

2-methyltetrahydrofuran (2-MeTHF) as a versatile green solvent for the synthesis of amphiphilic copolymers via ROP, FRP and RAFT tandem polymerizations.

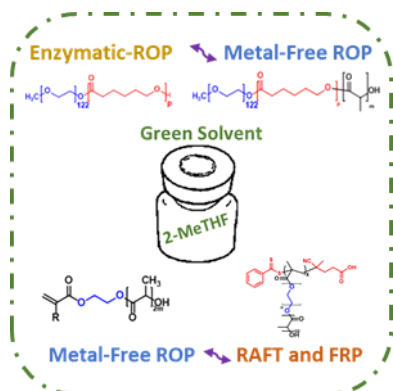
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Graphical Abstract



We have tested the suitability of 2-MeTHF as a green solvent for organo- and enzymatically catalyzed ROP of simple diblocks and in the production of more interesting A-B-C blockcopolymers using a single or double catalyst system. Labile-ester ROP initiators HEMA and PEGMA were also used to initiate LA macromonomers. To further demonstrate the versatility of 2-MeTHF as “multipolymerization” green solvent the produced macromonomers were tested in FRP and RAFT tandem polymerization.

Abstract

2-methyltetrahydrofuran (2-MeTHF) is a readily available, inexpensive, neoteric, bio-based solvent. It has been adopted across a wide range of chemical processes including the batch manufacture of fine chemicals, enzymatic polycondensations and ring opening polymerizations. To reduce the environmental burden related to the synthesis of pharmaceutical-grade polymers based on lactide and caprolactone, we envisaged the use of 2-MeTHF. For the first time, we combined a series of metal-free and enzymatic ROPs with free radical and controlled RAFT polymerizations (carried out separately and in tandem) in 2-MeTHF, in order to easily tune the chemistry and the architecture of the final polymers. After a simple purification, the amphiphilic polymers were formulated into nanoparticles (NPs) and tested for their cytocompatibility in three model cell lines, to assess their application as potential polymeric excipients for nanomedicines.

Introduction

Petrochemical solvents such as dichloromethane (DCM) and tetrahydrofuran (THF) are generally chosen for a variety of polymerization processes because they are relatively inexpensive. These conventional solvents dissolve a wide range of monomer precursors and the resulting polymer products, making them ideal solvents for homogeneous polymerizations. However, e.g., DCM is known to be carcinogenic to humans according to the World Health Organization IARC evaluations and it is recognized to contribute to the ozone depletion.[1] The European Union (EU) flagship guidance on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) has applied restrictions on the use of many petrochemical solvents, including carcinogens, mutagens and hazardous air pollutants.[1] The fundamental principles of green chemistry advocate to move towards 'greener' chemicals in place of more harmful analogues, a premise codified in EU legislation (Study for the strategy for a non-toxic environment of the 7th Environment Action Programme). The use of renewable feedstocks to replace petrochemicals represents one such 'greening' principles.

Economic pressure to use greener solvents in the chemistry sector is driving the substitution of petrochemicals with those produced from renewable bio-based feedstocks.[2],[3],[4] 2-Methyltetrahydrofuran (2-MeTHF) has been proposed as a suitable biologically produced alternative to petrochemical solvents.

2-MeTHF is a volatile cyclic ether generated by the chemo-catalytic treatment of biomass and has been touted as the most successful neoteric bio-based solvent.[5,6] It has been characterised for biological applications[7] and used in several laboratory-scale chemical processes.[8]

Selected solvent properties of 2-MeTHF are compared to those of conventional solvents in Table 1. As a synthetic solvent for polymerization, it is very close to the solvent power of conventional solvents; the Hildebrand solubility parameter for 2-MeTHF is reported as $16.9 \text{ MPa}^{1/2}$,[8] in line with the petrochemical solvents THF and DCM that range between $18\text{-}20 \text{ MPa}^{1/2}$, showing its potential as a good solvent for most ring opening polymerization (ROP). In addition, it exhibits a higher boiling point compared to DCM, THF and other cyclic solvents, which can allow for reactions to be conducted at a higher temperature, giving access to thermally initiated radical processes under ambient pressure. A further advantage is that 2-MeTHF shows a lower critical solution temperature with water,[9] minimising the effects of hydrolytic initiation caused by contaminant water, a crucial limitation in ROP synthesis. At the laboratory scale, the cost of 2-MeTHF is comparable to that of conventional solvents and can be expected to decrease with the economy of scale-up. These physical properties designate 2-MeTHF as an attractive solvent for a variety of reactions.

Table 1. Comparison of solvent properties

Solvent	Cost (£/L)*	$d_{\text{Hildebrand}} (\text{MPa}^{1/2})$	T_b (°C)*
2-MeTHF	51.5	16.9[8]	80.2
DCM	25.4	20.2**	39.8
THF	37.9-81.0	18.6**	65.0

*All costs and boiling points tabulated for laboratory grade reagents on Sigma-Aldrich website; ** Physical Properties of Polymers Handbook, 2nd ed. J. E. Mark.

Aliphatic polyesters have been explored for a range of biological applications including bone and tissue scaffolds, sutures and pharmaceutical encapsulation for drug and vaccine delivery systems.[8],[10],[11]

Specifically, the use of poly(lactide)s (PDLLA), poly (caprolactone)s (PCL) and poly(glycolide)s (PGA) and their copolymers have been the main focus for biomedical applications.[12],[13] Ideally, these sets of polyesters can also be produced from biological feedstocks, making them 'green' alternatives to petrochemical polymers. They are biocompatible, hydrolytically degraded for removal of their breakdown metabolites through natural excretory pathways in the human body, mechanically strong, sourced from abundant biological feedstocks and can be produced via multiple synthesis pathways.[14–17] It worth mentioning that most of these polyesters can be also synthesized in melt conditions (solvent-free) at relatively elevated temperature (>130°C) with high energy consumption, with a high risk to reduce the quality of the final polymer (e.g. side reactions) as well as difficulties to perform tandem reactions.[18]

The production of versatile biodegradable copolymers with hybrid architectures can be achieved by employing functional initiators such as (meth)acrylates, which introduce an end-chain reactive double bond to the final polymer product.[19] The functionality of the resultant copolymers can be explored further with a second synthesis step, such as Free Radical Polymerization (FRP) or a controlled mechanism as Reversible Addition-Fragmentation chain-transfer (RAFT) polymerization.[11]

Previously we demonstrated the suitability of 2-MeTHF for ROP process producing a library of PDLLA-based oligomers initiated by terpene-alcohols, achieving a totally sustainable chemical process. [9] In the present work, we are interested to expand upon this and assess the ability of 2-MeTHF as a "multipolymerization" solvent, allowing multiple and tandem reactions in similar conditions to produce common polymeric products and make green synthesis more accessible. To further reduce the environmental burden related to the synthesis of these pharmaceutical-grade polymers, we also explored 1,8-diazabicyclo[5.4.0]undec-5-ene (DBU) and *Candida antarctica* lipase B, also known as Nomozym 435 (lipase), as metal-free low-temperature catalysts for all the ROP reactions.

In the present work, various hydrophilic initiators, including methyl-polyethylene glycol 5000 Da (mPEG), polyethylene glycol methacrylate 300 Da (PEGMA) and hydroxyethyl methacrylate (HEMA), have been employed in the polymerization processes to generate amphiphilic block copolymers. mPEG initiated ROPs allowed the production of linear block copolymers; in particular mPEG-PDLLA, mPEG-PCL and mixed initiated m-PEG aliphatic block copolymers were designed alternating the use of DBU and lipase (separately or in tandem). Whereas PEGMA and HEMA produced grafted block (co)polymers. In both instances, the block copolymer architecture enables the resulting polymers to self-assemble into nanostructures in aqueous environments. These nanoparticles may be envisaged as functionalized polymeric carriers for drug delivery systems.

