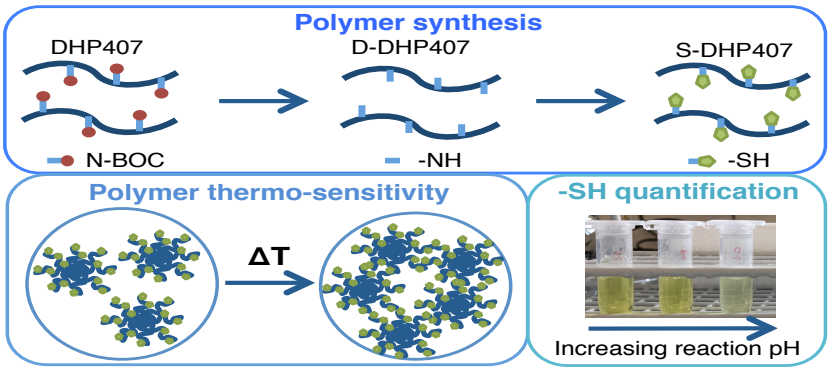
Polyurethane-based thiomers: a new multifunctional copolymer platform for biomedical applications

GRAPHICAL ABSTRACT



Polyurethane-based thiomers: a new multifunctional copolymer platform for biomedical applications

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ABSTRACT

During the past few decades, thiomers have attracted interest as responsive-polymers in the design of smart hydrogels, due to thiol reactivity to physical stimuli (i.e., pH, oxygen, UV/Vis irradiation). However, thiomer potentialities have been limited by their low thiolation degree and lack of multi-responsiveness to physical cues. In this work, a new class of thiomers with tailored thiolation degree and sensitivity to multiple physical stimuli was developed. To this aim, a polyurethane (33000 Da) was synthesised from Poloxamer® 407, 1,6-diisocyanatohexane and N-Boc diethanolamine. Secondary amino groups were exposed through Boc-removal (yield 80%, assessed by ^{1}H-NMR) giving up to $4.5x10^{20}\pm1.8x10^{19}$ –NH units/$g_{polymer}$ (Orange II Sodium Salt colorimetric assay) without polymer chemical degradation. Polymer thermo-responsiveness was proved by the increase in micelle hydrodynamic diameter upon system heating up to 45 °C and through estimated critical micellar temperatures. Thioglycolic acid grafting to exposed amines via carbodiimide chemistry was optimised to give up to $1.73x10^{19}$ thiols/$g_{polymer}$ (Ellman's method), while minimising disulphide bond formation. Finally, the best storage conditions against oxidation were investigated by quantifying free thiols at different time points.

KEYWORDS. Amphiphilic polyurethane; thiomers; responsive polymers; thermo-sensitivity; smart hydrogels.

1. Introduction

Thiolated polymers, also referred to as thiomers, are polymers containing pendant thiol groups along their backbone. Thiol groups easily react under mild pH and atmospheric conditions with a great variety of functional groups (e.g., thiols, hydroxyl groups, acrylates, methacrylates); thus, thiolated polymers have attracted a widespread interest in the research community for their potential applications in Tissue Engineering and Regenerative Medicine fields. Since the late 90s, the main application of thiomers has been aimed at improving polymer mucoadhesiveness by exploiting the interaction between thiolated polymers and thiol groups present in cysteine-rich mucus subdomains [1]. However, thiol's responsiveness to oxidative conditions, pH variations of the surrounding environment and UV/Vis irradiation paves the way to further prospective applications. Among them, the design of smart hydrogels for drug delivery systems or bio-inks

for 3D-printing applications are attracting widespread interest. For these purposes, over the past thirty years, many naturally derived and synthetic polymers have been modified to introduce thiol groups. Among natural polymers, chitosan [2], alginate [3] and hyaluronic acid [4] have been the most widely explored materials for the introduction of thiol moieties. Many works have been also published focusing on the design of thiolated hydrogels of synthetic origin, mainly based on poly(ethylene glycol), both in its linear and hyper-branched forms [5,6]. However, in all these studies, polymers were thiolated to be responsive to one physical stimulus affecting thiol reactivity. Hence, in order to obtain multifunctional materials in the form of multi-responsive hydrogels, thiolated polymers have been generally blended with polymers responsive to a different stimulus [7]. Only one attempt has been made to get multifunctional thiolated polymers through the grafting of thiol groups to an amphiphilic triblock copolymer (Pluronic® copolymers of poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) (PEO-PPO-PEO)) [7,8]. Indeed, these thiolated Pluronics® were then used to prepare temperature-sensitive hydrogels with additional responsiveness to another stimulus triggered by thiol groups. As a drawback, thiolated Pluronics could only be functionalised with two thiol groups at both end-functionalities, limiting their responsiveness to physical conditions affecting thiol reactivity.

Therefore, the purpose of the present work is to synthesise and characterise a new amphiphilic copolymer bearing a tuneable high amount of lateral thiol groups along its chains. Such multifunctional copolymers could be exploited to obtain hydrogels responsive to multiple physical stimuli: temperature, oxidative environment, pH or UV/Vis irradiation. To this aim, a new poly (ether urethane) (PEU) was first synthesised by exploiting the versatility of polyurethane chemistry. As building blocks, a PEO-PPO-PEO triblock copolymer of the previously mentioned Pluronic® family and an amino group-containing chain extender were selected to ensure thermo-sensitivity and to allow further functionalisation, respectively. Subsequently, upon amine exposure through removal of the caging group (i.e., tert-butyloxycarbonyl (BOC) protecting group), solvent-free carbodiimide chemistry was used to graft thioglycolic acid to the polymer backbone (Figure 1). To avoid the undesired formation of sub-products (i.e., disulphide bonds) during polyurethane functionalisation, different grafting conditions were tested. Then, for each tested parameter, the newly synthesised thiomer was chemically characterised. Lastly, the best storage conditions to protect against thiol oxidation were also investigated.

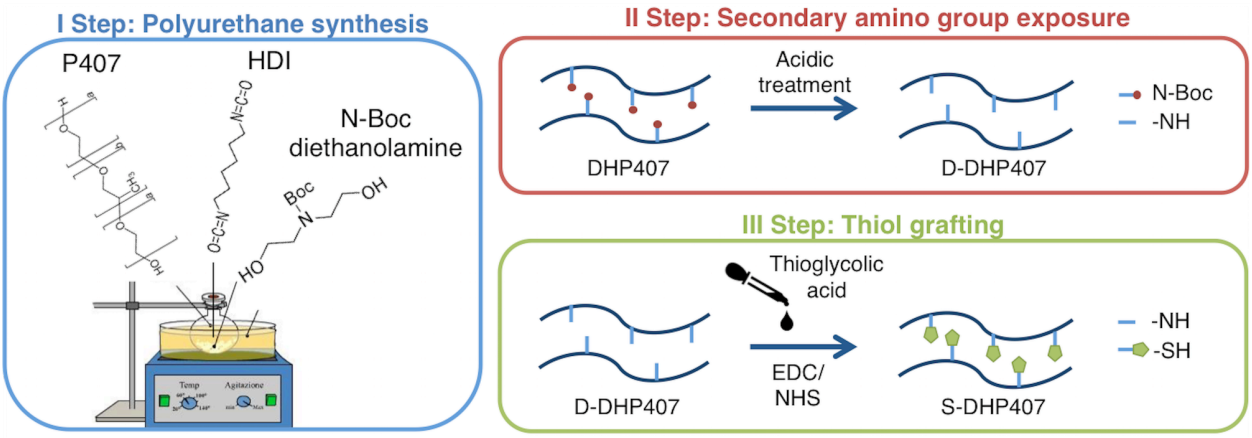


Figure 1. Schematic representation of the three main steps concerning the design of thiolated polyurethanes: synthesis (I), secondary amino group deprotection (II) and thiol grafting (III).

2. Experimental

2.1 Materials

Poloxamer® 407 (P407, PEO-PPO-PEO triblock copolymer, M_n = 12600 Da, 70% w/w PEO), 1,6-hexamethylene diisocyanate (HDI), dibutyltin dilaurate (DBTDL) and N-Boc-diethanolamine were purchased from Sigma Aldrich (Italy). Before use reagents were treated as follows in order to remove residual water and moisture: P407 was dried under reduced pressure at 100 °C for 8h and then cooled down at 40 °C under vacuum; HDI was distilled under reduced pressure; N-Boc-diethanolamine was kept at room temperature (RT) under vacuum in a desiccator and 1,2-dichloroethane (DCE) was poured over activated molecular sieves (3 Å, Sigma Aldrich, Italy) under nitrogen atmosphere overnight. All solvents were purchased from CarloErba Reagents (Italy) in analytical grade.

2.2 Poly(ether urethane) synthesis, secondary amine exposure and physico-chemical characterisation

2.2.1 Polyurethane synthesis

P407-based polyurethane was synthesised in a two-step procedure under nitrogen atmosphere according to the protocol recently published by Boffito, Pontremoli et al. [9]. However, some modifications resulting from protocol optimisation were required to maximise the number of

amines present along each polymeric chain (data not reported). Briefly, P407 was first solubilised at 15% w/V concentration in anhydrous DCE and equilibrated at 80 °C. HDI and DBTDL were then added to the macrodiol solution at 2:1 molar ratio and 0.1% w/w, respectively, with respect to P407, and the pre-polymerisation reaction proceeded for 45 minutes. At the end of the first step, the pre-polymer mixture was quickly equilibrated at 60 °C before the addition of the chain extender N-Boc diethanolamine (5% w/V in DCE) at 1:1 molar ratio with respect to P407. Subsequently, the chain extension reaction was carried on for 120 minutes. At the end of the second step, the reaction system was cooled down at room temperature, anhydrous methanol was added to stop the reaction and then the extended polyurethane was collected by precipitation in petroleum ether (4:1 vol ratio with respect to total DCE volume). Lastly, the synthesised PEU was dissolved at 20% w/V in DCE and purified by precipitation in a mixture of diethyl ether and methanol 98:2 V/V (5:1 vol ratio with respect to DCE volume). Finally, the polymer was collected by centrifugation (Hettich, MIKRO 220R) at 0 °C, 6000 rpm for 20 min, dried overnight at RT under a fume hood and finally stored at 5 °C under inert atmosphere.

