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ARTICLE

Supramolecular hydrogels based on custom-made poly(ether urethane)s and cyclodextrins as potential drug delivery vehicles: design and characterization

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The design of supramolecular hydrogels based on host-guest complexes represents an effective strategy to develop drug delivery systems. In this work, we designed SM hydrogels based on α-cyclodextrin and high-molar mass amphiphilic poly(ether urethane)s (PEUs, M_n 27 kDa) based on Poloxamer® 407 and differing in their chain extender. The successful formation of poly(pseudo)rotaxanes and their supramolecular interactions were chemically demonstrated. Then, self-healing (80-100% mechanical recovery) supramolecular hydrogels were developed by mixing PEU and α-cyclodextrin solutions at different concentrations. Stability in physiological-like environment and mechanical properties improved with increasing α-cyclodextrin content (9-10% w/v), meanwhile gelation time decreased. A synergistic effect of poly(pseudo)rotaxanes crystals and PEU micellar structures on gel properties was observed: the first were predominant at low PEU concentrations (1-5% w/v), while the latter prevailed at high PEU concentrations (7-9% w/v). Increasing PEU concentration led to gels with increased dissolution rate, not-fully developed networks and slight cytotoxicity, meanwhile residence time in aqueous media improved (> 7d). At low PEU concentrations (1- 5% w/v), cytocompatible gels (100% cell viability) were obtained, which maintained their shape in aqueous medium up to 5d and completely dissolved within 7d. PEU chemical composition affected PEU/α-cyclodextrin interactions, with longer gelation time and lower mechanical properties in gels based on PEU with pendant functionalities. Gels progressively released a model molecule (Fluorescein isothiocyanate-dextran) within 3-4 days with no initial burst release. We thus demonstrated the suitability of custom-made PEUs as constituent of SM hydrogels with α-cyclodextrin and the high potential of the resulting systems for drug delivery applications.

1. Introduction

Polymeric hydrogels are materials based on water-containing cross-linked macro-molecules. Many natural and synthetic polymers can be used as forming components of hydrogels for biomedical applications.[1–5]

Covalently cross-linked polymers can be designed to obtain stable water-based networks, which are often used to produce cellularized substrates for tissue engineering/regenerative medicine.[6] Chemical hydrogels have been widely used also to release specific drugs[7,8], but the formulation and payload release kinetics represent challenging aspects to be finely controlled. Differently, physically cross-linked hydrogels are developed by relying on reversible interactions (i.e., hydrogen, Van der Waals, hydrophobic or ionic bonds), thus widening the possibilities to engineer release kinetics and degradation/dissolution profiles.[9,10] Hence, hydrogels based on reversible bonds are often preferred to design drug releasing systems, with the additional advantage of providing a protective environment to the encapsulated cargo and minimizing the risks for drug/biomolecule degradation/denaturation due to the mild gelation conditions. However, the design of stable systems showing a sol-to-gel transition driven by physical interactions is not simple, since phase separation phenomena or weak network formation represent realistic issues.

A possible strategy to obtain tuneable physical hydrogels consists in inducing the sol to gel transition by exploiting specific interactions which can arise among the hydrogel components and finally lead to the formation of supramolecular (SM) structures.[11] In SM chemistry, cyclodextrins (CDs) are widely employed and investigated molecules, owing to their capability to form SM interactions with specific moieties. CDs are natural oligosaccharides with a toroidal geometry formed by glucopyranose units. CDs are coded as α-, β- or γ-CDs, depending on the number of glucopyranose units that constitute them (6, 7 or 8 units, respectively). Their discovery dates back to 1891 when Villiers obtained a white crystalline matter from the fermentation of starch.[12] Due to their shape, CDs can form SM host-guest inclusion complexes with a wide

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variety of natural or synthetic molecules.[13–15] Indeed, by exploiting their inner hydrophobic cavity, CDs can encapsulate hydrophobic small molecules[5] or thread along linear polymers based on aliphatic domains, thus forming substantial host-guest complexes.[16] Nowadays, it is well-known that CDs can form Van der Waals and hydrophobic interactions with polymer chains. The most studied and exploited interactions between CDs and linear polymers are those occurring with poly(ethylene oxide) (PEO)[17–20] and poly(propylene oxide) (PPO).[21] The resulting structures are known as polyrotaxanes (PRs) when stoppers (e.g., (α,α-dimethyl-3,5-dimethoxybenzyloxy) carbonyl groups,[22] dithiobenzoate groups,[23] N-triphenylmethyl groups[24]) are added to polymer ends or poly(pseudo)rotaxanes (PPRs) in case of free ends. Moreover, CDs can gather and form hydrogen bonds among hydroxyl groups of their heads and tails. The interactions occurring between CDs and amphiphilic triblock copolymers based on PEO and PPO (i.e., Poloxamer®, PEO-PPO-PEO) have been widely investigated in aqueous solutions.[25–27] In fact, α- and β-CDs can thread and pack on PEO and PPO domains, respectively, thus forming PPRs.[28] It has been thoroughly demonstrated that PPRs can then aggregate in water forming crystalline clusters characterized by a channel-like geometry.[29] This crystallization process results from the presence of hydrogen bonds between the external surfaces of threaded and packed CDs. Focusing on α-CDs and Poloxamers®, PPRs aggregation leads to the formation of viscous and turbid suspensions in water and other polar solvents, finally resulting in PPR-based SM-hydrogels. It has been supposed that the contribution of interacting hydrophobic regions could play a key role in the establishment of stable gels.[25,30] To support this hypothesis, many examples of SM-complexes based on properly synthesized amphiphilic polymers can be found in literature: poly(lactic acid) (PLA)-PEO,[31] poly(hydroxy butyrate) (PHB)-PEO,[32,33] poly(ε-caprolactone) (PCL)-PEO,[34,35] poly(L-lactide)-poly(dimethylamino ethyl methacrylate)-poly[(ethylene glycol) monomethyl ether methacrylate] (PLLA-DMAEMA-PEGMA)[36] and three-armed star copolymer based on PEG and PCL (mPEG-*acetal*-PCL-*acetal*-)$_3$.[37] Hydrogels prepared starting from these polymers were investigated as drug or DNA delivery systems *in vitro* and the release profiles were prolonged from days to weeks depending on the kind of interface settled between the samples and the surrounding medium (e.g., direct contact, indirect contact via transwell inserts) and the nature of the releasing aqueous medium itself (e.g., water, saline solution).[33,38,39] In addition to the previously discussed potential of SM gels as drug/biomolecule carriers for *in situ* localized release, CD-based self-assembled hydrogels have been also reported to exhibit remarkable mechanical properties and self-healing behaviour.[30,31,39–41] Indeed, being SM-hydrogels built on physical and hierarchical interactions, the involved crosslinks are reversible and allow the entire network to easily adapt to external mechanical stimuli (e.g., shear stresses). Hence, all the cited hydrogels were reported to be thixotropic and have the potential to be utilized in many processes, such as *in situ* injections.[42,43]