The resulting amphiphilic copolymers have been nanoprecipitated to investigate their self-assembling behaviour into nanoparticles (NPs) in an aqueous environment. Finally, the formulated nanoparticles were tested for their cytocompatibility in three model cell lines to evaluate their future use as drug delivery carrier-systems.

Experimental Section

Only commercially available chemical reagents were used and purchased from Merck, Sigma Aldrich or Fischer Scientific UK and used as received unless otherwise stated. Solvents were purchased from Fischer Scientific UK and used without further purification unless otherwise stated. Novozym 435 (Candida Antarctica lipase B immobilized on acrylic resin) was kindly donated by Novozymes A/S, Denmark.

Nuclear magnetic resonance spectroscopy: Conversion and degree of polymerization of each reaction were determined using ^1H nuclear magnetic resonance spectroscopy. CDCl_3 was used as common deuterated solvent and all samples were analysed using a Bruker DPX 400 MHz spectrometer operating at 400 MHz (^1H). Chemical shifts were assigned in parts per million (ppm). MestReNova 6.0.2 copyright 2009 (Mestrelab Research S. L.) was used for analysing the spectra.

Size Exclusion Chromatography (SEC) was performed in THF (HPLC grade, Fisher Scientific) as the eluent at 40 °C using two Agilent PL-gel mixed-D columns in series with a flow rate of 1 mL min⁻¹. A differential refractometer (DRI), were used for sample detection. The system was calibrated using polymethylmethacrylate (PMMA) standards.

Dynamic Light Scattering (DLS): Particle size analyses were performed by DLS utilizing a Zetasizer Nano spectrometer (Malvern Instruments Ltd) equipped with a 633 nm laser at a fixed angle of 173°. Nanoparticles were prepared at a concentration of 1 mg/mL adopting a simple solvent displacement methodology (acetone/water ratio 1:5).[10] Samples were equilibrated at 25°C for 20 seconds prior to measurements. All experiments were performed in duplicate averaging 15 scans per run of the same sample.

Transmission Electron Microscopy (TEM) samples at a concentration of 0.1 mg/mL in aqueous suspension (13 μL) were added to a copper grid (Formvar/carbon film 200 mesh copper [100]). The samples were left on the grid for 10 min and then the excess was removed with paper. The grid was allowed to dry under a fume hood for a minimum of 30 min prior to use. TEM images were recorded using FEI Tecnai BioTwin-12 TEM equipped with a digital camera at the Nanoscale and Microscale Research Centre of the University of Nottingham.

Synthetic Strategies

DBU-catalyzed mPEG-initiated ROP of lactide in 2-MeTHF (M/I ratio of 25)

The desired amount of LA and mPEG-initiator were weighed into a vial (pre-dried in an oven at 100 °C overnight). The [M]:[I] ratio was 25:1 or 125/1. for the synthesis of mPEG-LA₂₅ (25:1 M/I ratio) 0.55 mmol of mPEG and 13.9 mmol of LA were dissolved in 10 mL of 2-MeTHF, the vial was capped and the mixture was allowed to fully dissolve at 65 °C. DBU was then added at 2 % w/w compared to the monomer, to initiate the ROP reaction. After 20 min of reaction time, the process was stopped by precipitating the reaction mixture into cold heptane/diethyl ether (30-40 mL). The polymer was purified via two further precipitation steps and dried in a vacuum oven with quantitative conversion of monomer into polymer (from 80 to 98%). The same procedure was exploited to produce mPEG-LA₁₂₅ by altering the monomer/initiator feed-stock ratio to 125/1 and to extend the A-B block-macroinitiator based on mPEG-CL₅₀ to produce an A-B-C block copolymer. ^1H NMR (mPEG5000-(LA)₂₅ as model) (400 MHz, CDCl_3 , ppm): δ 5.19 (broad m, 48H), 3.66 (broad s, 492H), 3.02 (s, 3H), 1.59 (broad m, 144H).

Lipase-catalyzed mPEG-initiated ROP of caprolactone in 2-MeTHF (M/I ratio of 50)

The desired amount of ϵ CL and mPEG-initiator were weighed into a vial (pre-dried in an oven at 100 °C overnight). The [M]:[I] ratio was 25:1, 50:1 or 125:1. For the synthesis of mPEG-CL₂₅, 0.35 mmol of mPEG and 17.5 mmol of ϵ CL were dissolved in 10 mL of 2-MeTHF, the vial was capped and the mixture was allowed to fully dissolve at 65 °C. The heterogeneous Lipase catalyst was then added at 10 % w/w compared to the monomer, to initiate the ROP reaction. After 6h of reaction time, the process was stopped by precipitating the reaction mixture into cold methanol (30-40 mL). The polymer was purified via two further precipitation steps and dried in a vacuum oven with quantitative conversion of monomer into polymer (from 96 to 100%). This polymer was used as an A-B block-macroinitiator to be extended with a sequential, one pot DBU catalyzed ROP of LA. ¹HNMR (mPEG5000-(CL)₂₅ as model) (400 MHz, CDCl₃, ppm): δ 4.08 (broad t, 52H), 3.66 (broad s, 492H), 2.32 (broad t, 52H), 1.66 (broad m, 128H), 1.40 (broad m, 52H).

DBU-catalyzed PEGMA and HEMA-initiated ROP of lactide in 2-MeTHF (M/I ratio of 25)

The desired amount of LA and methacrylate-ester labile-initiator were weighed into a vial (pre-dried in an oven at 100 °C overnight). The [M]:[I] ratio was kept fixed at 25:1 for both PEGMA and HEMA. In the case of HEMA-LA₂₅, 0.55 mmol of HEMA and 13.9 mmol of LA were dissolved in 10 mL of 2-MeTHF in a capped vial and the mixture was allowed to fully dissolve at 65 °C. DBU was then added at 2 % w/w compared to the monomer, to initiate the ROP reaction. After 20 min of reaction time, the process was stopped by precipitating the reaction mixture into cold heptane/diethyl ether (30-40 mL). The polymer was purified via two further precipitation steps and dried in a vacuum oven with quantitative conversion of monomer into polymer (from 80 to 98%). The same procedure was exploited to produce PEGMA-LA₂₅. ¹HNMR of FRP-HEMA-LA₂₅ (400 MHz, CDCl₃) δ 6.14 (s, 1H), 5.62 (s, 1H) 5.18 (broad m, 48H), 4.42 (broad m, 4H), 1.90 (broad s, 3H) 1.59 (broad m, 156H).

FRP or RAFT polymerization of PEGMA-LA₂₅ and HEMA-LA₂₅ in 2-MeTHF

The desired amount (300mg) of macromonomer HEMA-LA₂₅ (or PEGMA-LA₂₅), RAFT agent (4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid, 1/15 mol ratio compared to monomer) and AIBN (1/5 mol ratio compared to RAFT) and were weighed into a vial and fully dissolved in 1.5 mL of 2-MeTHF. The reaction mixture was degassed by Ar bubbling in ice for 30min. Subsequently, the vials were placed in oil bath at 65 °C for 18h. The process was stopped by adding the reaction mixture into cold diethyl ether/THF (30-40 mL). The FRP strategy was performed following the same steps of the RAFT reactions without the addition of any RAFT agents and used a ratio of 0.5% w/w AIBN in relation to monomer. ¹HNMR of FRP-HEMA-LA₂₅ (400 MHz, CDCl₃) δ 5.18 (broad m, 48H), 4.30 (broad m, 2H), [2.19 (broad s, 6H) and 1.74 (broad m, 14H); CH₂, and CH₃ of polymer backbone and CHCH₃] 1.65 (broad m, 156H).

Biological Assays

Cell culture conditions

Caco-2 human epithelial colorectal cells, Calu-3 human epithelial lung cells and THP-1 human monocytes were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and were all used within a passage window of 5. Cells were routinely cultured in a growth medium at 37 °C with 5% CO₂ in 75 cm² culture flasks. The Caco-2 and Calu-3 cell growth medium consisted of Dulbecco's Modified Eagle Medium (DMEM) supplied with 10% (v/v) Fetal Bovine Serum (FBS), and 2 mM L-glutamine. Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) FBS and 2 mM L-glutamine was used for the culture of THP-1 cells.

