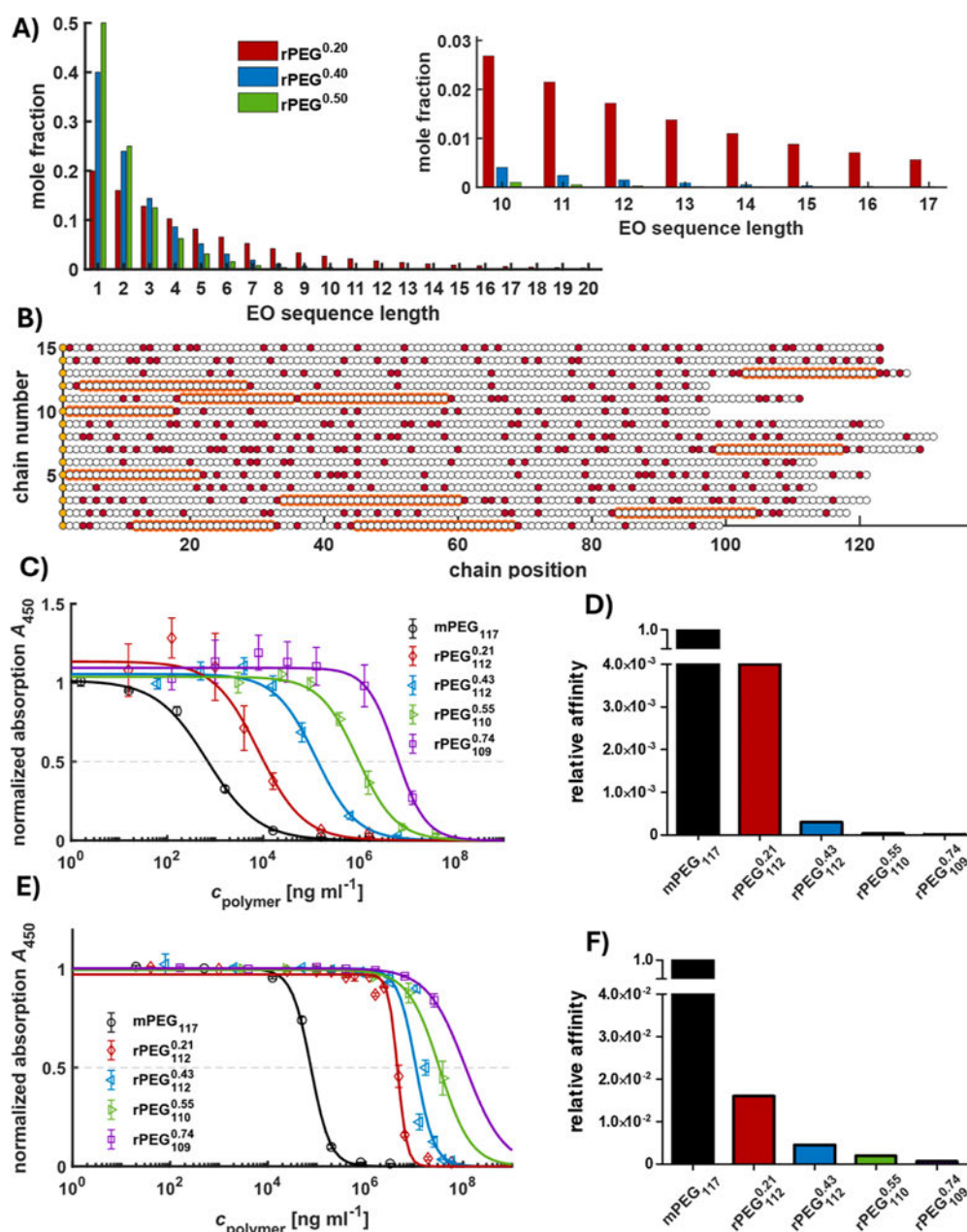


Figure 6. Calculation and influence of 'synthetic point mutations'. (A) Mole fractions of EO sequence lengths for different comonomer compositions; (B) section of 15 chains from the simulation (10⁴ chains) of EO (white) and GME (red) repeating unit distribution at different chain positions of rPEG₁₁₄^{0.20}; repeating patterns with 16 consecutive EO repeating units are highlighted in orange; yellow circles represent the initiator; (C) backbone-selective APA ELISA of investigated rPEGs and mPEG₁₁₇; the dashed gray line represents the half-maximal effective concentration (EC₅₀) value; (D) relative affinity (1/EC₅₀) of backbone-selective APA for investigated rPEGs and mPEG₁₁₇ based on EC₅₀ values obtained from backbone-selective APA ELISA; (E) end group-selective APA ELISA of investigated rPEGs and mPEG₁₁₇; the dashed gray line represents the EC₅₀ value; (F) relative affinity of end group-selective APA for investigated rPEGs and mPEG₁₁₇ based on EC₅₀ values obtained from end group-selective APA ELISA.

clearance) and safety (e.g., by activating the complement system and innate immune responses). This has become an increasingly severe issue over the years, since it renders the stealth effect of PEG ineffective. Recently, this issue has increased drastically, as the containment of the global COVID-19 pandemic strongly relied on PEGylated lipids for the transport of mRNA vaccines. We introduce the concept of random polyether copolymers that are structural PEG isomers,

i.e., randomized PEGs (rPEG), containing 'synthetic point mutations' as a key alternative to PEG for applications in therapeutic nanomedicine.