It is noticeable that all the already reported SM-hydrogels based on simple linear molecules and CDs require relatively high concentrations (generally >10% w/v) of the synthetic component to produce stable SM-gels. However, the minimization of synthetic polymer content in CD-based SM gels could represent a preferable approach, aiming at avoiding or at least reducing potential undesired interactions with the surrounding biological environment and increasing the probability for a better and complete regeneration of wounded or pathological tissues. However, weak and unstable systems are often obtained by lowering the concentration of the synthetic component of the systems. Starting from already available knowledge on the capability of aliphatic PEO-based poly(ether urethane)s (PEUs) to form PPR crystals in water,[44] and the aforementioned need to tune hydrogel composition minimizing synthetic polymer content, in this work novel α-CD-based SM hydrogels have been developed starting from custom-made PEUs. In detail, two different PEUs were synthesized starting from the commercially available triblock copolymer Poloxamer® 407 (P407, PEO-PPO-PEO, $\overline{M_n}$ 12600 Da), an aliphatic diisocyanate (1,6-hexamethylene diisocyanate, HDI) and two different chain extenders, namely an amino acid-derived diol (N-Boc Serinol) and an aliphatic cyclic diol (1,4-cyclohexanedimethanol). In previous works[45,46] aqueous solutions of these PEUs with concentration higher than a critical value (approx. 6% w/v) showed the capability to undergo a temperature-driven sol-to-gel transition with increasing temperature over their lower critical gelation temperature. Conversely, in this work their high PEO content (about 70% wt, similarly to P407) and the presence of other hydrophobic aliphatic domains (HDI and the chain extenders) in addition to the PPO block of P407 were exploited to form SM complexes with cyclodextrins resulting in SM hydrogels. In detail, SM hydrogels were designed at PEU and α-CD concentrations ranging from 1 to 9% w/v and from 7 to 10% w/v, respectively. The spontaneous self-assembly of PEUs and CDs into PPRs and the resulting crystals were characterized through X-Ray Powder Diffraction (XRD), Attenuated Total Reflectance - Fourier Transformed Infrared (ATR-FTIR) Spectroscopy and Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy. Gelation kinetics of SM hydrogels was qualitatively assessed through tube inverting test, meanwhile their swelling and stability in contact with a physiological-like aqueous environment were evaluated up to 7 days incubation. Moreover, a complete rheological characterization was performed to determine mechanical and self-healing properties of PEU-based SM hydrogels. As a proof of concept of the potential use of these hydrogels as drug/biomolecule carriers for localized delivery, release profiles of a model molecule (i.e., Fluorescein isothiocyanate-dextran, FD4, $\overline{M_w}$ 4000 Da) were characterized for systems with low PEU content (i.e., 1-5% w/v concentration range). Finally, cytocompatibility of SM hydrogels was evaluated on NIH 3T3 fibroblast cells using material extracts.

2. Experimental

2.1 Materials

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Poloxamer® 407 (P407, $\overline{M_n}$ 12600 Da, 70% PEO), 1,6-hexamethylene diisocyanate (HDI), N-Boc Serinol (NBoc), 1,4-cyclohexanedimethanol (CDM), dibutyltin dilaurate (DBTDL) and 3-(trimethylsilyl)propionic-2,2,3,3-d 4 acid sodium salt (TSP) were purchased from Merck/Sigma-Aldrich (Milan, Italy). α-CDs (from now on abbreviated with the acronym "CDs") were purchased from TCI Chemicals Europe (Zwijndrecht, Belgium). Before use, P407 was dehydrated for 8h at 100 °C under mild vacuum (approx. 200 mbar) and then equilibrated at 40 °C, while NBoc and CDM were dried at low pressure overnight at room temperature; HDI was distilled under vacuum. All the solvents were purchased from Carlo Erba Reagents in their analytical grade (Milan, Italy). The solvent used as medium for PEU synthesis, 1,2-dichloroethane (DCE, Carlo Erba Reagents, Milan, Italy), was anhydrified over activated molecular sieves (Sigma Aldrich, Milan, Italy) and N_2 atmosphere overnight before use.

2.2 Synthesis of poly(ether urethane)s

The two different PEUs were synthesized according to an already published two-step protocol.[45–47] In summary, P407 was first solubilized in anhydrous DCE (20% w/v) and equilibrated at 80 °C. HDI and DBTDL were then added at 2:1 molar ratio and 0.1% w/w with respect to P407, respectively. The first step was carried on under stirring (approx. 250 rpm) for 2.5 hours at 80 °C, thus obtaining an isocyanate-terminated pre-polymer. Subsequently, the solution containing the pre-polymer was cooled down at 60 °C, the chain extender was solubilized (3% w/v, 1:1 molar ratio with respect to P407) in anhydrous DCE and added. The second step continued for 1.5 hours at 60 °C. Then, once the solution was cooled down to room temperature, excess methanol was added and properly mixed to passivate any residual isocyanate group. The final solution containing the PEU was precipitated in petroleum ether (4:1 v/v ratio with respect to total DCE) under vigorous stirring. Then, the PEU was separated from the supernatant and dried overnight under a fume hood. Subsequently, the PEU was again solubilized in DCE (20% w/v) and purified in a mixture of diethyl ether (DEE) and methanol (98:2 % v/v and 5:1 v/v ratio with respect to DCE). The PEU was collected through centrifugation at 6000 rpm, 0 °C for 20 minutes (Hettich, MIKRO 220R), and dried overnight under the fume hood. Finally, the obtained PEU was stored in vacuum at 3° C until use. Both the performed precipitation procedures allowed the removal of unreacted reagents, low molecular weight by-products and catalyst molecules.

Figure S1 reports a schematic representation of PEU synthesis protocol.

Hereafter, the synthesized PEUs will be referred to with the acronyms NHP407 and CHP407, where P407 identifies the macrodiol Poloxamer® 407, H stands for the diisocyanate HDI, N and C refer to N-Boc Serinol and 1,4-cyclohexane dimethanol, respectively.

2.3 Chemical characterization of PEUs

2.3.1 Attenuated Total Reflectance – Fourier Transformed Infrared (ATR-FTIR) Spectroscopy

In order to prove the successful PEU synthesis, ATR-FTIR spectroscopy was performed at room temperature on PEU powder using a Perkin Elmer Spectrum 100 equipped with an ATR accessory with diamond crystal (UATR KRSS). Spectra resulted from 16 scans within the spectral region ranging between 4000 and 600 cm^{-1} at a resolution of 4 cm^{-1}. Registered data were analyzed through the Perkin Elmer Spectrum software. The ATR-FTIR spectrum of P407 was also registered according to the same protocol and used as reference.

2.3.2 Size Exclusion Chromatography (SEC)

NHP407 and CHP407 molar mass distribution was characterized using an Agilent Technologies 1200 Series (California, USA) SEC equipped with a Refractive Index detector and two Waters Styragel columns (HR1 and HR4). N,N-dimethylformammide (DMF, HPLC grade, Carlo Erba, Milan, Italy) added with LiBr (ReagentPlus®, >99%, Sigma Aldrich, Italy) at 0.1% w/v concentration was used as mobile phase at 0.5 ml/min flow rate. PEU number average molar mass ($\overline{M_n}$), weight average molar mass ($\overline{M_w}$) and dispersity index (D =$\overline{M_w}/\overline{M_n}$) were estimated using the Agilent ChemStation software and a specific calibration curve based on PEO standards ranging in $\overline{M_p}$ between 982 and 205500 Da. Samples were prepared by dissolving 2 mg of PEU in 1 ml of mobile phase and filtering the solution using 0.45 µm poly(tetrafluoroethylene) (PTFE) syringe filters (LLG International, Meckenheim, Germany).

2.4 Preparation and characterization of PEU- and CD-based SM complexes

2.4.1 Preparation of SM complexes

Inclusion complexes (ICs) were prepared in double distilled water by mixing PEU and CD solutions. Firstly, PEUs were weighted in the required amount and solubilized in water at 3 °C overnight. Then, a clear and transparent solution of CDs (14% w/v) was prepared, added to the PEUs samples and mixed using a vortex (at 40 Hz for 20-30 seconds) thus obtaining samples with 1% w/v PEU concentration and the amount of CD required to theoretically cover the 100% PEO domains (i.e., 7.6% w/v). Samples were then stored at room temperature (25 °C) for 72 hours until turbid and viscous suspensions of SM crystals were obtained. Self-assembled PPRs given by crystalline clusters of inclusion complexes were separated through centrifugation (Mikro 220R, Hettich, Germany) at 4500 rpm and 10 °C for 15 minutes. Collected PPRs were quenched and frozen with liquid nitrogen and freeze dried for 24 hours (Martin Christ ALPHA 2-4 LSC, Germany). Hereafter, the collected NHP407 and CHP407 supramolecular complexes will be referred to with the acronyms NHP407 SM_100% and CHP407 SM_100% to highlight that they were designed to allow CDs to cover all the PEO domains present in the PEUs.