Cytotoxicity experiments

The lactate dehydrogenase (LDH) release assay (Sigma-Aldrich, TOX7 kit) and the PrestoBlue cell viability assay (Thermo Fisher Scientific) were used to assess nanoparticle cytotoxicity via the assessment of cell membrane integrity and cellular metabolic activity, respectively. All cells were seeded at 1×10^4 cells/well in 96 well plates. THP-1 monocytic cells were seeded into plates with the addition of 100 ng/ml phorbol 12-myristate 13-acetate (PMA) to induce their differentiation into macrophages. Cells were cultured in plates for 24 h prior to assaying. Nanoparticle formulations were applied to cells in Hanks Balanced Salt Solution (HBSS) at concentrations of 0.5 mg/ml for 24 h. Triton X-100 (TX) applied at a concentration of 1 % (v/v) was used as a positive, cell death control and a vehicle control of HBSS containing no nanoparticles was used as a negative control. After exposure, 50 µl of supernatant was collected per well for analysis of LDH content. Cells were then washed twice with phosphate-buffered saline (PBS) and 100 µl 10% (v/v) PrestoBlue reagent diluted in phenol red free medium applied per well for 60 min. The resulting fluorescence was measured at 560/600 nm ($\lambda_{ex}/\lambda_{em}$) on a Tecan Spark M10 multimode plate reader. Relative metabolic activity was calculated by setting values from the negative control as 100% and positive control values as 0% metabolic activity. Detection of LDH release was performed according to the manufacturer's instructions and involved adding 100 µl LDH reagent to collected supernatant samples and incubating at room temperature shielded from light for 25 min. Absorbance was then measured at 492 nm. Relative LDH release was calculated with the negative control absorbance at 492 nm taken as 0%, and the positive control, assumed to cause total cell lysis, as 100%.

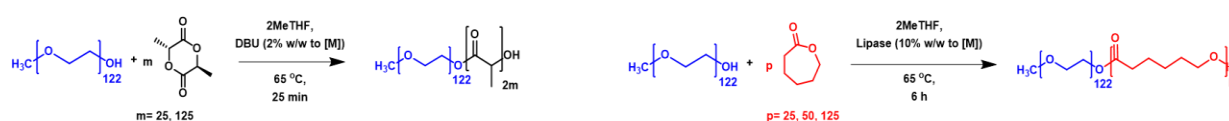
Results and Discussions

mPEG-CL, mPEG-LA and mix Pegylated-block synthesis in 2-MeTHF

2-MeTHF has a relatively high boiling point (80 °C) and showed a good dissolution ability for all the adopted reagents. This allowed us to carry out different polymerization strategies that commonly demand the utilization of different solvents and temperature conditions, directly in 2-MeTHF, without further alterations. We found that at 65 °C all the reagents were fully soluble at the used concentrations. This temperature was thus set as the reaction temperature for the metal-free ROP, enzymatic-ROP, FRP and RAFT synthetic steps in 2-MeTHF, thereby overcoming any solubility and reaction activation limitations.

There are a variety of catalysts (metal-based or metal-free) able to catalyse an ROP process, in solution or bulk.[20] Among the commercially available catalyst, the organocatalyst DBU and the enzyme Lipase are able to catalyse the ring opening of various cyclic monomers in mild and controlled conditions.[21],[22] Due to their different chemical affinity it is fundamental to combine the two catalysts for the production of mixed lactone-lactide copolymers. However, the reaction timeframe in which they act is completely different (minutes for DBU and hours for the biocatalyst) and difficulties can be encountered as well as side reactions may occur.[23] We have tested the suitability of 2-MeTHF as a solvent for the reaction of simple diblocks (single lactide and single lactone) and in the production of more interesting A-B-C block copolymers (lactone and lactide in the same chain) using a single or double catalyst system. The final material, if the sequential double catalysis reaction proves successful, would contain both caprolactone and lactide, envisaging a tunable (bio)degradability that is key in the production of smart polymeric excipients.

For the synthesis of pharmaceutically relevant, amphiphilic Lactide-PEGylated block copolymers in a single reaction step in 2-MeTHF, a metal-free ROP synthetic strategy catalysed by DBU was adopted (Scheme, Left).



Scheme 1. (Left) mPEG-initiated Lactide ROP in 2-MeTHF catalysed by DBU and (Right) mPEG-initiated Caprolactone eROP catalysed by Novozym 435 (Lipase) also in 2-MeTHF.

mPEG₅₀₀₀ was used as a macroinitiator and the initiator/monomer ratios were set to produce 25 and 125 LA units in order to establish the effect of the hydrophobic chain length on the final amphiphilic balancing of the resulting polymers. LA reached quantitative conversion into polymers within 25 mins (Figure S1 t0 vs purified), independent of the monomer/initiator ratio. The ¹H NMR of the purified copolymers show the characteristic peaks of the polymerized LA, in good agreement with the

monomer and initiator feed ratios (Table 2 and **Figure S1 GPC trace**). Control of the polymerization was confirmed by low dispersity values in the GPC analysis (<1.3 as from Table 2). DBU is an ideal catalyst for fast and controlled ROP of LA,[24] however, it has shown scarce activity in the ROP of lactones.[22,24] In order to alter the nature of the hydrophobic monomer, and with the aim of expanding the accessible library of amphiphilic materials synthesisable in 2-MeTHF, a lipase catalysed-ROP of ϵ -CL was performed. mPEG₅₀₀₀ (mPEG) was maintained as the macroinitiator and the initiator/monomer ratio was changed to achieve a final polymer chain length of 25, 50 and 125 units of ϵ -CL (Scheme 1, Right). ϵ -CL was quantitatively converted into polymers within 6h (Figure S2 t0 vs purified), with no discernible reactivity differences between each monomer/initiator feed-ratio. As expected, considering the bio process being heterogeneous, due to the use of immobilised lipase, as well as the different nature of the catalytic step the reaction time was longer than the DBU catalysed ROP. The enzyme was filtered out and the reaction mixture was precipitated in a solution of cold methanol. The ¹H NMR of the purified copolymers show the characteristic peaks of PCL (Table 2 and Figure S2). **Adequately controlled molecular weight dispersities were observed by GPC analysis (equal or below 1.3, Table 2) although a small shoulder in the GPC trace of the mPEG-CL₅₀ (Figure 1Cii) can be observed, which is likely due to some uncontrolled side reactions e.g. transesterification).** However, discrepancies in terms of molecular weight values (¹HNMR calculated versus measured via GPC, Table 2) were observed, likely due to the significant chemical differences between our amphiphilic materials and the PMMA standards. This observation is known from literature[25] and might be due to the presence of various polymer-column interactions and different solvated volumes. Differences between molecular weight calculated by ¹HNMR and GPC were noticed consistently throughout the series of amphiphilic and hybrid polymers generated in this paper.