Detailed in situ ¹H NMR kinetics experiments revealed an ideally random introduction of sterically demanding branching points in PEG by random copolymerization of EO and the comonomer glycidyl methyl ether (GME). The highly hydrophilic rPEGs demonstrate drastically reduced recognition

by both backbone- and end group-selective anti-PEG antibodies at pharmaceutically relevant concentrations in competitive enzyme-linked immunosorbent assays (ELISA). Simulations and calculations regarding the microstructure of such copolymers further support the experimental ELISA results. A wide structural diversity, due to the statistical copolymerization process, is present in rPEG samples, despite the precise control of chain length and excellent end group fidelity. This circumvents the formation of structural regularity within the polymer chains. Minimizing identifiable, repeated features should highly impede selective recognition of the polymer.

The presented rPEG strategy can rely on PEG technology at every stage, enabling the application of established good manufacturing practice (GMP) and integration into existing supply chains. Versatile conjugation chemistry has been established for PEG for more than three decades and is, in principle, fully transferable to rPEG. Furthermore, applying rPEGs exclusively for medical and pharmaceutical purposes, distinct from PEG's widespread use in everyday products such as surfactants, can be advantageous and help avoid undesirable side effects triggered by the anti-PEG antibodies. It remains to be determined whether the immune system can effectively adapt to a deliberately heterogeneous system and generate anti-rPEG antibodies. This important question is the focus of ongoing studies. We firmly believe that this unprecedented statistical variation approach bears universal potential to suppress undesired recognition for nanomedicine treatments ranging from protein bioconjugation to PEGylated nano-carriers such as LNPs.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.5c02716>.

Additional analytical data (NMR, SEC, MALDI TOF MS, HPLC), biocompatibility data (ELISA, cytokine release) and experimental procedures and methods and supplementary text and simulation plots (PDF)

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Notes

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Curriculum Vitae

Curriculum Vitae

Dominik Schulz

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Work experience

08/2021 – 06/2025

Research Scientist/PhD Student,

Johannes Gutenberg – University Mainz, Department Chemistry,
Research Group [REDACTED]

- Participation in cooperation project 1066 of the Collaborative Research Centre (CRC) – “Nanodimensional Polymer Therapeutics for Tumour Therapy”
- Advanced Training in MALDI TOF mass spectrometry
- Supervision of student lab courses (organic chemistry, medicinal polymers) as well as Bachelor’s and Master’s students

04/2021 – 08/2021

Graduate assistant,

Johannes Gutenberg – University Mainz, Department Chemistry,
Research Group [REDACTED]

- Laboratory operations
- Writing of scientific manuscripts

05/2015 – 04/2018

Retail work,

[REDACTED]

Further Education

10/2024 Examined basics course “**GxP – Intensivtraining**” by Pharma Akademie

09/2024 „**Teamwork and leadership for scientists**”

Educational Background

Since 08/2021 **PhD Thesis Macromolecular Chemistry,**
Johannes Gutenberg – University Mainz

10/2006 – 01/2021 **Chemistry Diploma,**
Johannes Gutenberg – University Mainz

07/2014 – 09/2019 Health related study hiatus,
completed recovery

Special Skills

IT **MS Office** / advanced proficiency
MestreNova, PSS WinGPC, ChemDraw, Reaxys, Citavi, Polyspace
MATLAB / basic proficiency

Language skills **German** / native
Englisch / business fluent
Spanish / basic knowledge

Conference attendance

- 09/2022 14th International Symposium on Ionic Polymerization (IP'22), Ghent, Belgium.
Poster presentation: Vinyl ether-based building blocks for the synthesis of polymers that are degradable at a pH of 6.
- 05/2023 7th International Symposium Frontiers in Polymer Science, Gothenburg, Sweden.
Poster presentation: Synthesis and functionalization of polyethylene glycol (PEG) isomers: reinventing a well-known polymer.
- 09/2023 Polymer Meeting 15 (PM15), Bratislava, Slovakia.
Poster presentation: Synthesis and functionalization of polyethylene glycol (PEG) isomers: reinventing a well-known polymer.
- 10/2023 14th European and Global Summit for Clinical Nanomedicine (CLINAM23), Basel, Switzerland.
Poster presentation: Synthesis and functionalization of polyethylene glycol (PEG) isomers: reinventing a well-known polymer.
- 04/2024 17th European Symposium on Controlled Drug Delivery, Egmond aan Zee, Netherlands.
Poster presentation: random PEGs (rPEGs) – a non-immunogenic PEG isomer for alternative treatment options.
- 07/2024 50th IUPAC World Polymer Congress, Warwick, United Kingdom.
Poster presentation: random PEGs (rPEGs) – a non-immunogenic PEG isomer for alternative treatment options.
- 09/2024 15th International Symposium on Ionic Polymerization (IP'24), Mainz, Germany (hosted by Frey Research group).
Poster presentation: rPEG - evading immune recognition through isomerization of PEG.
- 01/2025 International Symposium of the CRC 1066, Mainz, Germany.
Poster presentation: rPEG - evading immune recognition through isomerization of PEG.

Publications

Dreier, P.; Matthes, R.; Fuß, F.; Schmidt, J.; Schulz, D.; Linden, G. M.; Barent, R. D.; Schüttner, S.; Neun, B. W.; Cedrone, E.; Dobrovolskaia, M. A.; Bros, M.; Frey, H. Isomerization of Poly(ethylene glycol): A Strategy for the Evasion of Anti-PEG Antibody Recognition. *J. Am. Chem. Soc.* **2025**, *147* (25), 21538–21548

Deuker, M.; Schulz, D.; Koynov, K.; Morsbach, S.; Frey, H.; Landfester, K. Overcoming PEG Antigenicity: Statistical PEG Isomers Reduce Antibody Binding. *Adv. Sci. (Weinheim, Ger.)* 2026, *13* (7), e21061.

Schulz, D.; [REDACTED]
[REDACTED] H. Nanoscopic characterization of the thermal phase behavior of random poly(ethylene glycol – glycidyl methyl ether) copolymers (rPEGs), *under review at Macromolecules*