2.4.2 X-Ray powder diffraction (XRD) analysis

The diffractograms of NHP407 SM_100% and CHP407 SM_100% were acquired using an X Ray diffractometer Siemens D5005 with Bragg-Brentano geometry and vertical goniometer

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theta - theta (the sample holder is fixed, meanwhile tube and detector rotate). The diffractometer was equipped with a Ni-filtered Cu Kα (1.542 Å) source of radiation working at 40 kV, 40 mA. Analyses were performed in step scan mode within the 2θ range from 5° to 30° using an increment rate of 0.1°/step, and a scan speed of 10 s/step. NHP407 and CHP407 as such were also analysed according to the same protocol.

2.4.3 Attenuated Total Reflectance – Fourier Transformed Infrared (ATR-FTIR) Spectroscopy

To further characterize SM complexes, ATR-FTIR spectroscopy was also performed on NHP407 SM_100% and CHP407 SM_100%. Spectra resulted as an average of 32 scans within the spectral range from 4000 and 600 cm^{-1} at a resolution of 1 cm^{-1}. Analyses were performed at room temperature on three different NHP407 SM_100% and CHP407 SM_100% batches and registered spectra were averaged. The same analyses were also performed on PEUs and CDs as such for comparative purposes.

2.4.4 Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy

^{1}H-NMR analyses were carried out using an AVANCE III Bruker spectrometer equipped with an 11.75 T superconducting magnet (500 MHz ^{1}H Larmor frequency) and a Bruker BVT-3000 unit for temperature control. The NMR spectra were run at 300 K after waiting 10 min for sample temperature stabilization. Each spectrum was obtained as the average of 12 scans, with 10s relaxation time. 1 mM TSP in D_2O in a sealed capillary was inserted in the NMR tube and used as reference for the zero of the chemical shift scale.

Samples for ^{1}H-NMR spectroscopy were prepared by solubilizing NHP407 SM_100% and CHP407 SM_100% in deuterium oxide (D_2O, 99.8%, Sigma Aldrich, Italy) at a final concentration of 5 mg/ml. ^{1}H-NMR analyses were also conducted on self-assembling samples resulting from the mixture of PEUs (1% w/v in D_2O) and the required amount of CDs to cover the 50% of PEO domains (i.e., 3.8 % w/v in D_2O) in order to avoid the formation of a solid precipitating crystalline phase which could impede the analysis (acronyms for these samples are: NHP407 SM_50% and CHP407 SM_50%). CD, NHP407 and CHP407 samples were also prepared in the same conditions and analyzed as controls. The registered spectra were elaborated using MNova software (Mestrelab Research, S.L, Spain, www.mestrelab.com).

2.5 Preparation and characterization of PEU- and CD-based SM hydrogels

2.5.1 Preparation of SM hydrogels

Hydrogels were prepared in double distilled water (ddH_2O) by simply mixing PEU and CD solutions. Firstly, PEUs were weighed in the required amount in bijou sample containers (17 mm diameter, 7 ml, Carlo Erba Reagents, Milan, Italy) and solubilized in ddH_2O at 3 °C overnight. Then, a clear and transparent solution of CDs (14% w/v) was prepared in water and added to the PEUs samples to finally obtain a plethora of formulations differing in the final concentrations of both PEUs (1, 5, 7 and 9% w/v) and CDs (7, 8, 9 and 10% w/v). The mixtures were homogenized through a vortex (at 40 Hz for 20-30 seconds) and finally incubated in isothermal conditions (i.e., room temperature (25 °C) or 37 °C) to allow SM complexes formation and arrangement into an organized gel network.

Hereafter, SM hydrogels will be referred to with the acronym PEU X% - CD Y%, where PEU identifies the poly(ether urethane) which constitutes the synthetic counterpart of the hydrogel (i.e., CHP407 or NHP407), meanwhile X and Y define PEU and CD concentration within the formulation, respectively.

2.5.2 Qualitative evaluation of gelation time in isothermal conditions and phase-separation

Gelation time of SM hydrogels at room temperature (25 °C) and at 37 °C was qualitatively evaluated by visual inspection. Briefly, at predefined time intervals (every 1 and 5 minutes up to 1 hour observation and then every 10 minutes), the samples were inverted and the flowing of PEU-CD mixtures was assessed. The "gel" state was characterized by a "no-flow" condition within 30 s of vial inversion. Upon gelation, the samples were stored at the gelling temperature (25 or 37 °C) and visually checked every day to assess the occurrence of phase-separation phenomena.

2.5.3 Rheological characterization

Rheological tests on SM hydrogels based on NHP407 and CHP407 were performed using a stress-controlled rheometer (MCR302, Anton Paar GmbH, Graz, Austria) equipped with a Peltier system for temperature control and a 25 mm parallel plate configuration. SM gels (PEU concentrations ranging from 1 to 9% w/v and CD concentrations at 9 and 10% w/v) were loaded through injection (2 mm needle, approx. 0.4 ml) on rheometer lower plate previously equilibrated at 25 °C. For all the characterizations, the normal force was set at 0 N and the thickness of the sample was 0.6 mm. Strain sweep tests were performed at 37 °C and 1 rad/s angular frequency from 0.01 to 500% strain. Samples analyzed through strain sweep test were again subjected to the same analysis after 15 minutes in quiescent state at 37 °C in order to assess their capability to self-heal upon application of an increasing strain up to 500%. Frequency sweep tests were performed within the linear viscoelastic region (i.e., at 0.1% strain) at different temperatures (25, 30 and 37 °C) and angular frequency ranging between 0.1 and 100 rad/s. Self-healing strain tests were also conducted to study the recovery ability of SM hydrogels when a variable strain was applied cyclically over time at 37 °C, according to Wu et al. with slight modifications.[48] In detail, the samples were initially subjected to a constant strain (0.1 %, recovery phase) for 120 s at 1 Hz; then, in order to induce the complete breakage of hydrogel network, a higher strain (100 %, rupture phase) was applied for 60 s at 1 Hz. Each sample was subjected to this procedure for three cycles and then the starting strain (0.1 %, recovery phase) was applied again to register gel final mechanical properties.

2.5.4 Swelling and stability tests in physiological-like conditions

Swelling and dissolution behaviour of SM hydrogels (1 ml in bijou sample containers with 7 ml capacity and an inner diameter of 17 mm) was evaluated through incubation at 37 °C

in contact with phosphate buffered saline (PBS, pH 7.4) added with sodium azide (NaN_3, 0.1% w/v, Sigma Aldrich, Milan, Italy) to prevent mould growth. Initially, the hydrogels were weighed (W_{gel_i}) and equilibrated at 37 °C for 15 minutes. Then, PBS containing NaN_3 (1 ml, equilibrated at 37 °C) was added to each sample and the gels were incubated at 37 °C. PBS containing NaN_3 was completely refreshed trice a week. At predefined time steps (24h, 3 days, 5 days and 7 days) the residual PBS was thrown away and the remaining SM gels were weighed again (W_{gel_f}) to quantify the swelling ability of the hydrogels (PBS Absorption %) according to equation 1. The same samples were then freeze dried (Martin Christ ALPHA 2-4 LSC, Germany) and weighed again ($W_{dried\ gel\_f}$) in order to quantify hydrogel weight loss (Hydrogel dissolution %) using the formula reported in equation 2. To this aim, reference samples (i.e., as prepared SM gels, not undergoing incubation in aqueous media) were also prepared, freeze dried and weighed ($W_{dried\ gel\_i}$).

$$PBS\ Absorption\ \% = \frac{W_{gel_f} - W_{gel_i}}{W_{gel_f}} \times 100 \quad \text{(Eq.1)}$$

$$Hydrogel\ dissolution\ \% = \frac{W_{dried\ gel_f} - W_{dried\ gel_i}}{W_{dried\ gel_i}} \times 100 \quad \text{(Eq.2)}$$

Analyses were conducted in quintuplicate and results are reported as mean ± standard deviation.