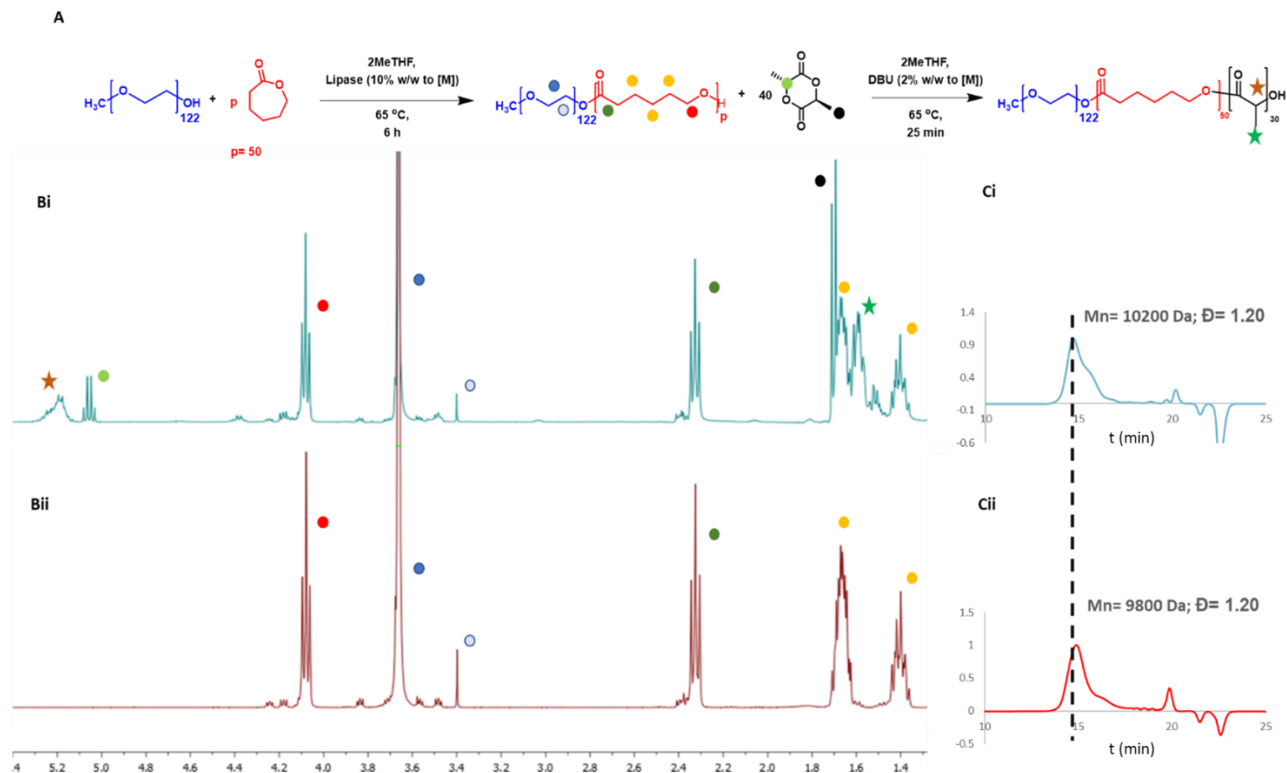


Figure 1. (A) Sequential ROP reaction scheme of mPEG-PCL₅₀ and mPEG-PCL₅₀-PDLLA₃₀, namely A-B and A-B-C block copolymers, in 2-MeTHF. **(Bi-Bii)** ¹HNMR spectra of mPEG-PCL₅₀-PDLLA₃₀ and mPEG-PCL₅₀ respectively with all the main polymeric and monomeric peaks assigned. **(Ci-Cii)** GPC traces of mPEG-PCL₅₀-PDLLA₃₀ and mPEG-PCL₅₀ respectively.

In order to produce an A-B-C (A: mPEG, B= εCL and C=LA) block copolymer, we exploited the stability of the produced polymer and the catalysts compatibility in 2-MeTHF (Figure 1A). To probe the feasibility of a sequential ROP, taking into account the different kinetics, the Lipase catalysed ring opening of εCL initiated by mPEG (M/I ratio of 50) was performed for the initial 6h as for entry 4 in Table 3 (and Figure 1A and 1Bii). Subsequently, without any purification step or catalyst removal, LA and DBU were added with a monomer (LA)/A-B macroinitiator feed ratio of 40/1. After 25 minutes (reaction time previously established for DBU catalysed ROP) the reaction mixture was analysed by ¹HNMR and the reaction was stopped. The enzyme was filtered out and the reaction mixture was precipitated in a solution of cold methanol and diethyl ether. The presence of peaks at 5.05 (quadruplet) and at 5.25 ppm (broad multiplet), related to the monomeric LA and the polymeric PDLLA CH group respectively, clearly show a slower kinetics of the third block growth. The conversion of LA into polymer, by ¹HNMR, was found to be around 80% (Figure 1Bi) compared to a full conversion in the same reaction timeframe for the diblock mPEG-LA shown previously. The slower kinetics might be due to the reduced flexibility and reactivity of the A-B macroinitiator compared to pure mPEG. **Nonetheless, the A-B-C block copolymer showed an asymmetric GPC peak (clear shoulder at lower molecular weight) indicating that the LA chain grew from the macroinitiator, however, a series of side reactions and/or unreacted macroinitiator may be present (Figure 1Ci and 1Cii). Further reaction optimization and different purification steps will be explored in future work.** The application of this polymer can be envisaged in the production of biomedical devices with a new set of physical properties and tunable biodegradability due to the coexistence of different hydrophobic polyesters.[26]

Table 2. Chemical characterization and NPs properties of mPEG PDLLA, PCL and PCL-PDLLA block copolymers synthesized in 2-MeTHF.

Entry and polymer label	M/I	Conversion (%) [*]	M _n (¹ HNMR) (Da) ^{**}	M _n (GPC) (Da) ^{***}	Đ _M	Size (nm) ^{****}	PDI
1.mPEG-LA ₂₅	25	96	8456	7000	1.21	40±5 ^a	0.250
2.mPEG-LA ₁₂₅	125	98	22568	11000	1.20	48±2 ^a	0.250
3.mPEG-CL ₂₅	25	100	7850	7600	1.20	60±2 ^a	0.140

4.mPEG- CL ₅₀	50	96	10472	9800	1.30	80±5 ^a	0.125
5.mPEG- CL ₁₂₅	125	99	19136	12400	1.26	79±2 ^a	0.100
6.mPEG- CL ₅₀ -LA ₄₀	(CL)50+(LA)40	96(CL)+78(LA)	14792	10200	1.30	72±2 ^a	0.130

* calculated by ¹HNMR

** Calculated from CH₂ mPEG-initiator backbone peak and CH PDLLA or -O-CH₂ PCL peaks ¹H NMR integration.

*** Compared to PMMA standards

**** DLS measurements

^a Average values from at least 2 sample replicates

All the materials were able to self-assemble in water into well-defined nanoparticles (NPs), with diameters ranging from approximately 40 nm up to 80 nm and PDIs equal or below 0.250 (Table 2), without the use of additional stabilizers during the nanoprecipitation step.[10] As a proof of concept, the *in vitro* cytotoxicity of mPEG-CL₅₀ and mPEG-CL₅₀-LA₂₅ was tested on Caco-2 (intestinal), Calu-3 (airway) and THP-1 (macrophage) cells at a fixed concentration of 500 µg/mL. Both particles showed no cytotoxicity in both assays carried out in all cell types studied similarly to the cell culture medium HBSS adopted as vehicle control (Figure S3 A and B).

By adopting 2-MeTHF as a solvent we were able to reduce the carbon footprint of the production of polymeric excipients with high-demand in the drug delivery and biomedical field.[15,27,28] We also confirmed the ability of these block copolymers to self-assemble into NPs along with their cytocompatibility. The use of 2-MeTHF also allowed for a sequential one-pot ROP of ε-CL and LA, crucial to produce a material with tunable biodegradability, circumventing the limitations of DBU and Lipase in the ROP of lactones and lactide respectively. Therefore, it was confirmed that the properties of the final materials produced in the ROP process were not compromised by the use of 2-MeTHF as the polymerization solvent

Synthesis of hybrid polymers via ROP-FRP or ROP-RAFT tandem reactions in 2MeTHF

The combination of ROP with controlled radical polymerizations has allowed the synthesis of amphiphilic hybrid polymers able to self-assemble into biodegradable NPs for biomedical applications. [29] One of the strategies to produce hybrid functionalized materials comprise the production of radically polymerizable macroinitiators via ROP. In particular, labile-ester initiators HEMA and PEGMA are well known to display a tendency to undergo transesterification and subsequently dimerization in the presence of active and bifunctional ROP catalysts such as lipase and TBD.[30–32] In previous

work, our group has assessed that the mild activity of DBU would prevent such transesterification side-reactions, even when targeting a DP_n below 35 was found to be unachievable with TBD.[19,30] This would allow for tandem polymerization, allowing for the production of polymers with a greater range of architectures and chemistries. To corroborate the versatility of 2-MeTHF and the ability of DBU to control polymerization reactions at 65 °C, rather than at room temperature as explored in the previous literature,[19] HEMA (Figure S4 as integrated spectra example) and PEGMA-initiated LA macromonomers were synthesized targeting a final DP_n of 25 (DP_n calculated from spectra integration as for Figure S4 and reference [19]). The control at such temperature would allow both total monomer and initiators solubility as well as the possibility to conduct radical polymerizations, AIBN initiated, in tandem simply in 2-MeTHF. In fact, the decomposition rate of AIBN at 65 °C produces a radical flux that is ideal for radical polymerizations.