Hereafter, the synthesised PEU will be referred to as DHP407, where D stands for the chain extender N-Boc diethanolamine, H refers to HDI and P407 identifies the macrodiol P407 (Figure 1).

2.2.2 Secondary amino group deprotection

To remove Boc protecting groups, thus exposing the secondary amino groups present along PEU backbone, DHP407 was subjected to an acidic treatment (Figure 1). In detail, 10 g of DHP407 powder were first solubilised in 225 mL of chloroform for 2h, at RT, under magnetic stirring (250 rpm) and nitrogen atmosphere. Afterwards, 25 mL of trifluoroacetic acid (TFA, Sigma Aldrich, Italy) were added and Boc cleavage reaction was carried on for 1h. Next, the deprotected DHP407 solution was concentrated in a rotary evaporator (Buchi Rotavapor Labortechnik AG) to remove both chloroform and TFA, washed twice with 100 mL of chloroform to completely remove residual TFA traces and dispersed in 200 mL of demineralised water (ddH_2O) under vigorous stirring at 4 °C overnight. Finally, deprotected PEU solution was dialysed (cut-off membrane 10-12 kDa, Sigma Aldrich, Italy) for 2 days against ddH_2O to wash Boc groups out and freeze-dried using a Martin Christ ALPHA 2-4 LSC.

Hereafter, PEU exposing free secondary amino groups will be referred to as D-DHP407.

2.2.3 Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) Spectroscopy

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy was conducted to verify the success of the synthesis, by assessing the appearance of the absorption peaks typical of PEU chemical bonds. Furthermore, the same analysis was also performed to verify PEU bond integrity after it was subjected to the previously described acidic treatment. For both analyses, a Perkin Elmer Spectrum 100 equipped with an ATR accessory (UATR KRSS) with diamond crystal was used. Spectra resulted from 32 scans in the spectral range from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}. The analyses were performed at RT and elaborated using the Perkin Elmer Spectrum software.

2.2.4 Size Exclusion Chromatography (SEC)

In order to investigate DHP407 and D-DHP407 molecular weight distribution, an Agilent Technologies 1200 Series (CA, USA) Size Exclusion Chromatography was used. The instrument was equipped with a refractive Index (RI) detector and two Waters Styragel columns (HR1 and HR4) conditioned at 55 °C. N,N-dimethylformammide (DMF, CHROMASOLV Plus, inhibitor-free, for HPLC, 99.9%, CarloErba Reagents, Italy), added with 0.1% w/V lithium bromide (LiBr, Sigma Aldrich, Italy), was used as mobile phase. Number average molecular weight (M_n) and polydispersity index (D) were estimated using the Agilent ChemStation software starting from a calibration curve based on poly(ethylene glycol) standards with peak molecular weight (M_p) in the range 4000-200000 Da. Before analyses, samples were dissolved in the mobile phase at 2 mg/mL and filtered through a 0.45 μm syringe filter (Poly(tetrafluoroethylene) membrane, Whatman).

2.2.5 Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy

To establish whether the adopted deprotection protocol is able to successfully remove Boc protecting groups, Proton Nuclear Magnetic Resonance spectroscopy was conducted on both DHP407 and D-DHP407 samples. In details, 10 mg of PEU were first dissolved in 750 μL of deuterated dimethyl sulfoxide (d6-DMSO, Sigma Aldrich, Italy) and then analysed using a Bruker Avance III spectrometer equipped with 11.74 T magnet (500 MHz Larmor Frequency) and a Bruker BBFO direct probe. All the analyses were performed at 300 K using a Bruker BVT

3000 unit for temperature control. Each spectrum resulted from 32 scans, while the residual d6-DMSO proton signal at 2.5 ppm was used for ^{1}H chemical shift scale.

2.2.6 Secondary amino group quantification through Orange II Sodium Salt

Secondary amino groups exposed along polymer backbone were quantified through a colorimetric assay (Orange II Sodium Salt, Sigma Aldrich, Italy), adapting to water-soluble polymers the method proposed by Noel, Liberelle et al. [10]. In detail, Orange II Sodium Salt was first dissolved at 0.175 mg/mL in ddH_2O and then solution pH was adjusted to 3 with HCl 1M. Subsequently, D-DHP407 samples were solubilised at 0.04% w/V in the dye solution and the electrostatic coupling reaction between the cationic PEU-NH and the anionic Orange molecules was carried on for 18 hours, at RT and in the dark. Then, samples were put in dialysis (cut-off membrane 10-12 kDa, Sigma Aldrich, Italy) against ddH_2O for 3 days to remove the unreacted dye and freeze-dried using a Martin Christ ALPHA 2-4 LSC. Control samples (DHP407) were also subjected to the same protocol to evaluate the amount of absorbed dye. Lastly, grafted/absorbed Orange molecules were desorbed by solubilising 10 mg of the lyophilised samples in 1 mL of ddH_2O adjusted to pH 12 with NaOH 1 M. After incubation in the dark at RT for 2 hours, samples were centrifuged at 15 °C, 6000 rpm for 10 min to separate the polymer. Extract absorbance was measured at 485 nm using an UV-Vis spectrophotometer (PerkinElmer, Lambda 25) and secondary amino groups were finally quantified referring to a calibration curve based on Orange molecules dissolved at predefined concentrations (range: 1.75 – 29.2 μg/mL) in ddH_2O at pH 12. The colorimetric assay was performed in triplicate on samples belonging to three different deprotected batches and results are reported as mean ± standard deviation.

2.2.7 Dynamic Light Scattering (DLS) measurements

To investigate whether the presence of Poloxamer 407 allowed the arrangement of polyurethane chains into micelles upon temperature variations, Dynamic Light Scattering (DLS – Zetasizer Nano S90, Malvern Instruments, Worcestershire, UK) measurements were performed on D-DHP407 samples at 25 °C, 30 °C, 37 °C and 45 °C. Specifically, the polymer was first dissolved in physiological saline solution (0.9% NaCl) at 0.5 and 1% w/V concentrations to avoid solution turbidity. Subsequently, samples were equilibrated at the test temperature for 15

min and analysed according to the method published by Pradal et al. [11]. At each tested condition, the reported hydrodynamic diameter of the polymeric structures resulted from the average of three different analysed samples.

2.2.8 Critical Micellar Temperature (CMT) investigation

To estimate the temperature at which PEU chains arrange into organised polymeric structures, Critical Micellar Temperature (CMT) of D-DHP407 systems prepared at 0.5 and 1% w/V concentrations in physiological saline solution (0.9% NaCl) was estimated using the fluorescent dye 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma-Aldrich, Italy). DPH was first dissolved in methanol (0.4 mM) and then added to each sample in order to reach a final concentration of 10 µL/mL. As reported by Alexandridis et al. [12], the absorbance intensity of this fluorescent dye at 350-360 nm increases when dispersed into micelle hydrophobic core. Hence, micellisation was investigated by heating the systems from 5 °C to 40 °C (5 min of equilibration time at each temperature, 1 °C temperature increase at each step) and measuring the absorption peak at 356 nm with an UV-Vis spectrophotometer (PerkinElmer, Lambda 25). CMT was finally defined at the first inflection of the sigmoidal curve obtained by plotting absorbance at 356 nm *vs* temperature. For each tested polymer concentration, analyses were performed in triplicate and results are reported as mean ± standard deviation.

2.3 Bulk functionalisation of D-DHP407 to expose thiol groups and physico-chemical characterisation

2.3.1 Grafting of thioglycolic acid to secondary amino groups

Thiols were grafted to polymer chains through carbodiimide chemistry by exploiting the high reactivity of PEU-secondary amino groups with COOH-containing molecules under mild conditions (Figure 1). Thioglycolic acid (TGA, ≥ 99%, Sigma Aldrich, Italy) was selected as the grafting molecule to introduce thiols along PEU backbone. The coupling reaction through carbodiimide chemistry consisted of two steps: (i) TGA-COOH activation at acidic pH and (ii) amide bond formation between TGA-COOH and PEU-NH. Specifically, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, TCI Europe, Belgium) and N-hydroxysuccinimide (NHS, Sigma Aldrich, Italy) were first dissolved at 100 mg/mL and 50 mg/mL, respectively, in

ddH_2O. Then, TGA was added at 1:1 molar ratio to EDC/NHS solution, pH was adjusted to 5 and the activation reaction was carried on for 1 hour, at 4 °C, under vigorous stirring. After that, D-DHP407 solution (previously dissolved at 18% w/V in ddH_2O and equilibrated at 4 °C) was added to TGA solution to reach a final –NH/-COOH molar ratio of 1:20, meanwhile stirring was maintained at 450 rpm. In order to investigate the influence of pH value and reaction time on coupling efficiency and disulphide bond formation, three different pH values (pH 4, 5 and 7) and two reaction times (6h and 24h) were tested for the coupling phase. At the end of the grafting step, samples were put in dialysis (cut-off membrane 10-12 kDa, Sigma Aldrich, Italy) against ddH_2O adjusted at pH 4 with HCl 1 M for 2 days at 4 °C and in the dark. Finally, samples were freeze-dried using a Martin Christ ALPHA 2-4 LSC.

Hereafter, thiolated PEU will be referred to as S-DHP407_pHX_Yh, where X stands for the pH value adopted for the grafting step, while Y indicates the reaction time.

2.3.2 Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) Spectroscopy

To verify the integrity of polyurethane bonds after thiol grafting as well as to assess the success of the thiolation procedure, S-DHP407_pHX_Yh spectra were acquired following the previously described protocol. For each condition, analyses were performed on three different samples belonging to three different batches. Results are reported as averaged spectra.