2.5.5 Cytotoxicity evaluation

Cytotoxicity was evaluated on all the developed SM hydrogels, with PEU concentration ranging from 1 to 9% w/v and CD concentration at 9 or 10% w/v. Briefly, SM hydrogels (250 mg) were prepared in bijou sample containers (7 ml, 17 mm inner diameter), sterilized through UV light irradiation (290 nm) for 30 minutes and incubated at 37 °C for 15 minutes. Then, 1 ml of Dulbecco's Modified Eagle Medium (37 °C, high glucose, Carlo Erba Reagents, Italy) containing foetal bovine serum (10% v/v, Carlo Erba Reagents, Italy) was added every 100 mg of SM hydrogel. The samples were incubated for 24 hours at 37 °C and subsequently the eluates were collected. Simultaneously, NIH 3T3 cell line (Atcc, USA) were cultured in the same medium (37 °C, 5% CO_2) on a 96 well plate (20'000 cell/well, 100 µl cell culture medium) for 24 hours. The eluates were filtered (0.22 µm, LLG Labware) and then administered to the cultured cells (100 µl/well) for 24h at 37 °C. Then, the medium was completely refreshed with fresh one (100 µl) containing resazurine (0.1 mg/ml, Sigma Aldrich, Milan, Italy) and the plate was incubated again for 1 hour at 37 °C. Cell viability was quantified through a multimode plate reader (Perkin Elmer Victor X3) by measuring the reduced resazurine at 595 nm after excitation at 530 nm. Three independent experiments were performed in order to assess cell viability with respect to control samples (i.e., cultured cells in fresh medium not containing gel extracts).

2.5.6 Release study of fluorescein isothiocyanate dextran (FD4)

FD4 release profile from SM hydrogels was investigated on samples with 1 or 5% w/v PEU concentration and CD at 10% w/v concentration. FD4 ($\overline{M_w}$ 4000 Da, Sigma Aldrich, Milan, Italy) was selected as model molecule of drugs and macromolecules, such as antibiotics and proteins. In order to load the molecule with SM gels, a CD solution in water (14% w/v) was used as solvent for FD4; then, an aliquot of this solution was mixed with PEU solutions previously prepared in ddH_2O in bijou sample containers (7 ml capacity, inner diameter 17 mm, Carlo Erba Reagents, Milan, Italy) in order to reach a CD and FD4 concentration of 10% w/v and 1 mg/ml, respectively, and a final sample volume of 1 ml. The hydrogels were incubated at room temperature (25 °C) for 48 hours to ensure their complete supramolecular gelation. Then, they were incubated at 37 °C for 15 minutes and PBS (1 ml, 37 °C) was added to each sample as releasing medium. At predefined time steps (2, 4, 6, 24, 48 and 72 h), the eluates were taken and the samples were added with fresh PBS (1 ml). The quantification of the released FD4 was performed through a multimode plate reader (Perkin Elmer Victor X3) by measuring the absorbance at 490 nm on a 96 well plate (200 µl for each well) and referring to a calibration curve obtained from standard samples with FD4 concentration within the range 0.05-1 mg/ml. Five samples were prepared for each investigated formulation. Data are shown as average ± standard deviation.

2.6 Statistical Analysis

Statistical analysis was conducted through GraphPad Prism 8 for Windows 10 (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). Two-way ANOVA analysis coupled with Bonferroni multiple comparison test was performed to compare results, assessing the statistical significance according to Boffito *et al*.[45]

3. Results

3.1 Chemical characterization of as synthesized PEUs

ATR-FTIR spectroscopy and SEC were conducted to prove the successful synthesis of CHP407 and NHP407 poly(ether urethane)s. Figure 1 reports the ATR-FTIR spectra of the synthesized PEUs and P407 as reference. In both NHP407 and CHP407 ATR-FTIR spectra, the appearance of new bands related to urethane domains was observed at 1720 cm^{-1} due to the stretching vibration of free carbonyl groups (C=O), and at 1530 cm^{-1} due to the concurrent bending and stretching of N-H and C-N bonds, respectively. N-H stretching was also observed at 3350 cm^{-1}. Moreover, the absence of any peak around 2200 cm^{-1} was a clear demonstration of a complete conversion of isocyanate groups. PEU spectra also exhibited the typical absorption peaks related to the presence of P407 as building block at 2880 and 1250 cm^{-1} due to CH_2 stretching and rocking, respectively, and at 1100 cm^{-1} ascribed to C – O – C stretching vibration.

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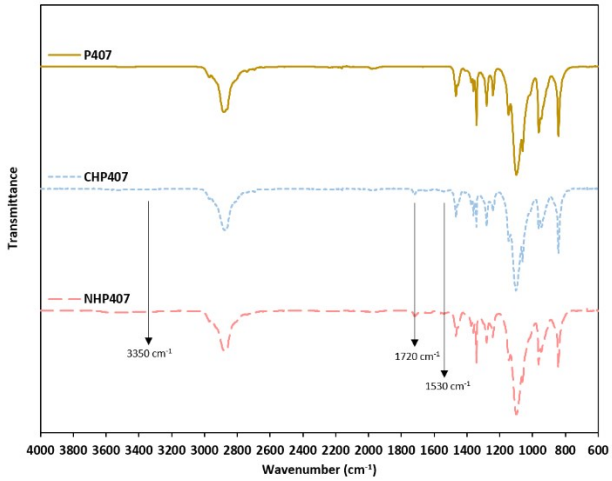

Figure 1: ATR-FTIR spectra of P407 (ocher continuous line), CHP407 (light blue dotted line) and NHP407 (light red dashed line). Absorbance peaks demonstrating the successful synthesis of poly(ether urethane)s are evidenced at 3350, 1720 and 1530 cm^{-1}.

The synthesized poly(ether urethane)s showed number average molar mass ($\overline{M_n}$) and dispersity index of *ca.* 27 000 Da and 1.8, respectively, with no significant differences induced by the two chain extenders (i.e., N-Boc serinol and CDM for NHP407 and CHP407, respectively) used for their synthesis.

3.2 Characterization of SM complexes

3.2.1 X-Ray powder diffraction (XRD) characterization

XRD measurements were conducted to confirm the formation of SM complexes and characterize the possible crystalline phase resulting from the self-assembly of PPRs. XRD pattern of P407 triblock copolymer is characterized by two peaks at *circa* 19°-19.3° and 23.1°-23.5°, resulting from the presence of PEO blocks (approx. 70% wt).[49] As reported in figure 2, NHP407 and CHP407 spectra were characterized by two relevant peaks at 2θ of 19.1° and 23.2°, thus indicating the presence of crystalline domains similar to P407. Interestingly, the increased molar mass of the synthesized PEUs and their more complex chemical structure was probably not significantly affecting the crystallization process of these macromolecules with respect to simple P407-based systems.

In the case of linear PEG-based polymers, it is known that typical peaks of a hexagonal channel-like complexation with CDs appear at 2θ values around 7.4°-7.6°, 12.8°-13° and 19.6°-20°.[38,49,50] XRD measurements on PEU-based SM complexes clearly evidenced the formation of crystalline domains given by PPR assembly (figure 2). In detail, a peak at 2θ equal to 19.8° appeared in the XRD spectra of both NHP407 SM_100% and CHP407 SM_100%. Moreover, the presence of additional peaks at 7.6° and 13° was a further confirmation of the formation of hexagonal channel-shaped SM crystals. These data demonstrated that also macromolecules containing P407 moieties as building blocks can undergo SM crystallization process. Additionally, no peaks related to the conformation of the native PEUs and typical CD multiple reflections (highly crystalline) appeared in XRD patterns of PEU-based SM complexes. In the specific conditions investigated in this work, the total mass yield of precipitated crystalline phase was 58 ± 0.5% and 30 ± 0.5% for CHP407- and NHP407-based SM complexes (average of three measurements), respectively. The significantly higher mass collected from CHP407-based SM complexes production could be related to the presence of pendant BOC groups in NHP407 poly(ether urethane). In fact, although NHP407 contains a less rigid chain extender (i.e., serinol) with respect to the cyclic monomer that characterizes CHP407 (i.e., cyclohexane), the steric hindrance of BOC pendant groups in NHP407 most likely interfered with CD threading along polymer chains.