For both methacrylate initiators no transesterification occurred in the reaction time frame of 25 mins (Figure 2 inset) and a quantitative conversion (>96%) of monomer into polymer was observed (Figure 2). The final DP_n was confirmed, via 1H NMR, to be equal to 25 as targeted in the initial feed ratio.

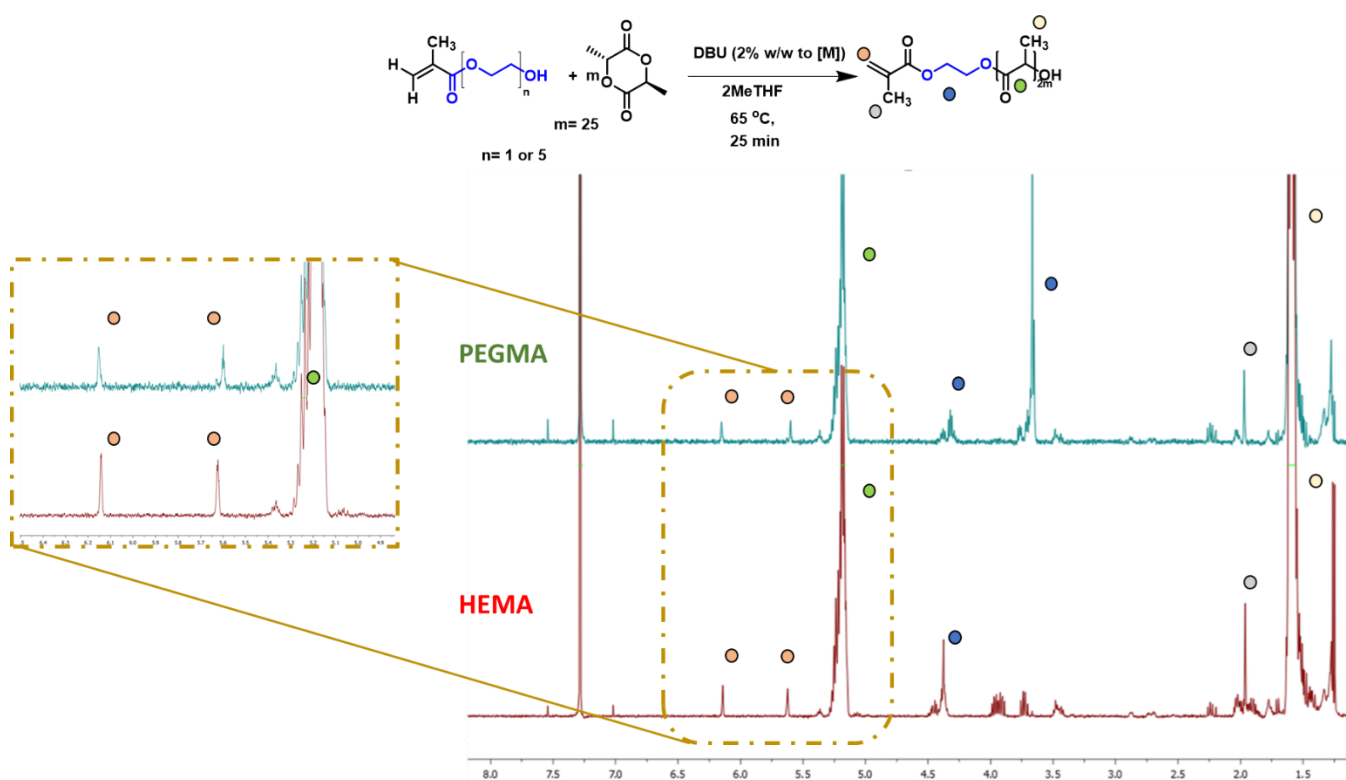


Figure 2. (Top) ROP reaction scheme of methacrylate-Lactide hybrid macromonomers in 2-MeTHF. **(Bottom)** 1H NMR of the final purified macromonomers (PEGMA and HEMA initiated) and main macromolecule peak assignments. The targeted DP (ideal number of LA units in the final oligomer) was 25, as reported in the stoichiometry of the reaction scheme. In this case the number of LA CH protons is equal to the number of repetitive units. However, LA bears two protons per full unit and thus the final DP is considered dividing by two the total number of LA CH protons.

To further demonstrate the versatility of 2-MeTHF as “multipolymerization” solvent the produced macromonomers were tested in FRP and RAFT tandem polymerization, keeping the reaction temperature of 65 °C as for the ROP stage (Figure 3A). Both FRP and RAFT model reactions yielded grafted amphiphilic biodegradable hybrid-polymers. These hybrid materials have the potential to show altered degradation profile and reduced toxicity compared to polymers synthesized simply by radical polymerizations.[29] Full conversion of macromonomers (PEGMA-LA₂₅ and HEMA-LA₂₅) into grafted-polymer was observed for the FRP reactions, while a conversion of around 70% was calculated for the two polymers polymerized by RAFT (when DP 15 was targeted) after the same reaction timeframe of 18 h (Figure 3), therefore the theoretical M_n was calculated to be 39 kDa for HEMA-LA₂₅ and 41 kDa for PEGMA-LA₂₅. GPC analysis detected a single (although slightly asymmetric, possibly indicative of minimal amount of side reactions) polymeric species only for the RAFT synthesised polymers (Figure 3C-top), indicating that the macromonomers were compatible to the tandem ROP controlled radical reaction cycle, while broad bimodal peaks were detected in both the FRP prepared polymers (Figure 3C-bottom). The discrepancy between M_n from ¹HNMR and M_n from GPC of the controlled RAFT synthesized chains might be ascribed to the spatial restriction of the long-grafted chains, along the new formed backbone, leading to a more confined solvated volume inside the GPC system. Either way, experimental M_n was lower than the theoretical M_n for RAFT polymerizations, while M_n was significantly higher for the FRP polymerizations, which is evidence of the RAFT agent controlled the reaction. The same behavior was observed for the RAFT and FRP polymers made with PEGMA-LA₂₅ (Table 3).