2.3.3. Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy

To further confirm the success of the thiolation procedure, Proton Nuclear Magnetic Resonance spectroscopy was conducted on S-DHP407_pHX_Yh samples according to the previously described protocol. D-DHP407 sample was also analysed and its spectrum was used as control.

2.3.4 Carbon Nuclear Magnetic Resonance (^{13}C-NMR) spectroscopy

Due to the high reactivity of thiol groups with oxygen, the formation of disulphide bonds cannot be completely avoided. Hence, Carbon Nuclear Magnetic Resonance spectroscopy was conducted on S-DHP407_pHX_Yh samples as well as on D-DHP407 as control, to distinguish the contribution of –SH moiety from that of S-S one. Specifically, samples were first prepared by dissolving 10 mg of polymer in 750 µL of d6-DMSO and then analysed according to the

previously described protocol. However, differently from ^{1}H-NMR analyses, in this case spectra resulted from 1024 scans. Finally, the d6-DMSO signal at 39.5 ppm was used for ^{13}C-NMR chemical shift scale.

2.3.5 Thiol group quantification through Ellman's method

The quantification of free thiols grafted to PEU amino groups was performed using the colorimetric Ellman's method by measuring the absorbance of yellow-coloured reaction products between PEU-SH and Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid or DTNB, Sigma Aldrich, Italy). The protocol used in this work was an adaptation of that published by Anitha et al. [13]. Briefly, 2.5 mg of S-DHP407_pHX_Yh were first dissolved in ddH_2O at 4 °C and then 250 µL of dibasic sodium phosphate buffer 0.5 M adjusted at pH 8 were added to the solution. Subsequently, 500 µL of Ellman's reagent solution (1.5 mM in dibasic sodium phosphate buffer 0.5 M) were added to each sample followed by 3h of incubation at RT and in the dark to allow the coupling reaction. Finally, samples were centrifuged at 7 °C, 10000 rpm for 10 minutes and extract absorbance was analysed at 412 nm using an UV-Vis spectrophotometer (PerkinElmer, Lambda 25). Control samples (D-DHP407) were subjected to the same protocol. To quantify the number of free sulfhydryl groups, a calibration curve based on TGA was prepared in the range 0.5 mM – 20 µM. The colorimetric assay was performed in triplicate on samples belonging to three different batches and results are reported as mean ± standard deviation.

2.3.6 Shelf life studies

Due to the high reactivity of sulfhydryl groups with oxygen, thiols easily form disulphide bonds, progressively lowering the number of free –SH available for further applications. Hence, in order to minimise oxidation phenomena during storage, shelf life studies were conducted. In detail, for each tested thiolation protocol, S-DHP407 samples were stored under vacuum or in inert atmosphere (under nitrogen) at 4 °C. Subsequently, at pre-defined time points (15d, 30d and 60d) thiol groups were quantified through the Ellman's method according to the previously described protocol. Quantification was performed in triplicate on samples belonging to three different batches and results are reported as mean ± standard deviation.

2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.00 for MacOSX (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). Two-way ANOVA analysis followed by Bonferroni's multiple comparison test was used to compare results. The statistical significance of each comparison was assessed as reported by Boffito et al. [14].

3. Results and discussion

3.1 Chemical characterisation of as synthesised (DHP407) and deprotected (D-DHP407) polyurethane

The newly synthesised PEU was first characterised by means of Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy, Size Exclusion Chromatography (SEC) and Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy to assess the success of the synthesis and –NH deprotection protocols. Then, prior to thiol-bearing molecules grafting to –NH groups, amino group quantification was performed by adapting to water soluble polymers a well-established colorimetric assay usually exploited to quantify amines exposed on water insoluble polymer surfaces.

3.1.1 *DHP407 chemical characterisation*

Successful PEU synthesis was confirmed by the appearance of new bands in DHP407 ATR-FTIR spectrum, compared to P407 one, ascribed to the formation of urethane bonds [15] at: 1720 cm^{-1} (stretching vibration of free C=O), 1537 cm^{-1} (bending vibration of N-H and stretching vibration of C-N) and 3332 cm^{-1} (stretching vibration of N-H) (Figure 2). These findings are in agreement with previous results published by Boffito et al. [14]. On the other hand, the band at 1633 cm^{-1} could be attributed to the stretching vibration of the carbonyl groups belonging to urea bonds. Indeed, reactions carried out at high temperatures (i.e., higher than 65 °C) in the presence of DBTDL as catalyst and/or residual water coming from reagents and/or solvents could lead to urea by-product formation, as reported by Yildiz et al. [16] and Semsarzadeh et al. [17]. Furthermore, the peak at 3525 cm^{-1} can be attributed to the presence of hydrogen bonds among polymeric chains. Lastly, the absence of unreacted isocyanates was also confirmed by the lack of the band at 2200 cm^{-1}.

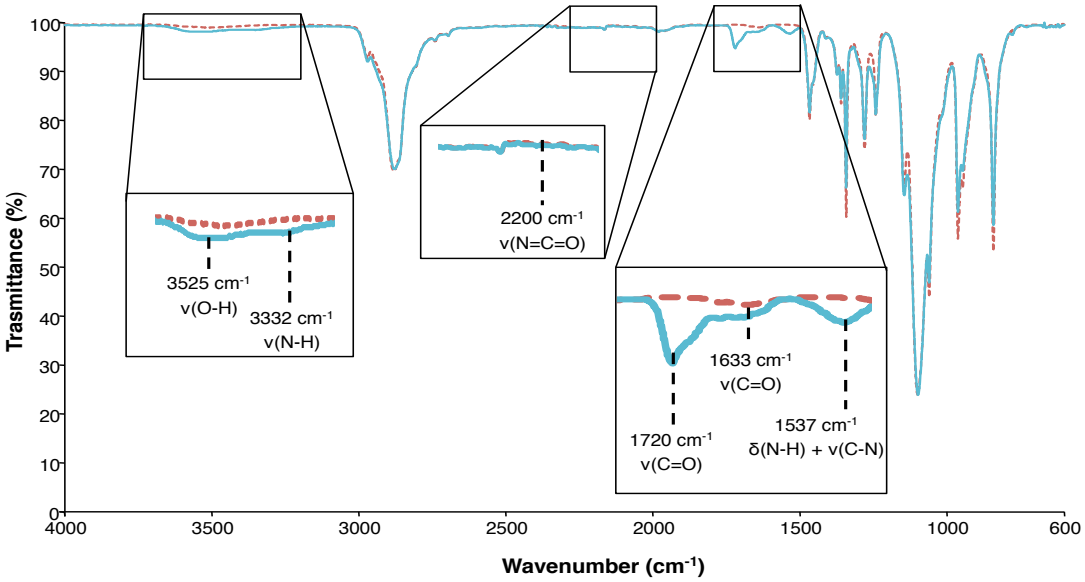


Figure 2. ATR-FTIR spectra of P407 (red – dashed line) and DHP407 (blue – continuous line). New adsorption bands confirming the success of polyurethane synthesis are shown in magnified inserts.

DHP407 exhibited a number average molecular weight (M_n) of 33000 - 35000 Da and a polydispersity index of 1.6.

3.1.2 D-DHP407 chemical characterisation

In order to verify the integrity of urethane bonds after Boc removal, ATR-FTIR characterisation was also performed on D-DHP407 samples. As reported in the Supporting Information (Figure S1), DHP407 and D-DHP407 spectra were perfectly overlapped, thus confirming that the here-applied protocol for amino group exposure did not chemically alter PEU structure.

Further confirmation of polyurethane integrity was provided by Size Exclusion Chromatography (Figure S2).

However, no clear conclusion about the effective exposure of secondary amino groups could be achieved from ATR-FTIR spectra as only negligible differences in transmission percentage of amine-related peaks were detected.

To demonstrate the exposure of secondary amino groups, thus validating the deprotection method proposed in this work, Proton Nuclear Magnetic Resonance (^{1}H-NMR) was performed

on DHP407 and D-DHP407 samples (Figure 3). After deprotection, a considerable reduction of the peak intensity at 1.38 ppm (ascribed to the Boc methyl groups) was observed, suggesting a strong although not complete removal of Boc groups (magnified insert C in Figure 3). Considering the maximum theoretical number of amino groups, the deprotected –NH amount was calculated to be around 80%. As a consequence, the adopted deprotection treatment allowed the exposure of 4 - 5 free amino groups per each D-DHP407 chain.

Additionally, the bands at 5.67 ppm and 7.1 ppm in the ^{1}H-NMR spectra of both DHP407 and D-DHP407 could be ascribed to –NH groups belonging to urea and urethane bonds, respectively, according to Qin et al. [18]. Hence, ^{1}H-NMR characterization further confirmed the formation of urea as by-product of PEU synthesis as previously suggested by the ATR-FTIR analyses (Figure 2). However, by comparing the intensities of the urethane and urea bands (magnified inserts A and B in Figure 2, respectively), the latter turned out to be present in a lower amount. Hence, although some by-products formed during the synthesis, they did not interfere with the effective accomplishment of a high molecular weight polymer with a narrow molecular weight distribution as confirmed by SEC results.