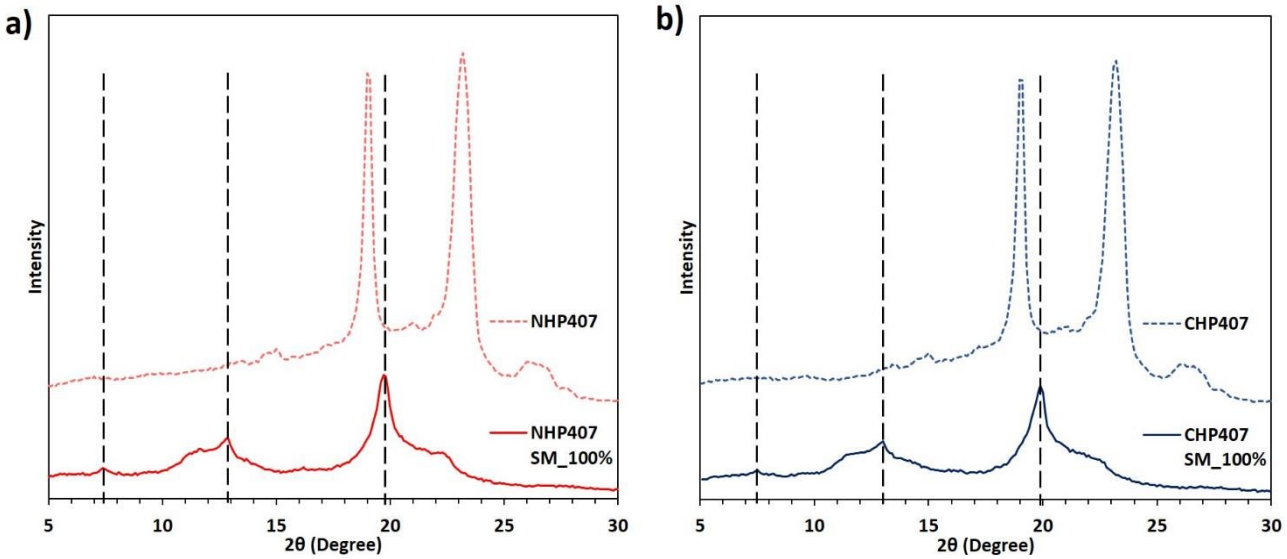

Figure 2: XRD spectra of PEUs and their SM crystals. a) NHP407 and NHP407 SM_100% (red dashed and continuous lines, respectively), and b) CHP407 and CHP407 SM_100% (blue dashed and continuous lines, respectively). As synthesized PEUs showed diffractograms similar to P407, thus indicating a common conformation of PEO in both the starting macrodiol (P407) and the chain-extended macromolecules (PEUs). The peaks related to SM self-assembly into hexagonal channel-like crystals are highlighted by vertical black dashed lines (2θ equal to 7.6°, 13° and 19.8°). No peaks related to pure PEU conformation and typical CD multiple reflections (highly crystalline) appeared in XRD patterns of SM complexes.

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3.2.2 Attenuated Total Reflectance – Fourier Transformed Infrared (ATR-FTIR) spectroscopic analyses

ATR-FTIR analyses of the SM crystalline powders (i.e., CHP407 SM_100% and NHP407 SM_100%) showed relevant differences with respect to control samples based on pure PEUs and CDs (figure 3). The co-presence of absorption peaks characteristic of the native PEUs and CDs in the ATR-FTIR spectra of SM complexes proved the occurrence of an evident self-assembly process. For instance, the peaks at 1075, 1155 and 2954 cm^{-1} in the ATR-FTIR spectra of SM complexes are typical of CDs and can be ascribed to C-O, C-O-C and CH stretching vibrations, respectively. Another important signal in CD spectrum is located at 3308 cm^{-1}, which is due to the symmetric and asymmetric stretching vibration of –OH groups.[51,52] When CDs were involved in SM crystals with PEUs, this peak upshifted to 3340 cm^{-1}. This significant variation could be correlated to a different crystalline conformation of CDs, which probably were not in a cage-like packing structure upon assembling with the PEUs.[18,53,54] Indeed, it has been supposed that CDs could be packed along PEU chains forming hydrogen bonds between –OH groups of the interacting outer faces of the aligned cavities. This particular conformation could cause a different vibration of –OH groups with respect to the cage-type packing, by lowering the strength of the spontaneous association of CDs. This hypothesis was further supported by the shift observed in the typical absorption peak due to the stretching of urethane carbonyl groups (C=O), which moved from 1720 cm^{-1} to 1740 cm^{-1}. This shift was most likely a consequence of the inhibition of PEU chain crystallization resulting from CD threading along PEU chains. These observations were in accordance with previous studies[44] and the previously commented XRD results. As a matter of fact, the observed peak shifts in ATR-FTIR spectra further supported the hypothesis of a channel-like conformation of complexed PPRs, with a potential contribution given also by urethane bonds.

3.2.3 Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopic analyses

In addition to XRD and ATR-FTIR analyses, ^{1}H-NMR characterization was also performed on NHP407 SM_100% and CHP407 SM_100% upon their solubilization in D_2O. The co-presence of the characteristic peaks of both PEUs (within the chemical shift range from 1.60 to 0.95 ppm and at 3.75 ppm) and CDs (within the range between 4.05 and 3.55 ppm and at 5.07 ppm) (Figure 4) further proved that the collected crystals resulted from a self-assembly process between PEUs and CDs, as previously demonstrated also by XRD and ATR-FTIR characterizations. Detailed signal assignment to corresponding protons in CD, NHP407 and CHP407 ^{1}H-NMR spectra has been reported in Supporting Information file (Figures S2, S3 and S4).

In order to exploit ^{1}H-NMR spectroscopy to thoroughly investigate the possible interactions occurring between PEUs and CDs, analyses were also conducted on self-assembling samples (i.e., NHP407 SM_50% and CHP407 SM_50%) properly designed to avoid the formation of a solid precipitating crystalline phase. However, due to the complex chemical composition and the relatively high molar mass of NHP407 and CHP407, no evident shifts of the typical peaks of CD hydrogens were generally observed, in contrast with data already reported for similar PEUs with lower molar mass ($\overline{M}_n$ 3100 Da).[44] In fact, only a negligible shift in CD inner protons (H3 and H5) signals