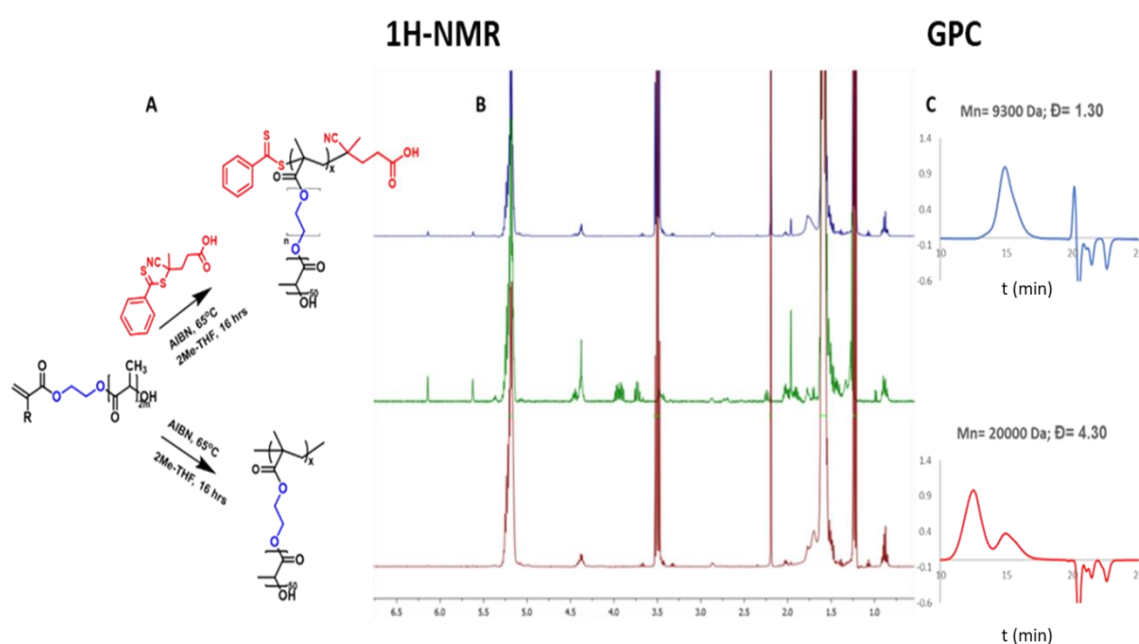


Figure 3. (A) RAFT and FRP reaction scheme of hybrid HEMA-LA₂₅ macromonomer in 2-MeTHF. **(B)** ¹HNMR spectra of FRP-HEMA-LA₂₅ (absence of residual double bond-related peaks) **(bottom)**, macromonomer starting material **(centre)** and RAFT-HEMA-LA₂₅ (residual double bond-related peaks in the circled region) **(top)**. **(C)** GPC monomodal trace of RAFT-HEMA-LA₂₅ **(top)** and GPC bimodal trace of FRP-HEMA-LA₂₅ **(bottom)**.

Moreover, considering the amphiphilicity of the grafted copolymers prepared from the tandem polymerization steps, we investigated the ability for the materials to self-assemble nanoparticles that could be utilized for pharma applications.

Table 3. Chemical characterization and NPs properties of PEGMA and HEMA macromonomers polymerized by FRP and RAFT strategies in 2-MeTHF.

Entry and polymer label	Conversion (%) [*]	M _n (¹ HNMR) (Da) ^{**}	M _n (GPC) (Da) ^{**}	Đ _M	Size (nm) ^{****}	PDI
7.FRP-PEGMA-LA ₂₅	100	-	11000	5.14	117±2 ^a	0.120
8.RAFT-PEGMA-LA ₂₅	70	27300	9800	1.40	95±2 ^a	0.140
9.FRP-HEMA-LA ₂₅	100	-	20000	4.30	125±2 ^a	0.080
10.RAFT-HEMA-LA ₂₅	70	26110	9300	1.30	93±2 ^a	0.090

* calculated by ¹HNMR

** Calculated from CH PEGMA- HEMA-initiator acrylic peak and CH PDLLA ¹H NMR integration.

*** Compared to PMMA standards

**** DLS measurements

^a Average values from at least 2 sample replicates

All the hybrid-polymers produced well-defined nanoparticles in water when nanoformulated following conventional nanoprecipitation technique, as for the block copolymers described previously. Both RAFT-PEGMA-LA₂₅ and RAFT-HEMA-LA₂₅ showed smaller particles sizes, 95 and 93 nm respectively, when compared to the two counterparts synthesised via FRP, 117 and 125 nm (Table 3). This trend may hint to an improved packing of the hydrophobic core when similar chain length are in contact, during the nanoprecipitation event, and a consequent lower degree of interaction among the grafted chains with different length as in the case of the FRP-synthesised polymers (Table 3). However, the sizes of the grafted materials were constantly bigger than the ones of NPs produced from linear diblock (Table 2). This is can likely be explained by a less efficient packing arrangement of the hydrophobic section as a result of the higher steric hindrance of the long lactide side chains. In addition, to confirm the self-assembling of these novel materials and the shape in the dry state, as example, RAFT-PEGMA-LA₂₅ was analysed by TEM (Figure S5). Globular particles with sizes ranging from 41 to 59 nm (50 ± 9 nm) can be seen confirming the nature of these NPs. Considering these novel NPs are intended for drug delivery applications an initial assessment of their *in vitro* toxicity on model

cell lines was conducted (Figure S6). For this purpose, intestinal epithelial (Caco-2 cells) and lung epithelial (Calu-3 cells) were selected to model administration *via* oral and inhalation routes, respectively. Furthermore, a macrophage cell line (activated THP-1 cells) was also chosen to investigate potential cytotoxicity on innate immune cells. Cytocompatibility of the NPs was investigated at a fixed, and relatively high concentration[11] of 500 $\mu\text{g}/\text{mL}$ and the four particles showed no signs of cytotoxicity in both assays carried out and in all cell types studied (Figure S6). This included no decrease in cellular metabolic activity over 24 h (Figure S6A), and no membrane disruption, as indicated by a lack of LDH release (Figure S6B).

The high solubility of all the starting materials and the higher boiling point compared to DMC and THF allowed the use of 2-MeTHF for both ROP and radical-initiated reactions.

The utilization of a single “greener” solvent, produced from biomass that has shown negative genotoxicity and mutagenicity,[7] for all the tandem polymerizations, the easy NPs production, the proved cytocompatibility and the well-understood hydrolytical clearance and biodegradation, render these hybrid-grafted materials and their synthetic pathways promising to be adopted in drug delivery or other biomedical applications.[33–35]

Conclusions

By adopting 2-MeTHF as a solvent we have been able to reduce the carbon footprint of the production of polymeric excipients with high-demand in the drug delivery and biomedical field. We have demonstrated that the green 2-MeTHF is effective as a reaction solvent for lactide, caprolactone, block copolymers macroinitiators and hybrid methacrylate-ester macroinitiators in ROP, eROP, FRP and RAFT polymerizations as both a single process and in tandem.

The use of 2-MeTHF also allowed for a sequential one-pot ROP of ϵ -CL and LA, crucial to produce a material with tunable biodegradability, circumventing the limitations of DBU and Lipase in the ROP of lactones and lactide respectively.

Labile-ester ROP initiators HEMA and PEGMA were used to initiate LA macromonomers. For both methacrylate initiators, no transesterification occurred in the reaction time frame and a quantitative conversion of monomer into polymer was observed. To further demonstrate the versatility of 2-MeTHF as “multipolymerization” green solvent the produced macromonomers were tested in FRP and RAFT tandem polymerization. Keeping the reaction temperature at 65 $^{\circ}\text{C}$ as for the ROP stage, both FRP and RAFT model reactions yielded grafted amphiphilic biodegradable hybrid-polymers. Due to the temperature requirements for many radical processes to allow for adequate radical initiation, the use of 2-MeTHF clearly showed the potential to conduct a wider range of polymerizations compared to solvent free conditions as well as the more commonly used lower boiling point solvents, DCM and THF.

We confirmed the ability of all the amphiphilic materials produced, linear block and hybrid-grafted copolymers, to self-assemble into NPs without the use of any stabilizer. Moreover, *in vitro* toxicity testing demonstrated that the produced polymers in nanoparticle formulation are non-toxic and appropriate vehicles for future investigation in oral, inhalation and systemic drug delivery studies.

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Support Information

2-methyltetrahydrofuran (2-MeTHF) as a versatile green solvent for the synthesis of amphiphilic copolymers via ROP, FRP and RAFT tandem polymerizations.