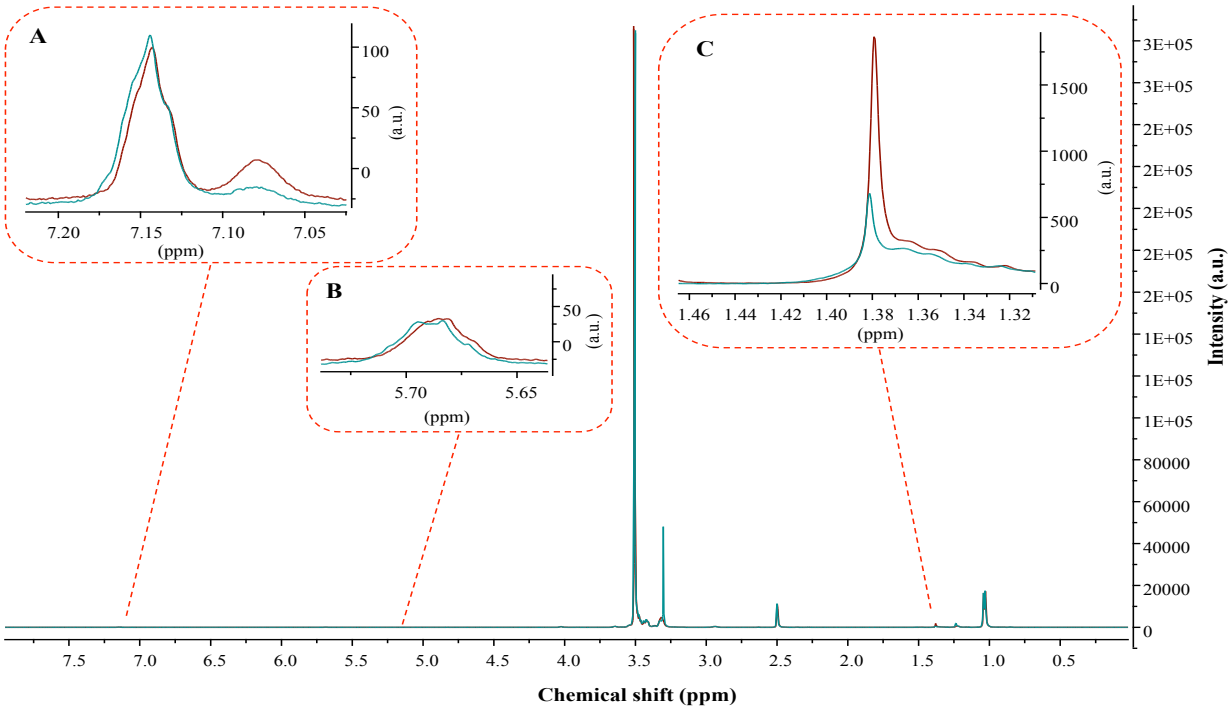


Figure 3. ^{1}H-NMR spectra of DHP407 (red) and D-DHP407 (green). Magnified inserts A and B highlight the bands attributed to the –NH groups belonging to the urethane and urea bonds,

respectively. Magnified insert C highlights differences between the spectra at 1.38 ppm ascribed to the Boc methyl groups.

3.1.3 Secondary amino group quantification

To determine the number of secondary amino groups exposed along D-DHP407 chains, the colorimetric assay Orange II Sodium Salt was applied on both DHP407 and D-DHP407. As shown in Figure S3, the dark orange colour of D-DHP407 samples after the desorption reaction suggested the successful exposure of secondary amino groups. Control samples (DHP407) subjected to the same protocol showed a light orange colour, due to partial dye adsorption by polyurethane chains.

To further assess the validity of the proposed deprotection method and to verify the repeatability of this process, Orange II Sodium Salt colorimetric assay was conducted in triplicate on samples belonging to three different D-DHP407 batches. Such colorimetric assay, previously used to quantify the number of primary amino groups exposed by water insoluble polymers, was effectively adapted to determine the number of secondary amino groups in water-soluble polymers. Assuming that one orange molecule electrostatically interacts with one –NH, the total number of free amino groups, as average of three different deprotection procedures, was measured to be $4.5 \times 10^{20} \pm 1.8 \times 10^{19}$ units/g of D-DHP407. This value was about the 80% of the theoretical number of potentially exposed functional groups along PEU chains, and was in agreement with the amount of deprotected amino groups calculated from ^{1}H-NMR data. Indeed, the peak at 1.38 ppm, ascribed to Boc methyl groups, in D-DHP407 spectrum showed an intensity that turned out to be approx. the 20% of the initial one. Hence, although the adopted protocol was not able to completely remove the caging groups, it was considered to be a good compromise between the concurrent needs of exposing amines for further functionalisation and preserving the polymer from potential degradation phenomena. Furthermore, the amount of functional groups exposed by using this protocol resulted to be highly repeatable among different deprotection procedures.

3.2 Polyurethane thermo-sensitivity evaluation

Due to its amphiphilic nature, Poloxamer® 407 is a thermo-sensitive polymer as demonstrated first by Bermudez et al. [19]. Therefore, the presence of P407 as a repeating unit in D-DHP407

PEU should ensure the capability of polyurethane chains to arrange into organised structures as a consequence of temperature variation. Hence, the thermo-responsiveness of this material was first assessed by estimating the variation of micelle hydrodynamic diameter upon temperature increase. Subsequently, as a further confirmation, the critical micellisation temperature of D-DHP407-based aqueous solutions was also investigated.

3.2.1 *Dynamic Light Scattering measurements*

D-DHP407 micelle formation and nucleation were analysed by studying the variation of their hydrodynamic diameters upon heating in the 25 °C – 45 °C range. Figure 4 reports the intensity (A, C, E and G) and volume (B, D, F and H) patterns of the polymeric structures contained in D-DHP407 aqueous solution at 0.5% w/V concentration measured at different temperatures.

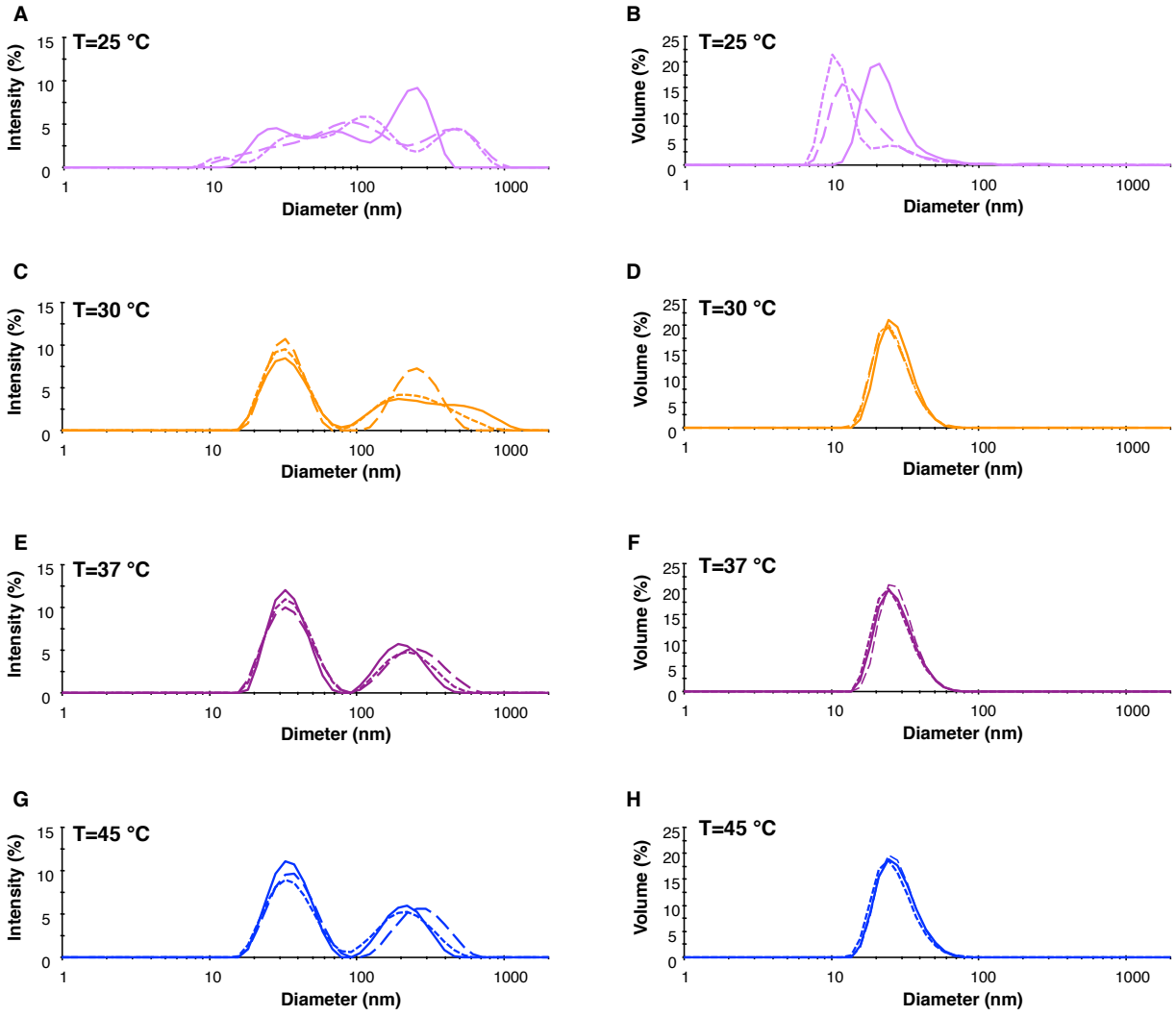


Figure 4. Distribution by intensity (A, C, E, G) and by volume (B, D, F and H) of the hydrodynamic diameter of the polymeric micelles and aggregates in D-DHP407 solutions with 0.5% w/V concentration measured at different temperatures (25 °C – 30 °C – 37 °C and 45 °C). Continuous, dash and dot lines represent measurements conducted on three different samples prepared in the same conditions.

Due to the high instability of the system and the absence of a well-defined chain organisation, a clear dimensional distribution was not detectable at 25 °C (Figure 4_A). At 30 °C, a well-defined double peak appeared. Particularly, the peak centred at 35.27±1.15 nm (Figure 4_C) suggested temperature-driven chain arrangement, leading to the formation of single micelles, in agreement with findings by Boffito et al. [14]. On the contrary, the second peak centred at about 280 nm was attributed to micelle aggregation, and showed high variability, due to an establishing equilibrium between initial aggregates and disaggregated structures. At 37 °C (Figure 4_E), two distinct peaks were identified at 36.21±1.54 nm and 237.59±20.55 nm again ascribed to the presence of single micelles and clusters, respectively. This bimodal chain organisation was further confirmed at 45 °C (Figure 4_G) with the presence of two peaks at 37.38±0.62 nm and 246.76±6.98 nm.