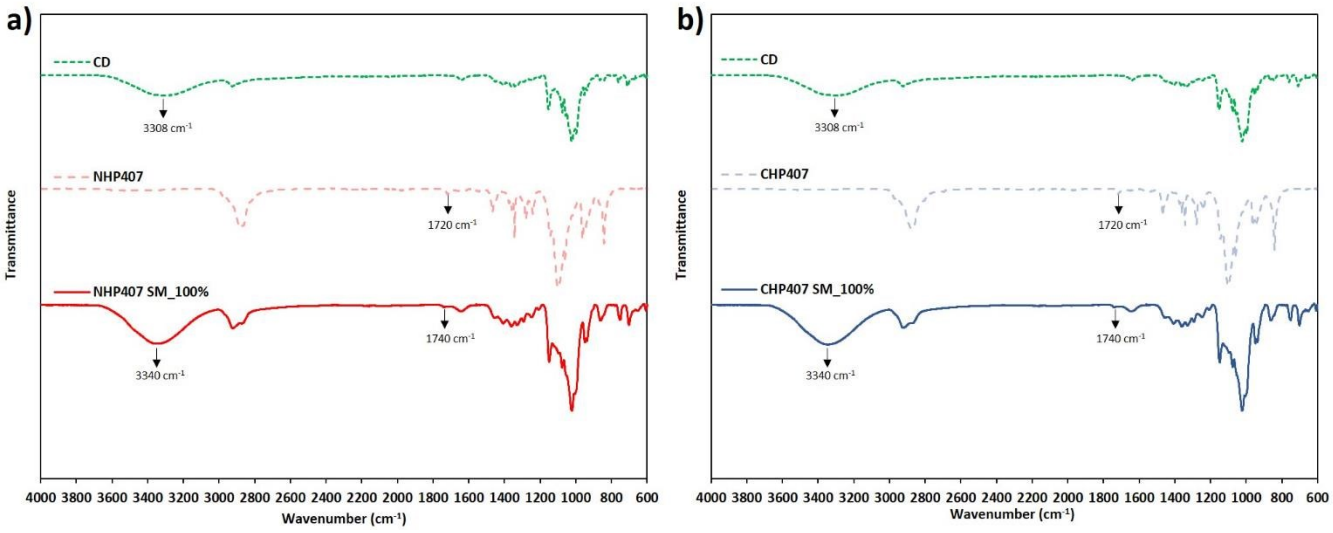

Figure 3: ATR-FTIR spectra of CD, PEUs and SM complexes. a) Comparison among CD (green dashed line), NHP407 (red dashed line) and NHP407 SM_100% (red continuous line), and b) Comparison among CD (green dashed line), CHP407 (blue dashed line) and CHP407 SM_100% blue continuous line). The co-presence of PEU and CD bands in the ATR-FTIR spectra of SM samples was a further demonstration of the formation of a SM compound. The evident shifts of –OH (from 3308 to 3340 cm^{-1}) and C=O (from 1720 to 1740 cm^{-1}) bands are evidenced by arrows in spectra of NHP407 and CHP407 at the SM conformation.

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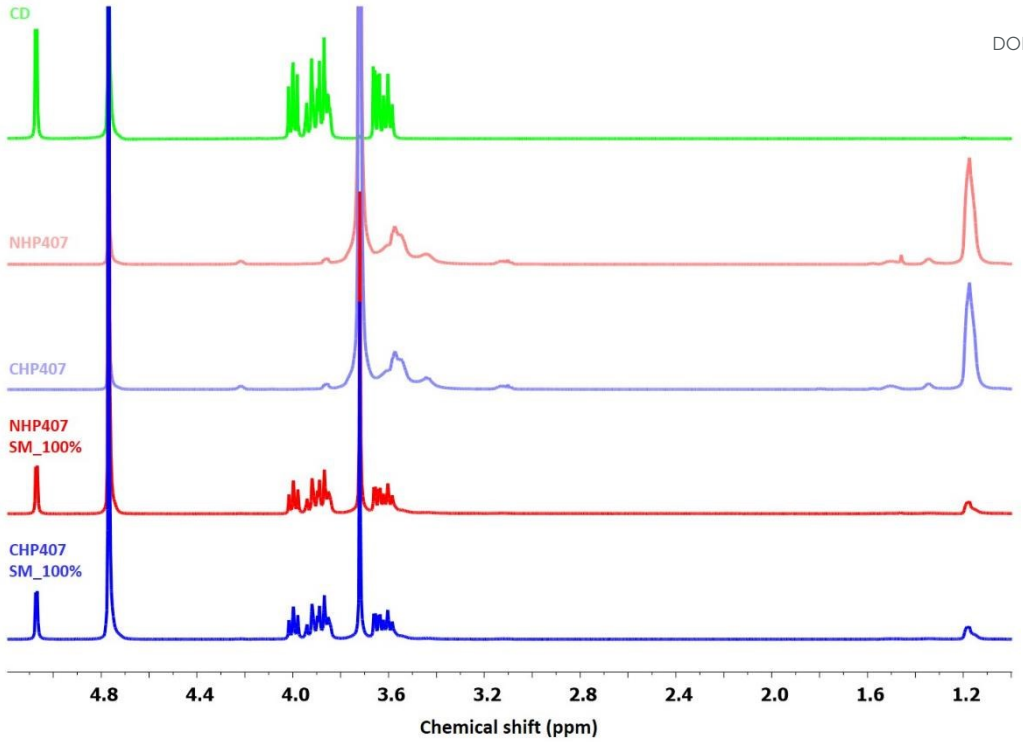

Figure 4: ^{1}H-NMR spectra of CDs (green), CHP407 (light blue), NHP407 (light red), CHP407 SM_100% (blue), NHP407 SM_100% (red). The co-presence of CD and PEU in the collected SM crystals is proved by the appearance of the typical peaks of both the components in CHP407 SM_100% and NHP407 SM_100% spectra.

were observed in both NHP407 SM_50% and CHP407 SM_50% (Figure 5a). Moreover, in accordance with Hasan *et al.,*[44] the shifts of the characteristic peaks of PEO domains were not quantifiable due to non-uniform and too wide peaks, as reported in figure 5a. These aspects can be explained by the likely lower yield of SM self-assembly occurring when macromolecules, such as the here-developed PEUs, are involved in SM complex formation. Additionally, a lower stability of the formed complexes cannot be completely excluded, as a consequence of a hindered threading of CDs along PEU chains. Hence, in both NHP407 SM_50% and CHP407 SM_50% CDs are present mainly in the free form and the amount of threading CDs is too small to induce an appreciable shift in the CD ^{1}H-NMR signals. On the other hand, the peaks related to the HDI portion of the PEUs (in the chemical shift range from 1.55 to 1.30 ppm) were found to broaden in ^{1}H-NMR spectra of the complexed samples (Figure 5b and 5c). This observation could be an evidence of the interaction occurring between urethane domains and CDs, as previously hypothesized from ATR-FTIR analyses results. Finally, for the remarkable complexity of the studied systems, it was not possible to perform NMR shift titration analyses to calculate constants of association.

3.3 Supramolecular Hydrogel characterization

3.3.1 SM hydrogel formulation and gelation kinetics

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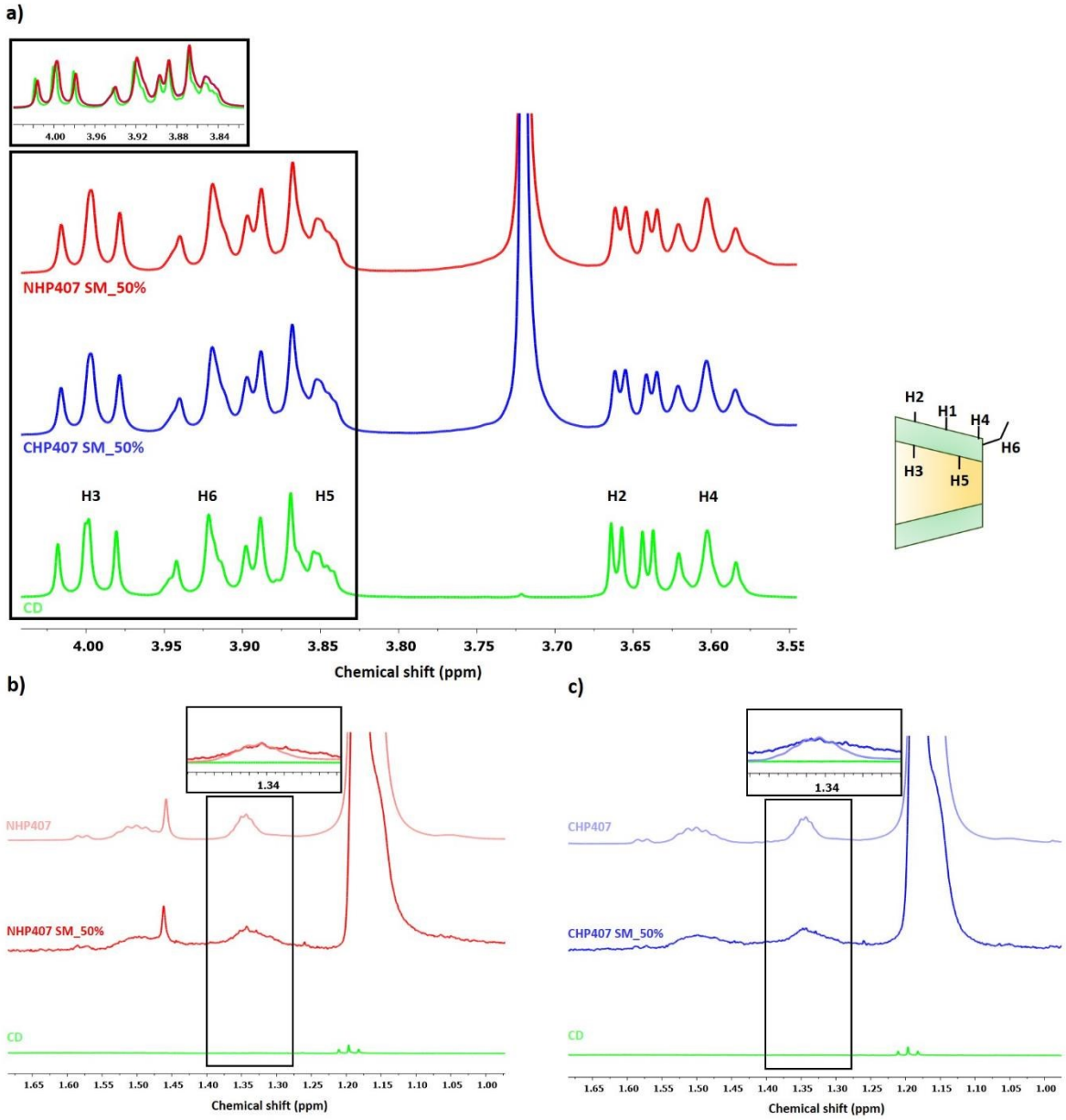