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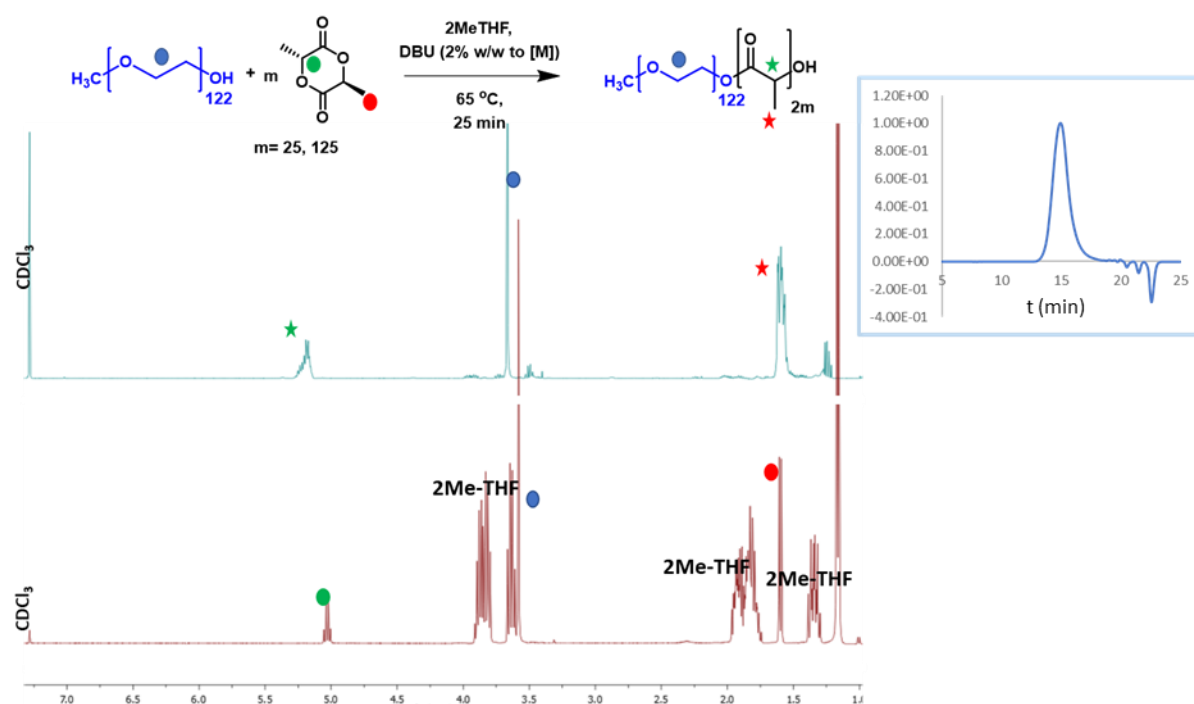


Figure S1. (Top) ROP reaction scheme of A-B block copolymers mPEG-LA. **(Bottom)** Stacked ¹H NMR spectra of the starting reagents mixture in 2-MeTHF (t=0, before DBU addition) and purified final polymer (**GPC trace, Right**). Clear shift and variations of Lactide-related peaks, methine CH at 5.05 ppm (quadruplet, monomer) and at 5.25 ppm (broad multiplet, polymer) and methyl group CH3 at 1.60 ppm (sharp doublet, monomer) and at 1.59 ppm (broad multiplet, polymer).

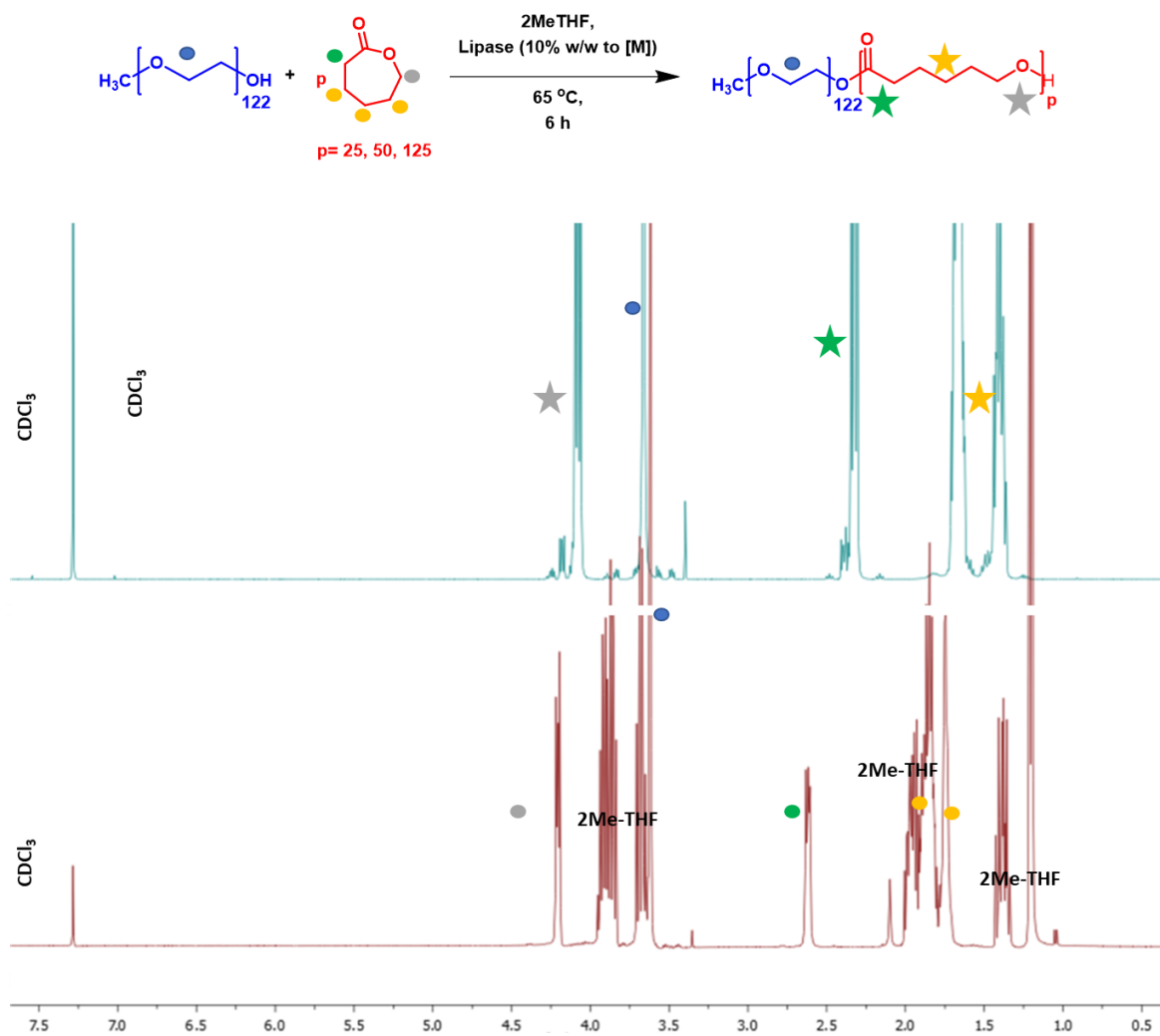


Figure S2. (Top) ROP reaction scheme of A-B block copolymers mPEG-CL. (Bottom) Stacked ¹H NMR spectra of the starting reagents mixture in 2-MeTHF (t=0, before Lipase addition) and purified final polymer. Clear shift and variations of Caprolactone-related peaks can be observed.