Although two different polymeric structures were identified from intensity patterns of hydrodynamic diameter at 30 °C, 37 °C and 45 °C (Figure 4_C, E and G), suggesting the simultaneous presence of micelles and aggregates, when referred to volume patterns (Figure 4_D, F and H) only one peak was present at about 35 nm. This means that, even though the system included both micelles and clusters, the amount of aggregates present in the samples was negligible (< 1% for all tested temperatures).

Similar results were obtained for 1% w/V concentrated D-DHP407 aqueous solutions with slight differences at 30 °C as the peak ascribed to micelle aggregates was more intense and less variable (Figure S4). This result suggested that D-DHP407 chain organisation also depended on polymer concentration, as previously shown by Boffito et al. [14].

In addition, the comparison between the intensity profiles of D-DHP407 samples analysed at 37 °C and 45 °C further proved polymer responsiveness to temperature (Figure S5): the intensity of the peak ascribed to the presence of single micelles decreased while that of the peak attributed

to micelle clusters increased. In detail, for D-DHP407 at 0.5% w/V concentration the intensity of the peak ascribed to micelles varied from 64.4% at 37 °C to 61.5% at 45 °C, while the intensity of the peak centred at about 280 nm increased from 35.6% to 38.5%. Hence, the presence of P407 blocks in polyurethane chains allowed polymer thermo-sensitivity, in accordance with previously reported data [20, 21].

3.2.2 Critical Micellar Temperature evaluation

The critical micellar temperature (i.e., the temperature at which micelle nucleation process begins) was estimated for D-DHP407 aqueous solutions with 0.5% and 1% w/V concentrations containing DPH. Solution absorbance intensities at 356 nm were measured at different temperatures within the 5-40 °C range. The detection of the absorbance was due to DPH solubilisation into the micelle hydrophobic core, suggesting chain arrangement into micelles upon temperature increase. Figure 5 shows the UV-Vis spectra of D-DHP407 samples at 0.5% and 1% w/V concentrations registered in the spectral range 330 – 400 nm at different temperatures.

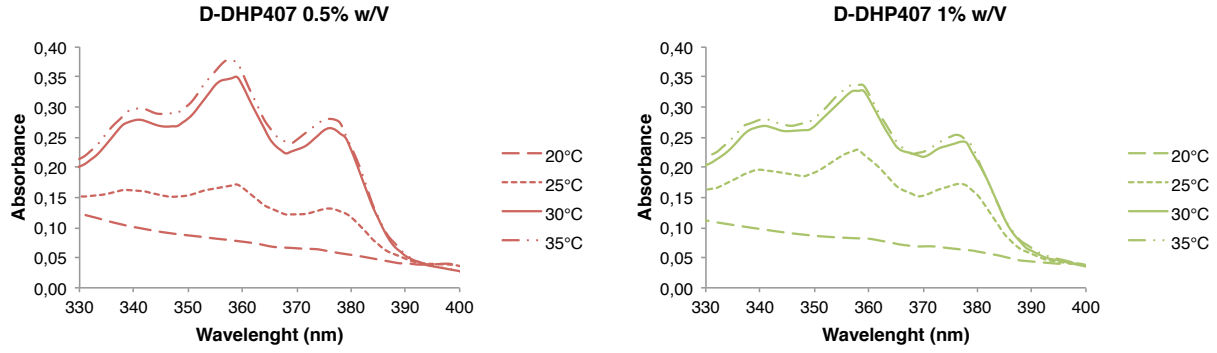


Figure 5. UV/Vis spectra of D-DHP407 solutions at 0.5% (red, on the left) and 1% (green, on the right) w/V concentrations, containing DPH, recorded in the spectral range 330 – 400 nm upon temperature increase.

For both the analysed formulations, the UV-Vis spectra did not show any absorbance at temperatures up to 20 °C, suggesting that polymeric chains were present as unimers. Upon temperature increase to 25 °C, an absorption peak at 356 nm appeared, evidencing DPH encapsulation within micelles. Upon further heating, a sharp increase in the absorbance intensity

at the same wavelength was registered, confirming the rearrangement of the polymeric chains into micelles in response to temperature variation. These results supported the occurrence of a temperature-driven polymeric chain organisation, as suggested also by DLS analyses (Figure 4_C and E).

Finally, for each sample the CMT was estimated by considering the first inflection of the sigmoidal curve obtained by plotting the absorbance intensity at 356 nm as a function of temperature (Figure 6_A and B) [14].

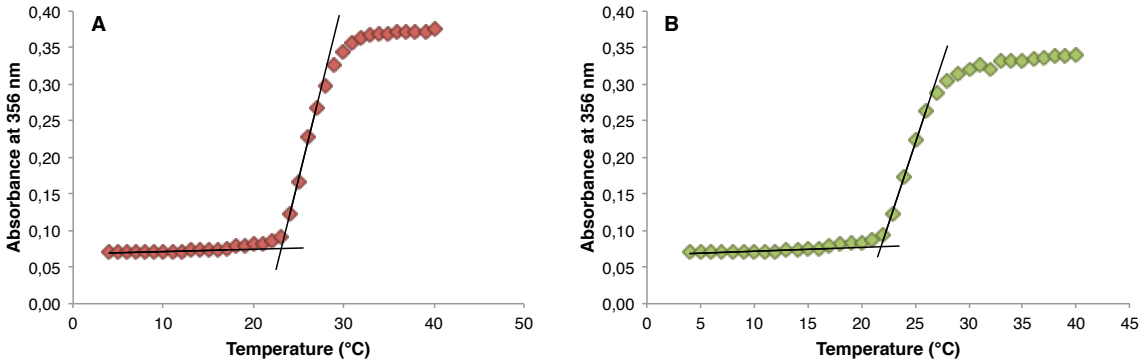


Figure 6. Measured absorbance at 356 nm as a function of temperature in the 5 °C – 40 °C range for D-DHP407 samples prepared in physiological saline solution at 0.5% (A) and 1% w/V (B) concentrations.

The temperature at which the nucleation process began was 23.2 ± 0.1 °C and 21.9 ± 0.1 °C ($0.0001 < p < 0.001$) for D-DHP407 solutions at 0.5% and 1% w/V concentrations, respectively, thus confirming the hypothesis that chain organisation was dependent on both temperature and polymer concentration.

3.3 Optimisation of the thiolation protocol through carbodiimide chemistry

To introduce pendant sulfhydryl groups along polyurethane chains, carbodiimide mediated grafting reaction was exploited, due to its simplicity, high reaction rate and mild chemistry. As water is used as reaction solvent, carbodiimide chemistry can be also considered one example of green chemistry.

To the best of our knowledge, this kind of chemistry has been usually used to react carboxylic groups with primary amino groups. However, in the present study, carbodiimide chemistry has

been used for the first time to graft –COOH groups with secondary amino groups exposed along the previously synthesised polyurethane.

A different number of thioglycolic acid molecules could be grafted along PEU backbone, depending on the reaction pH: at progressively higher pH compared to the pK_a of the –NH-containing molecules, reactivity should increase. However, the complexity of polyurethane structure did not allow the determination of D-DHP407 pK_a value. Furthermore, due to the presence of unprotected thiol groups in the grafted molecules, particular care must be taken in the selection of reaction conditions in order to avoid the formation of side-products (i.e., disulphide bonds). Therefore, although a basic pH could theoretically improve amide bond formation by carbodiimide chemistry, a pH value higher than 7 was not considered, as it would enhance the formation of S-S bonds [13]. Hence, the pH of the grafting step was varied in the range 4 - 7 in order to optimise the grafting reaction and, at the same time, to minimise the formation of disulphide bonds.

3.3.1 Attenuated Total Reflectance Fourier Transform Infrared spectroscopy

To assess the success of the thiolation procedure, thiomers synthesised according to each tested condition were first analysed by ATR-FTIR spectroscopy. Averaged ATR-FTIR spectra of S-DHP407_pHX_6h and S-DHP407_pHX_24h are reported in Figure 7 and Figure S6, respectively.

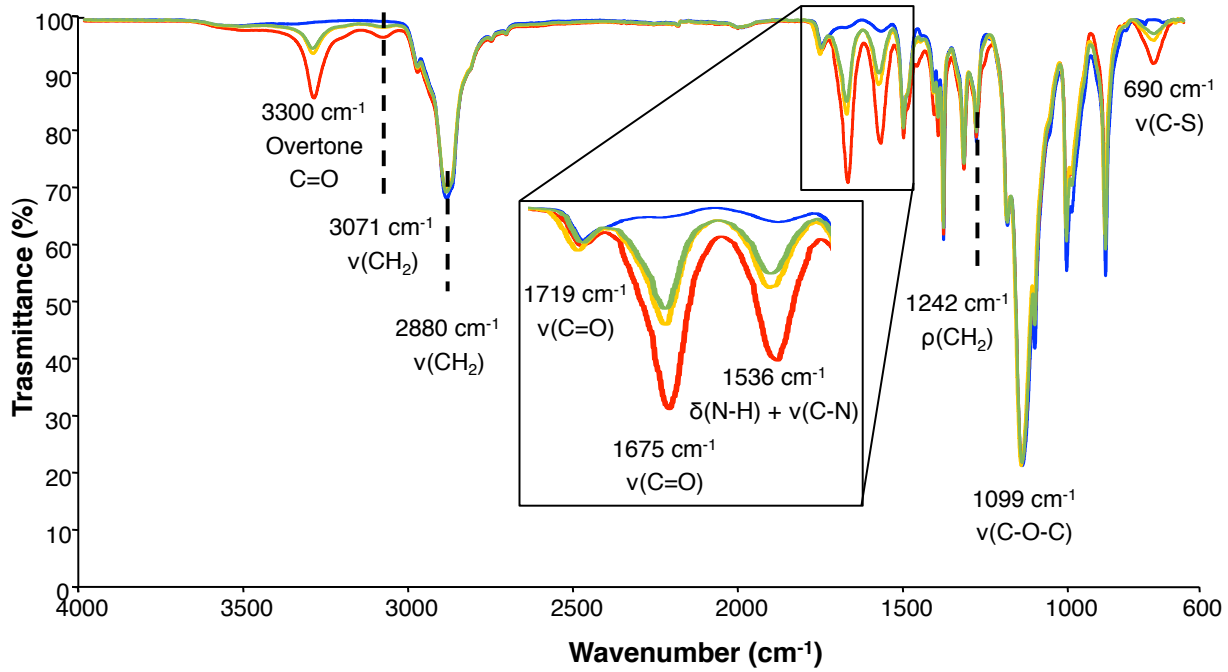


Figure 7. Averaged ATR-FTIR spectra of D-DHP407 (blue) and S-DHP407 after TGA grafting at pH 4 (red), 5 (yellow) and 7 (green) and 6h reaction time. Differences between spectra are reported as magnifications.