Figure 5: a) ^{1}H-NMR spectra of NHP407 SM_50%, CHP407 SM_50% and CD as reference. Insert on top left reports overlapped ^{1}H-NMR spectra within 3.81 and 4.4 ppm highlighting only negligible shifts for CD inner protons (H3 and H5) signals in NHP407 SM_50% and CHP407 SM_50% compared to CD as such. b) ^{1}H-NMR spectra of NHP407 SM_50%, NHP407 and CD within the range 1.70-0.95 ppm. Insert on top reports overlapped ^{1}H-NMR spectra within 1.25 and 1.55 ppm highlighting the broadening of signals ascribed to the HDI portion of the polymer. c) ^{1}H-NMR spectra of CHP407 SM_50%, CHP407 and CD within the range 1.70-0.95 ppm. Insert on top reports overlapped ^{1}H-NMR spectra within 1.25 and 1.55 ppm highlighting the broadening of signals ascribed to the HDI portion of the polymer in CHP407 SM_50% compared to native CHP407.

Gelation potential and timing were studied on PEU and CD mixtures at different concentrations, as reported in Table 1. Mixtures with the same compositions were also prepared using P407 as synthetic component, for comparison purposes. Gelation tests were conducted at both 25 and 37 °C to assess the influence of temperature on the gelation process of the formulations.

Interestingly, a complete sol-to-gel transition was observed in formulations containing CD at 9 or 10% w/v and PEU at very low concentrations (1 and 3% w/v). Previous studies reported that 1 and 3% w/v concentrated aqueous solutions of PEUs with similar composition were unable to undergo a sol-gel transition even upon thermal stimulus.[45,55] This feature indirectly proved the key role exerted by CDs in allowing the gelation of low concentrated PEU aqueous solutions. In addition, the possibility to obtain gels from polymer/CD mixtures containing a low amount of synthetic counterpart could represent an effective strategy for the design of highly cell-friendly and biocompatible formulations for biomedical applications. Up to 5% w/v PEU concentration, the systems showed remarkably faster gelation at 25 °C with respect to 37 °C, irrespective of their CD content. This behaviour could be related to the well-known ability of

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PEUs to form stable complexes based on micelles and micelle aggregates with increasing temperature,[45] which could obstruct the threading of CDs along their polymer chains, as suggested also by the slower appearance of the typical turbidity of supramolecular complexes at 37 °C compared to 25 °C. Nevertheless, the formulations at 7 and 9% w/v PEU concentrations were characterized by faster gelation when incubated at 37 °C compared to 25 °C. This different trend could be ascribed to the predominant temperature-driven gelation of these systems over SM complex formation and physical crosslinking. Indeed, at concentrations higher that a critical value (namely the critical gelation concentration -CGC- which has been reported to be around 5% w/v for aqueous solutions of PEUs with similar composition[45,47]), PEU thermo-sensitivity and the capability of their aqueous solutions to undergo temperature-driven sol-to-gel transition and significant viscosity increase in response to temperature increase probably played the main role in the gelation observed in 7 and 9% w/v PEU concentrated formulations. Nevertheless, a slight formation of turbidity was observed even in this unfavourable condition, thus indicating that a partial assembly of free PEO domains of PEUs and CDs into PPR-based crystals was occurring. Irrespective of sample incubation temperature and PEU concentration, gelation time decreased with increasing CD concentration within the hydrogels as a consequence of the increased SM crosslinking occurring at higher CD contents (9 and 10% w/v). In more detail, systems with CD concentrations of 7 and 8% w/v were characterized by extremely slow gelation kinetics (typically over 3 days), while CDs at 9 and 10% w/v concentrations opened the possibility to form turbid hydrogels within 40 minutes or few hours.

At equal PEU and CD concentrations, CHP407-based samples exhibited faster gelation when incubated with CDs with respect to NHP407-based ones, with the exception of 9% w/v CD concentrated samples containing PEUs at 1, 3 and 5% w/v concentration incubated at 37 °C. This particular behaviour could be correlated to the molecular conformation of CHP407: the absence of pendant groups along its backbone (NHP407 chains present pendant Boc moieties) made CD threading along their chains easier and faster also in unfavourable conditions (i.e., at 37 °C). Additionally, it is likely that CHP407 chains exhibited slightly slower rate of micellization compared to NHP407 ones due to the higher rigidity of their backbone induced by the cyclic group of CDM. For this reason, in 7 and 9% w/v PEU concentrated formulations, CHP407 chains probably better interacted with CD also at 37 °C, as highlighted by the faster gelation of CHP407-based formulations compared to NHP407-based ones at that temperature (e.g., CHP407 7% - CD 9% and NHP407 7% - CD 9% underwent gelation within 12 and 33 minutes at 37 °C, respectively). A further evidence of the enhanced interaction between CHP407 and CD molecules was also provided by the higher yield of SM crystal recovery achieved in CHP407 SM_100% compared to NHP407 SM_100% (approx. 2-fold higher).

At synthetic polymer concentration up to 5% w/v, PEU-based SM hydrogels were characterized by slower gelation with respect to simple P407-based systems at 37 °C. Indeed, in these conditions, the higher molar mass and complexity of PEUs compared to P407 were responsible for their slower interactions with CDs and SM physical crosslinking leading to gel network formation. These data were also in agreement with the enhanced micellization potential of PEU-based aqueous solutions compared to P407 based ones,[45] being micelle formation an obstacle to CD threading phenomena as previously stated. Differently, at 37 °C incubation temperature and 7 and 9% w/v polymer concentration, the trend was opposite with PEU-based systems undergoing faster gelation than P407-based ones. This behaviour is ascribable to the capability of PEU aqueous solutions at these concentrations to undergo temperature-driven gelation and quick viscosity increase differently from P407-based ones (CGC of P407-based aqueous solutions has been measured to be around 18% w/v[45]). As a matter of fact, at 25 °C, i.e. at a temperature significantly lower that the lower critical gelation temperature of PEU-based aqueous solutions with 7% and 9% w/v concentration,[45] irrespective of synthetic polymer concentration within the formulation, P407-containing samples exhibited significantly faster gelation compared to PEU-based ones. In addition, with regard to P407-based SM hydrogels, relatively high concentrations of P407 (> 10% w/v) turned out to be required to achieve stable hydrogel networks[33,38,56] having good mechanical properties and avoiding crystal precipitation under mechanical stimulus. Differently, phase-separation phenomena were quite rare in PEUU-based systems. Only after different days (within 5 days) of incubation at 37 °C, samples with PEU concentration of 5%, 7% and 9% w/v and CD concentration at 7% w/v exhibited precipitation of a crystalline phase. Differently, PEU 1% - CD 8% formulations did not show sol-to-gel transition at neither 25 nor 37 °C and underwent phase separation at both the investigated incubation temperatures. Beyond these few exceptions, the majority of the samples that underwent a complete gelation did not show phase separation phenomena at any temperature (25 or 37 °C) up to 1 month incubation time. This indicated that the formed SM gels were physically stable in the long term.