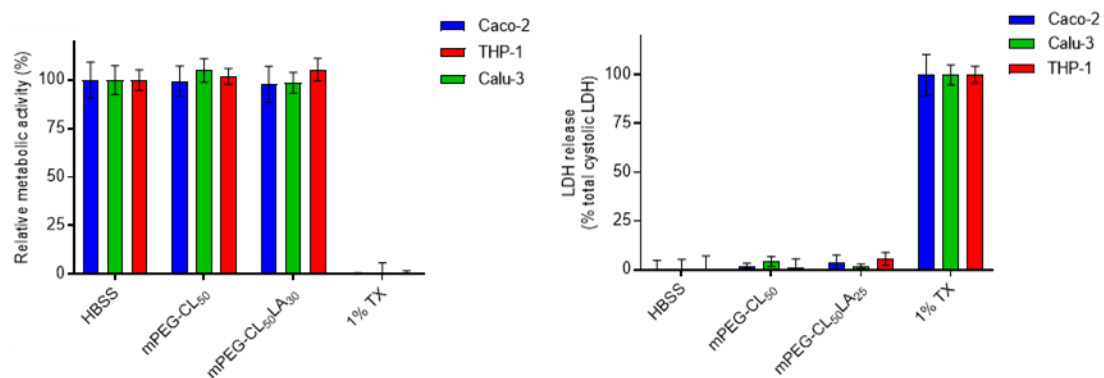


Figure S3. Cytocompatibility of NPs on Caco-2 (intestinal), Calu-3 (airway) and THP-1 (macrophage) cells. Cytotoxicity was determined by (Left) PrestoBlue metabolic activity and (Right) LDH release as an indicator of membrane damage. Particles (0.5 mg/ml) were applied to cells in HBSS and exposed for 24 hours to cells. HBSS treatment represents the vehicle control and Triton X-100 (TX) applied at 1% (v/v) was used as the cell death control. Data are presented as mean \pm S.D (n=3).

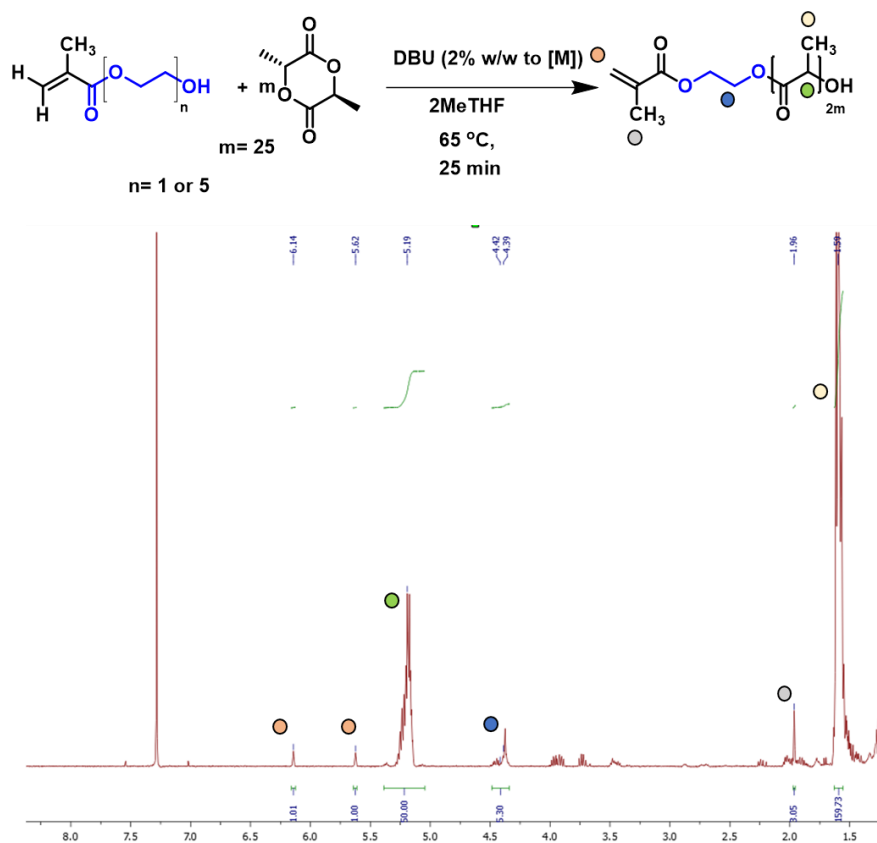


Figure S4. (Top) ROP reaction scheme of methacrylate-Lactide hybrid macromonomers in 2-MeTHF. **(Bottom)** Integrated ¹H NMR of the final purified macromonomers HEMA initiated and main macromolecule peak assignments. The targeted DP (ideal number of LA units in the final oligomer) was 25, as reported in the stoichiometry of the reaction scheme. In this case the number of LA CH protons is equal to the number of repetitive units. However, LA bears two protons per full unit and thus the final DP is considered dividing by two the total number of LA CH protons.

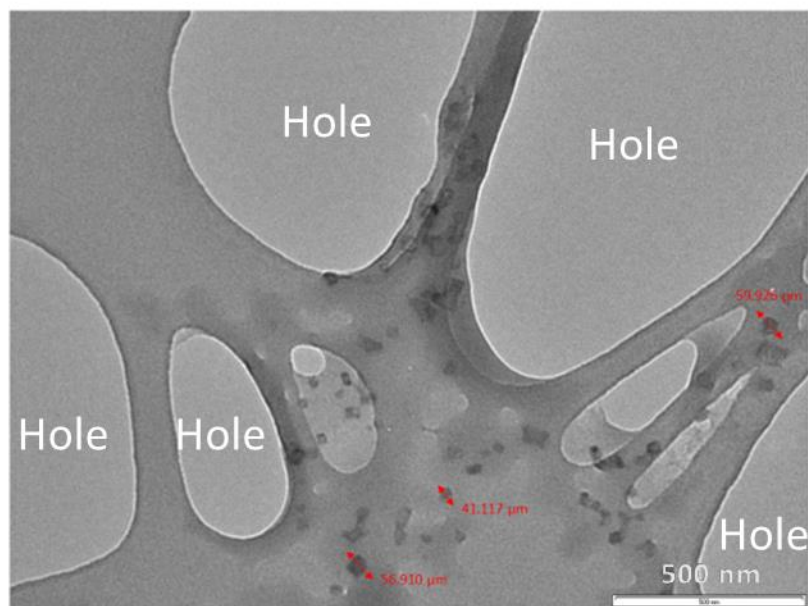


Figure S5. TEM picture of RAFT-PEGMA-LA25.

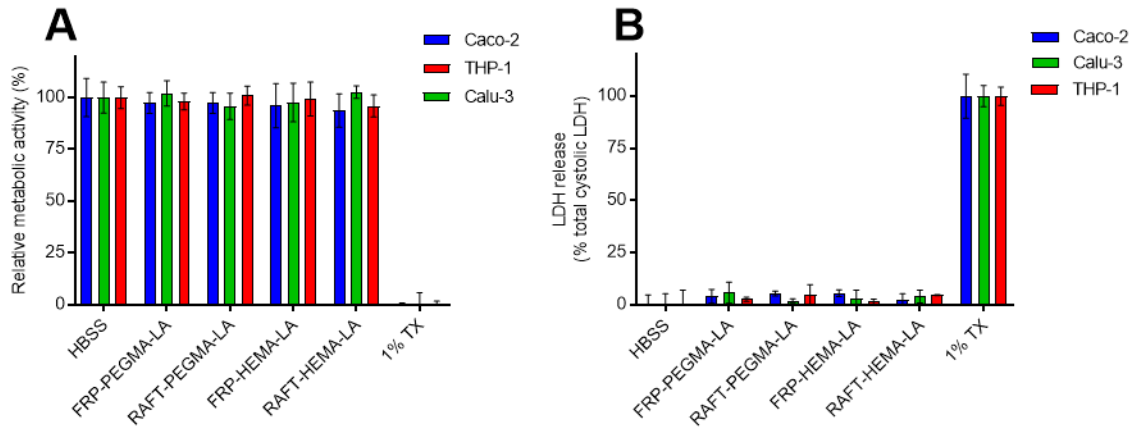


Figure S6. Cytocompatibility of NPs on Caco-2 (intestinal), Calu-3 (airway) and THP-1 (macrophage) cells. Cytotoxicity was determined by (A) PrestoBlue metabolic activity and (B) LDH release as an indicator of membrane damage. Particles (0.5 mg/ml) were applied to cells in HBSS and exposed for 24 hours to cells. HBSS treatment represents the vehicle control and Triton X-100 (TX) applied at 1% (v/v) was used as the cell death control. Data are presented as mean \pm S.D (n=3).