Irrespective of the reaction conditions, a sharp increase in the intensity of the peaks ascribed to amide bonds was observed if compared to the control spectrum (D-DHP407). In detail, significant differences were registered in the peaks at 1719 cm^{-1} and 1675 cm^{-1}, which are attributed to carbonyl group (C=O) stretching vibrations. Specifically, the band centred at 1675 cm^{-1} can be ascribed to the carbonyl groups belonging to the amide bonds (Amide I). The increased absorption intensity at 1675 cm^{-1} with increasing reaction time, compared to the control spectrum, further confirmed the formation of amide bonds between TGA-COOH and PEU-NH. In addition, the appearance of a new band centred at 3300 cm^{-1} can be ascribed to an overtone of the carbonyl groups belonging to the amide bonds, while the weak peak at 3071 cm^{-1} could be referred to the stretching vibration of CH_2 groups belonging to TGA. The absorption band at 1536 cm^{-1} can be attributed to the stretching vibration of C-N bonds together with the bending vibration of N-H groups belonging to the urethane bonds [15]. In addition, the appearance of a new band at 690 cm^{-1}, attributed to the stretching vibration of C-S bonds, further confirmed the presence of TGA along polymer chains. However, due to the typical weakness of the S-H band between 2600 cm^{-1} and 2550 cm^{-1} and the strong CH_2 stretching vibration at 2800 cm^{-1}, the presence of free thiols was not clearly evident.

For what concerns the influence of pH value over the efficiency of the grafting reaction, a reduced intensity of the bands attributed to the newly formed amide bonds (1675 cm^{-1} and 1536 cm^{-1}) was observed by increasing the pH from 4 to 7. This trend was independent on the reaction time (6h – Figure 7 and 24h – Figure S6).

Hence, conversely to what theoretically expected, the coupling degree lowered upon pH increase. This phenomenon may be attributed to the complex chemical structure of D-DHP407, showing several-charged components, which can modify the common reaction equilibrium. This aspect, together with the impossibility to experimentally determine PEU pK_a, makes very difficult to theoretically predict the most promising pH for the grafting step.

However, the use of low pH values for both the activation and the grafting steps is also advantageous to avoid thiol oxidation, which would lead to the formation of disulphide bonds.

The influence of reaction time over TGA grafting was better elucidated by comparing the S-DHP407 ATR-FTIR spectra acquired after 6h and 24h grafting reaction at different pH conditions (Figure S7_A, B and C). The reaction time of the second-step of the carbodiimide mediated grafting minimally affected TGA grafting degree when using pH 5 and 7 (Figure S7_B and C, respectively), with a slight increase in the intensity of the bands ascribed to amide and C-S bonds after 6h as compared to 24h reaction time. However, when the reaction was conducted at pH 4, the same absorption bands were significantly increased after 6h compared to 24h reaction (Figure S7_A). These results could be correlated to the decreased activity of EDC coupling reagent at low pH: in acidic conditions, EDC easily forms urea by-products, thus lowering the number of activated carboxylic groups suitable for amide bond formation [22]. Hence, the highest efficiency of grafting was reached by performing the reaction for 6h at pH 4, conditions that could also contribute to minimise the oxidation of thiol groups leading to disulphide bonds.

3.3.2 Nuclear Magnetic Resonance spectroscopy to evaluate thiol grafting

To further confirm the success of thioglycolic acid grafting to PEU secondary amino groups, S-DHP407_pHX_Yh samples were first analysed through proton nuclear magnetic resonance spectroscopy. D-DHP407 samples were prepared according to the same protocol and analysed as control condition. If compared to D-DHP407 ^{1}H-NMR spectrum (Figure 8), S-DHP407_pHX_Yh spectra showed the appearance of two new peaks at 3.25 and 2.20 ppm ascribed to the methylene and thiol groups, respectively. Additionally, the newly formed peak at 7.85 ppm could be attributed to the –NH groups of amide as reported by Qin et al. [18]. Hence, ^{1}H-NMR confirmed the presence of TGA and, thus, the success of thiol grafting through the formation of amide bonds between PEU secondary amino groups and TGA carboxylic moieties in all the analysed conditions.

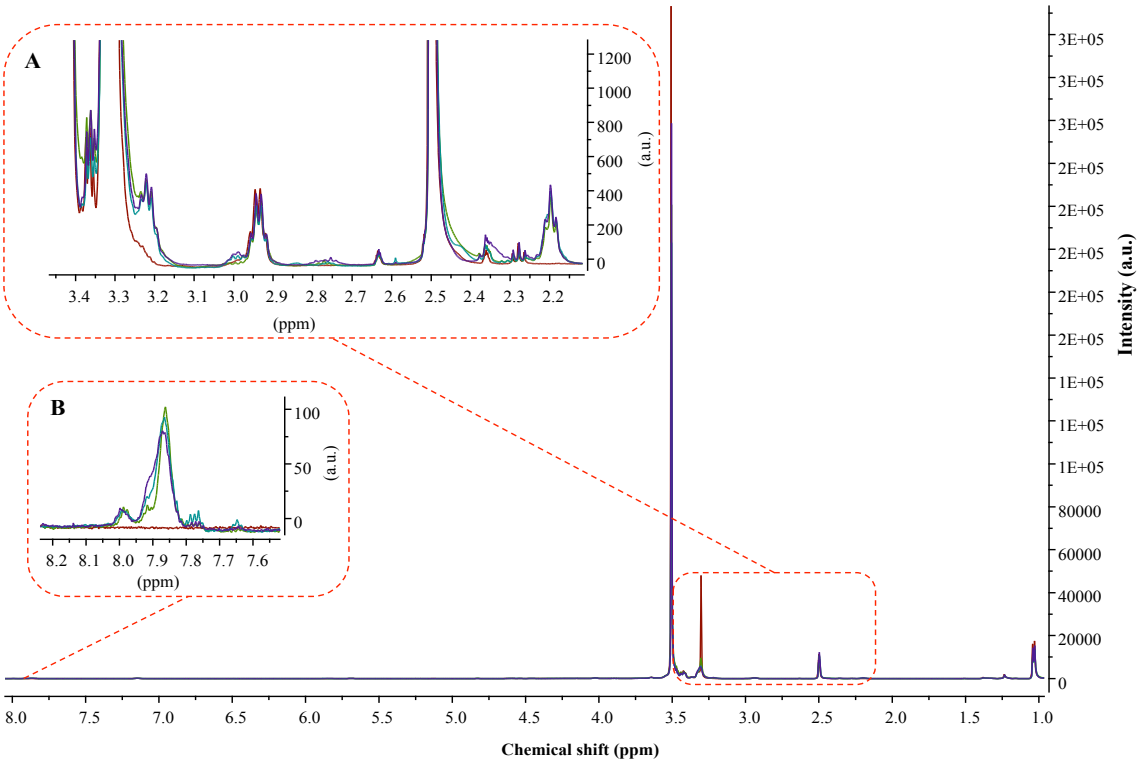


Figure 8. ^{1}H-NMR spectra of D-DHP407 (red), S-DHP407_pH4_6h (green), S-DHP407_pH7_6h (violet) and S-DHP407_pH4_24h (blue). Magnified inserts highlight differences between the spectra.

However, ^{1}H-NMR spectra did not allow the investigation of the influence of grafting conditions over disulphide bond formation, because the ^{1}H-NMR signals of the methylene protons of the thioglycolic moiety in the thiolic or disulphide forms were overlapped.

For this reason, in order to verify the presence of disulphide bonds as a consequence of the grafting reaction and to study the influence of coupling conditions on the formation of this sub-product, ^{13}C-NMR spectra of D-DHP407 and S-DHP407_pHX_Yh were also acquired (Figure 9 and Figure 10). Additionally, ^{13}C-NMR characterisation provided a further confirmation of the occurrence of side-reactions during poly(ether urethane) synthesis as the band at 158.6 ppm can be ascribed to the carbonyl zone belonging to urea (Figure 9_insert B) [18]. However, the intensity of this band was negligible with respect to that ascribed to the urethane bonds at 156.6 ppm (Figure 9_insert C), thus suggesting that only a small amount of urea was contained in the polymer chains. Nevertheless, the presence of urea bonds did not interfere with the intended properties that the designed polymer should exhibit. Indeed, polymer thermo-responsiveness was

ensured by the macrodiol (Figure 4 and 5), while thiol group grafting involved the –NH groups exposed along polymer chains as a consequence of Boc removal.

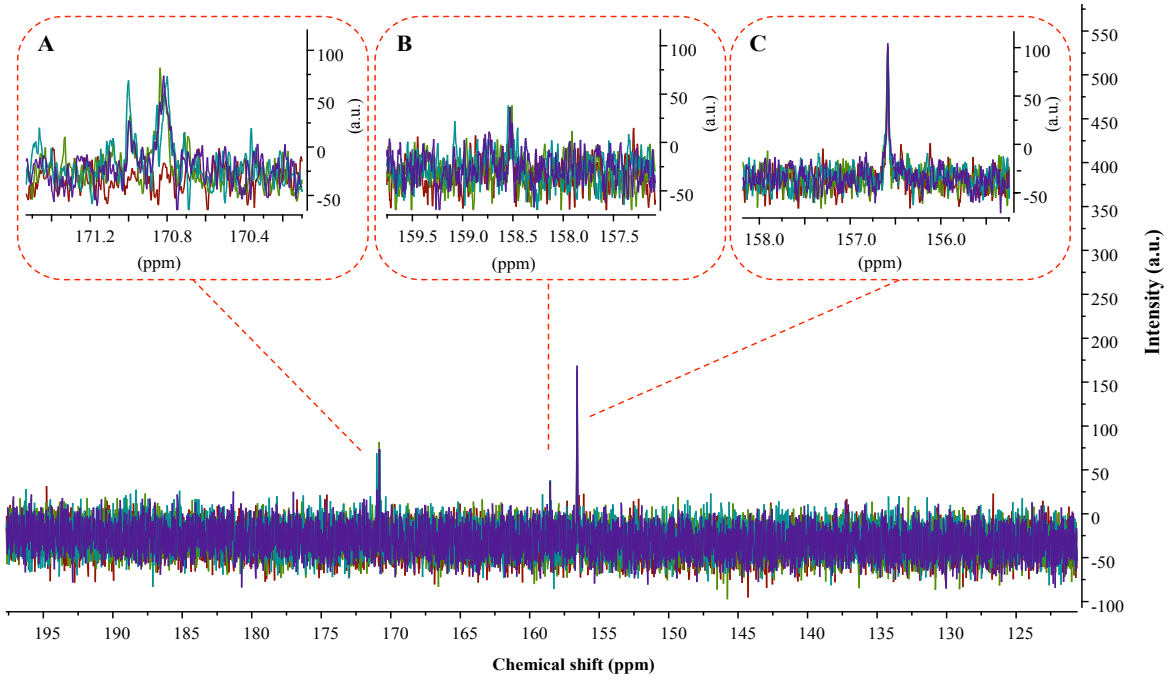


Figure 9. ^{13}C-NMR spectra of D-DHP407 (red), S-DHP407_pH4_6h (light green), S-DHP407_pH7_6h (violet) and S-DHP407_pH4_24h (dark green). Magnified inserts highlight differences between the spectra.

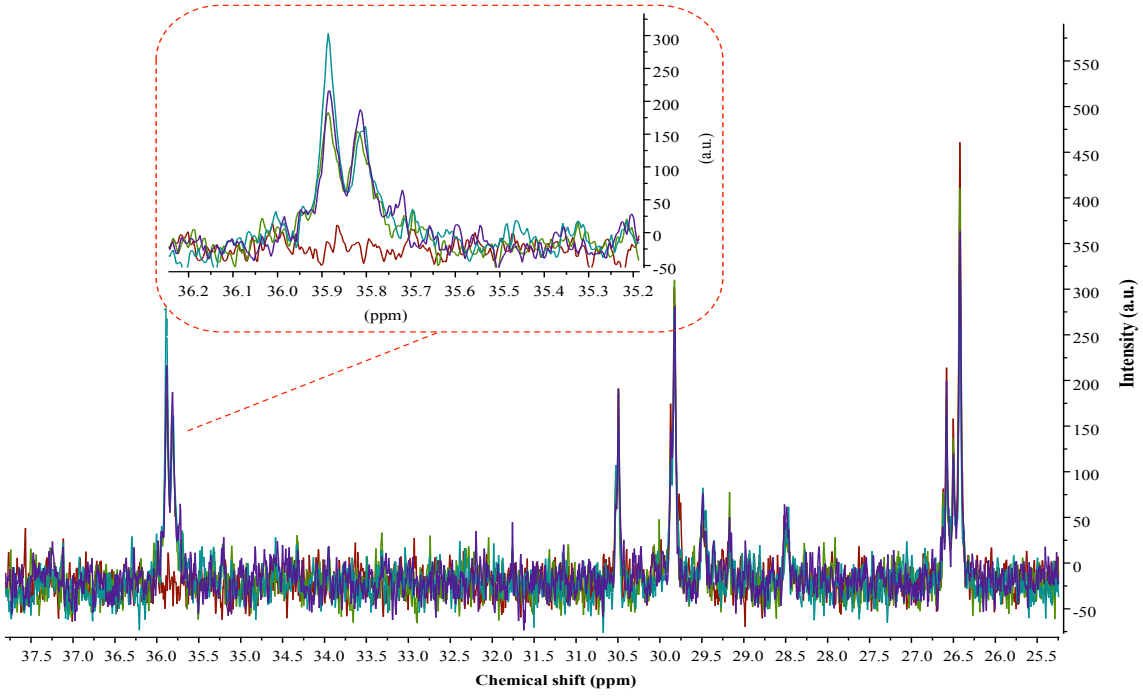
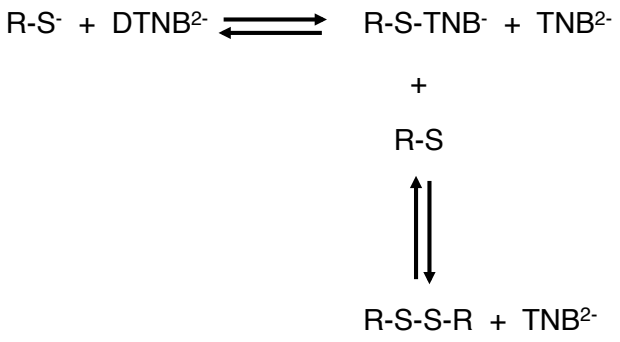


Figure 10. ^{13}C-NMR spectra of D-DHP407 (red), S-DHP407_pH4_6h (light green), S-DHP407_pH7_6h (violet) and S-DHP407_pH4_24h (dark green). Magnified insert highlights differences between the spectra in the range 35.2 – 36.2 ppm.

If compared to the control (D-DHP407), ^{13}C-NMR spectra of S-DHP407_pHX_Yh showed the appearance of two new sets of signals after the grafting process, further proving TGA coupling to PEU-NH. The first set of signals (Figure 9) consisted of two peaks at 171.0 ppm and 170.9 ppm that could be attributed to the carbonyl group involved in the amide bond between D-DHP407-NH and thioglycolic acid preserving the free -SH moieties, or converted into S-S bonds, respectively. The second group of signals (Figure 10) involved two resonances at 35.8 ppm and 35.9 ppm corresponding to the methylene carbons of the thioglycolic acid preserving the free -SH moieties, or converted into S-S bonds, respectively. Hence, ^{13}C-NMR spectra confirmed the presence of disulphide bonds showing different contributions depending on the pH value set during the grafting step. In addition, ^{13}C-NMR spectra allowed a rough estimation of the thiol/disulphide ratio by comparing the intensities of the two methylene NMR signals at 35.8 (-SH) and 35.9 (-S-S-) ppm. As evidenced in Figure 10, upon variation of reaction conditions, different ratios of thiol/disulphide bonds were obtained. Among the tested conditions, pH 4 and 6h reaction time (light green spectrum in Figure 10) were considered to be the optimal parameters to graft the highest number of thiol groups while minimising the amount of formed disulphide bonds.

3.3.3 Thiol quantification by Ellman's Method

Sulfhydryl groups grafted along D-DHP407 chains were quantified through thiol-disulphide exchange reactions between PEU-SH and Ellman's reagents (Scheme 1), leading to the formation of a yellow-coloured product in their oxidised form (TNB^{2-}).



Scheme 1. Schematic representation of Ellman's method applied to S-DHP407_pHX_Yh to quantify free sulfhydryl groups.

To investigate the influence of grafting pH and reaction time on –SH oxidation into disulphide bonds, thiol quantification was performed on all S-DHP407_pHX_Yh samples (Figure 11_A and B).

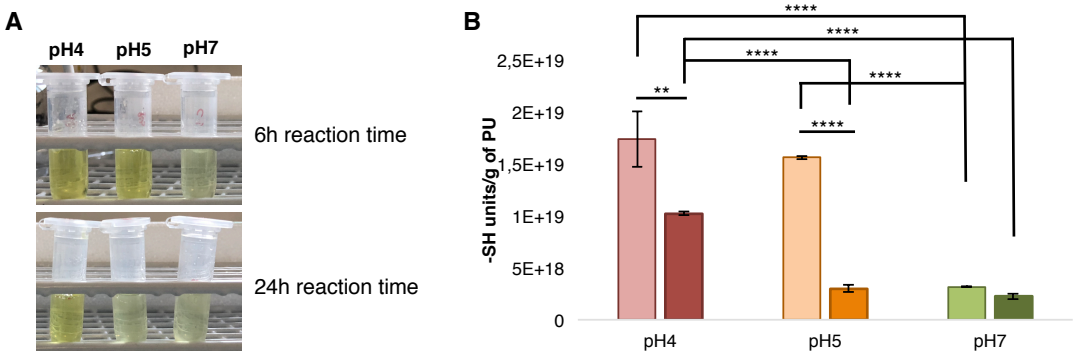


Figure 11. Ellman's colorimetric assay performed on S-DHP407_pHX_Yh (A). The different yellow intensity stands for different amounts of exposed thiols as a consequence of adopted pH and reaction times during the grafting step. The bar graph reports the number of thiol units/g of polymer (B) obtained at pH 4 (red), 5 (orange) and 7 (green) for 6h and 24h of reaction (light and dark colours, respectively).

Irrespective of the pH adopted for the coupling reaction of TGA to D-DHP407, the number of free thiols decreased from 6h to 24h reaction time, in agreement with spectroscopic results in the case of grafting reaction carried out at pH 4. Although any clear dependence of grafting efficiency over reaction time were detected from ATR-FTIR spectra of S-DHP407 prepared at pH 5 and pH 7 (Figure S7_B and C, respectively), the observed differences in free thiol units assessed through Ellman's assay can be attributed to oxidation phenomena, which occurred during the grafting step. Hence, at longer reaction time for S-DHP407_pHX_24h, the amount of grafted thiols decreased. Indeed, the minimal quantity of thiols was obtained for S-DHP407_pH5_24h and S-DHP407_pH7_24h with $2.97 \times 10^{18} \pm 3.57 \times 10^{17}$ and $2.26 \times 10^{18} \pm 3.05 \times 10^{17}$

units/g of polymer, respectively. Regarding S-DHP407_pH 4_Yh, the difference between 6h and 24h reaction time could not be exclusively attributed to thiol oxidation as a lower amount of grafted TGA was also measured at higher reaction time (Figure S7_A).

The pH of the medium influenced the available free –SH groups along polymer chains: at both 6h and 24h reaction time, the number of thiol units decreased with increasing the pH values. Indeed, at neutral or alkaline conditions, sulfhydryl groups easily form disulphide bonds. Thiol oxidation cannot be completely avoided; however, acid pH and short reaction times for the coupling reaction can minimise the formation of disulphide bonds. Hence, the highest amount of thiols ($1.7x10^{19}\pm2.73x10^{18}$ units/g of polymer) was reached by using pH 4 and 6h during grafting reaction.

To further validate these results and to verify the repeatability of the thiolation process, thiol quantification was performed on S-DHP407_pHX_Yh belonging to three different syntheses. Independently from the synthesis, the amount of thiol units/g of polymer was approximately the same for each analysed condition. For instance, –SH groups were measured to be $1.2x10^{19}\pm1.9x10^{17}$, $9.1x10^{18}\pm8.5x10^{17}$ and $9.9x10^{18}\pm4.1x10^{17}$ for three different S-DHP407_pH4_6h batches, respectively, with an average number of $1x10^{19}\pm1.3x10^{18}$ sulfhydryl groups for each g of PEU.

3.3.4 Shelf Life studies

Shelf life studies were conducted on all S-DHP407_pHX_Yh samples to investigate the degree of thiol oxidation under storage conditions. To this purpose, thiolated samples were prepared and stored at 4 °C, in the dark, under vacuum (approx. 2-5 mbar) or under nitrogen. Subsequently, at predefined time points (15d, 30d and 60d) thiols were quantified through the Ellman's method. Results for S-DHP407_pHX_6h are reported in Figure 12 as percentage variation respect to initial –SH content. A similar trend was observed in S-DHP407_pHX_24h samples (data not reported).

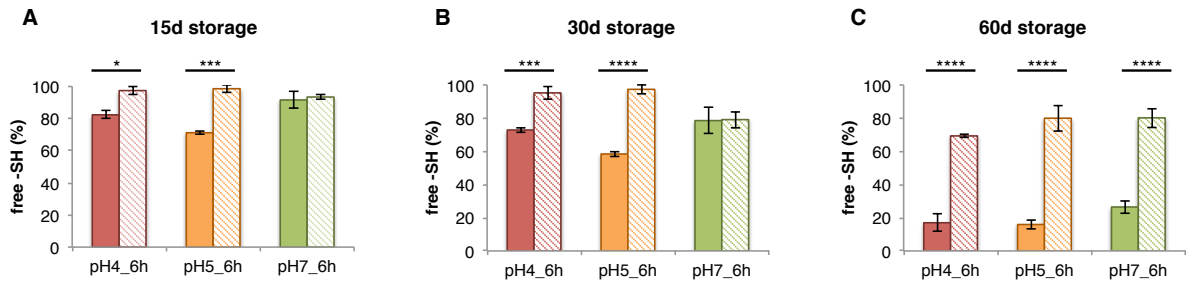


Figure 12. Thiol quantification in S-DHP407_pH4_6h (red), S-DHP407_pH5_6h (orange) and S-DHP407_pH7_6h (green) after 15d (A), 30d (B) and 60d (C) storage in the dark, at 4 °C under vacuum (dark colour) or nitrogen atmosphere (light colour).

Irrespective of the pH adopted for the grafting reaction of TGA to D-DHP407, the number of sulfhydryl groups tended to decrease over time, confirming that thiol oxidation could not be completely avoided neither under vacuum nor under nitrogen atmosphere. Specifically, samples stored under vacuum immediately showed a reduction of pendant thiols, reaching the 20-30% of the initial available functional groups after 60d. On the other hand, storage under nitrogen led to the formation of a lower number of disulphide bonds, with approx. the 70% - 80% of the initial functional groups still in the –SH form after 60d storage. Hence, sample storage under nitrogen turned out to be the most preserving storage condition against thiol oxidation.

4. Conclusion

Due to the high reactivity of thiol groups under mild external stimuli, thiomers are attracting increasing interest as responsive polymeric component in the preparation of smart hydrogels. With the final aim to design a dual-stimuli responsive polymer and to expose a high number of sulfhydryl groups along each polymeric chain, in this work an amphiphilic thiolated polyurethane was successfully synthesised. Specifically, by exploiting the versatility of polyurethane chemistry, a PEU containing Poloxamer 407 as the macrodiol and an amino-group bearing chain extender was synthesised. Subsequently, secondary amino groups were successfully Boc-deprotected through an acidic treatment as proved by ^{1}H-NMR spectroscopy and the colorimetric Orange II sodium salt assay, measuring $4.5 \times 10^{20} \pm 1.8 \times 10^{19}$ –NH units/g of PEU. Then, polymer thermo-responsiveness was confirmed by investigating the changes in

micelle hydrodynamic diameter upon system heating up to 45 °C and through critical micellar temperature estimation. Lastly, solvent-free carbodiimide chemistry was exploited to introduce thiol groups along polymer chains, through amide bond formation between TGA-COOH and PEU-NH. Among the tested conditions, pH 4 and 6 hours of grafting reaction turned out to allow the exposure of the highest amount of –SH groups ($1.7x10^{19}\pm2.73x10^{18}$ units/g of polymer) while minimising disulphide bond formation as demonstrated through ^{1}H-NMR, ^{13}C-NMR, ATR-FTIR and Ellman's colorimetric assay. Therefore, the presence of P407 as building block, which ensures thermo-sensitivity, and the grafting of a high amount of thiols make the amphiphilic thiolated polyurethane developed in this work a powerful responsive material able to overcome the limits of commercially available responsive polymers. The double responsiveness of this PEU opens the possibility to prepare hydrogels simultaneously sensitive to temperature and to another physical stimulus using only one polymeric component. For example, potential applications of the thiolated amphiphilic PEU could be the design of thermo-sensitive and photo-curable bio-inks or post-injection cross-linkable thermo-sensitive systems or hydrogels with improved cell-adhesiveness.

Author Contributions

The manuscript was written through contributions of all authors. Last authors equally contributed to the supervision of Rossella Laurano first author. All authors have given approval to the final version of the manuscript.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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Figure Captions

Figure 1. Schematic representation of the three main steps concerning the design of thiolated polyurethanes: synthesis (I), secondary amino group deprotection (II) and thiol grafting (III).

Figure 2. ATR-FTIR spectra of P407 (red – dashed line) and DHP407 (blue – continuous line). New adsorption bands confirming the success of polyurethane synthesis are shown in magnified inserts.

Figure 3. ^{1}H-NMR spectra of DHP407 (red) and D-DHP407 (green). Magnified inserts A and B highlight the bands attributed to the –NH groups belonging to the urethane and urea bonds, respectively. Magnified insert C highlights differences between the spectra at 1.38 ppm ascribed to the Boc methyl groups.

Figure 4. Distribution by intensity (A, C, E, G) and by volume (B, D, F and H) of the

hydrodynamic diameter of the polymeric micelles and aggregates in D-DHP407 solutions with 0.5% w/V concentration measured at different temperatures (25 °C – 30 °C – 37 °C and 45 °C). Continuous, dash and dot lines represent measurements conducted on three different samples prepared in the same conditions.

Figure 5. UV/Vis spectra of D-DHP407 solutions at 0.5% (red) and 1% (green) w/V concentrations, containing DPH, recorded in the spectral range 330 – 400 nm upon temperature increase.

Figure 6. Measured absorbance at 356 nm as a function of temperature in the 5 °C – 40 °C range for D-DHP407 samples prepared in physiological saline solution at 0.5% (A) and 1% w/V (B) concentrations.

Figure 7. Averaged ATR-FTIR spectra of D-DHP407 (blue) and S-DHP407 after TGA grafting at pH 4 (red), 5 (yellow) and 7 (green) and 6h reaction time. Differences between spectra are reported as magnifications.

Figure 8. ^{1}H-NMR spectra of D-DHP407 (red), S-DHP407_pH4_6h (green), S-DHP407_pH7_6h (violet) and S-DHP407_pH4_24h (blue). Magnified inserts highlight differences between the spectra.

Figure 9. ^{13}C-NMR spectra of D-DHP407 (red), S-DHP407_pH4_6h (light green), S-DHP407_pH7_6h (violet) and S-DHP407_pH4_24h (dark green). Magnified inserts highlight differences between the spectra.

Figure 10. ^{13}C-NMR spectra of D-DHP407 (red), S-DHP407_pH4_6h (light green), S-DHP407_pH7_6h (violet) and S-DHP407_pH4_24h (dark green). Magnified insert highlights differences between the spectra in the range 35.2 – 36.2 ppm.

Figure 11. Ellman's colorimetric assay performed on S-DHP407_pHX_Yh (A). The different yellow intensity stands for different amounts of exposed thiols as a consequence of adopted pH and reaction times during the grafting step. The bar graph reports the number of thiol units/g of

polymer (B) obtained at pH 4 (red), 5 (orange) and 7 (green) for 6h and 24h of reaction (light and dark colours, respectively).

Figure 12. Thiol quantification in S-DHP407_pH4_6h (red), S-DHP407_pH5_6h (orange) and S-DHP407_pH7_6h (green) after 15d (A), 30d (B) and 60d (C) storage in the dark, at 4 °C under vacuum (dark colour) or nitrogen atmosphere (light colour).

Scheme Captions

Scheme 1. Schematic representation of Ellman's method applied to S-DHP407_pHX_Yh to quantify free sulfhydryl groups.