Hereafter, in order to make gel suitable for preparation in different laboratories and practical scenarios, SM systems were prepared by incubating PEU/CD mixtures at 25 °C.

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Table 1: *gelation time of the studied bioartificial formulations, differing in the concentration of synthetic polymer (CHP407, NHP407 and P407 as control) (1, 3, 5, 7 and 9% w/v) and CDs (7, 8, 9 and 10% w/v). ON: overnight gelation; x: non gelling systems; (*): phase separation within 5 days.*

T = 25 °C														
CHP407	**CD 7%**	**CD 8%**	**CD 9%**	**CD 10%**	**NHP407**	**CD 7%**	**CD 8%**	**CD 9%**	**CD 10%**	**P407**	**CD 7%**	**CD 8%**	**CD 9%**	**CD 10%**
1%	x	x (*)	24h	ON	**1%**	x	x (*)	24h	ON	**1%**	360'	360'	360'	360'
3%	x	4d	ON	4h30'	**3%**	x	4d	ON	4h30'	**3%**	110'	90'	65'	45'
5%	x	4d	ON	4h	**5%**	x	4d	ON	4h10'	**5%**	90'	60'	45'	45'
7%	x	4d	ON	3h40'	**7%**	x	4d	ON	3h50'	**7%**	90'	55'	45' (*)	40' (*)
9%	x	4d	ON	2h30'	**9%**	x	4d	ON	3h	**9%**	75' (*)	50' (*)	45' (*)	40' (*)
T = 37 °C														
CHP407	**CD 7%**	**CD 8%**	**CD 9%**	**CD 10%**	**NHP407**	**CD 7%**	**CD 8%**	**CD 9%**	**CD 10%**	**P407**	**CD 7%**	**CD 8%**	**CD 9%**	**CD 10%**
1%	x	x (*)	4d	ON	**1%**	x	x (*)	ON	ON	**1%**	ON	ON	ON	ON
3%	x	4d	4d	ON	**3%**	x	4d	ON	ON	**3%**	ON	ON	70'	70'
5%	x (*)	4d	4d	ON	**5%**	x (*)	4d	ON	ON	**5%**	ON	ON	60'	45'
7%	33' (*)	12'	12'	10'	**7%**	20' (*)	22'	33'	12'	**7%**	ON	160'	50'	25'
9%	12' (*)	7'	7'	6'	**9%**	16' (*)	16'	12'	7'	**9%**	ON	160'	45'	22'

3.3.2 Rheological characterization of SM gels

Based on tube inverting test results, rheological characterization was performed on SM hydrogels with PEU concentration of 1%, 5%, 7% and 9% w/v, meanwhile CD concentrations of 9% and 10% w/v were selected for the relatively fast gelation observed in samples with these CD contents. Strain sweep tests were performed to characterize gels in terms of resistance to applied deformation and identify their region of viscoelastic linearity (LVE). Within the LVE region, all samples showed constant elastic and viscous moduli (G' and G'', respectively), with G' greater than G", thus proving that all samples were in the gel phase at the testing temperature (i.e., 37 °C). Upon achievement of a critical value of strain (γ_L), G' started to decrease, suggesting the appearance of cracks within the gel network finally leading to sample rupture, with G'' becoming higher than G', as typical of fluid systems. In general, better mechanical properties (i.e., higher G' values within the LVE, G'_{LVE}, Table 2) were observed for samples with PEU concentration ranging between 1% and 5% w/v and CDs at 10% w/v concentration with respect to those prepared starting from PEU/CD mixtures with CD at 9% w/v concentration. This is probably due to the achievement of better assembled supramolecular networks based on crystallized PPRs at low PEU concentration and high CD content, which favoured the interactions between PEU chains and CD molecules, as previously suggested. By increasing PEU concentration within the formulations, no remarkable differences were observed between samples containing CDs at 9% or 10% w/v concentration. This behaviour could be correlated to the predominant contribution of temperature-driven micellization phenomena over PPR formation and assembly in the overall gel network of these formulations, in agreement with our previous findings from tube inverting tests. As a matter of fact, the trend of G' evaluated within the LVE as a function of PEU concentration within the systems at a fixed CD concentration was characterized by a pyramidal shape suggesting that the effects of PPR crystals and micelle-based structures were reciprocal: the first being predominant at lower PEU concentration (1% and 5% w/v), and the latter becoming prevalent at higher PEU concentrations (7% and 9% w/v). γ_L values of the characterized formulations increased with increasing PEU content within the hydrogels (e.g., for NHP407-based systems at 10% w/v CD concentration γ_L increased from 0.4% to 4.5% with increasing PEU concentration from 1% to 9% w/v), suggesting that increasing PEU content within the systems resulted in an enhanced capability of the gels to withstand applied deformations. No significant differences between NHP407- and CHP407-based formulations were observed in terms of resistance to applied strain (e.g., NHP407 5% - CD 9% and CHP407 5% - CD 9% exhibited γ_L of 0.7 and 0.4%, respectively). Similarly, no clear dependence of γ_L over CD content within the samples was assessed (e.g., CHP407 5% - CD 9% and CHP407 5% - CD 10% showed γ_L of 0.4 and 0.3%, respectively). Strain sweep tests were also exploited to evaluate a potential self-healing behaviour of the gels by repeating the analysis on the same sample after 15 minutes of quiescence at 37 °C. Interestingly, for all the tested formulations a recovery greater than 80% of the starting storage modulus was observed, with no differences between CHP407- and NHP407-based samples (Table 2). As an example, G' and G'' trends as a function of applied deformation for CHP407-based systems containing

CDs at 10% w/v are reported in Figure 6, as measured before and after recovery (i.e., quiescence at 37 °C for 15 min.).

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Table 2: Storage and Loss moduli within the LVE region (G'_{LVE} and G''_{LVE}, respectively), and percentage of recovery in G' upon gel rupture followed by self-healing at 37 °C for 15 minutes (G'recovery) of SM hydrogels, as assessed through strain sweep tests at 1 Hz frequency and 37 °C.

	G'_{LVE} (Pa)	G''_{LVE} (Pa)	G' recovery (%)		G'_{LVE} (Pa)	G''_{LVE} (Pa)	G' recovery (%)
NHP407 1% - CD 9%	665	50	90	**CHP407 1% - CD 9%**	1100	70	83
NHP407 1% - CD 10%	2300	150	94	**CHP407 1% - CD 10%**	2690	150	87
NHP407 5% - CD 9%	6840	1110	86	**CHP407 5% - CD 9%**	8670	1120	87
NHP407 5% - CD 10%	14320	1880	81	**CHP407 5% - CD 10%**	17580	3230	92
NHP407 7% - CD 9%	5520	1510	91	**CHP407 7% - CD 9%**	7700	2790	96
NHP407 7% - CD 10%	5170	1530	96	**CHP407 7% - CD 10%**	18750	3910	89
NHP407 9% - CD 9%	4400	2650	88	**CHP407 9% - CD 9%**	3000	1570	93
NHP407 9% - CD 10%	4890	2770	94	**CHP407 9% - CD 10%**	4400	2160	93

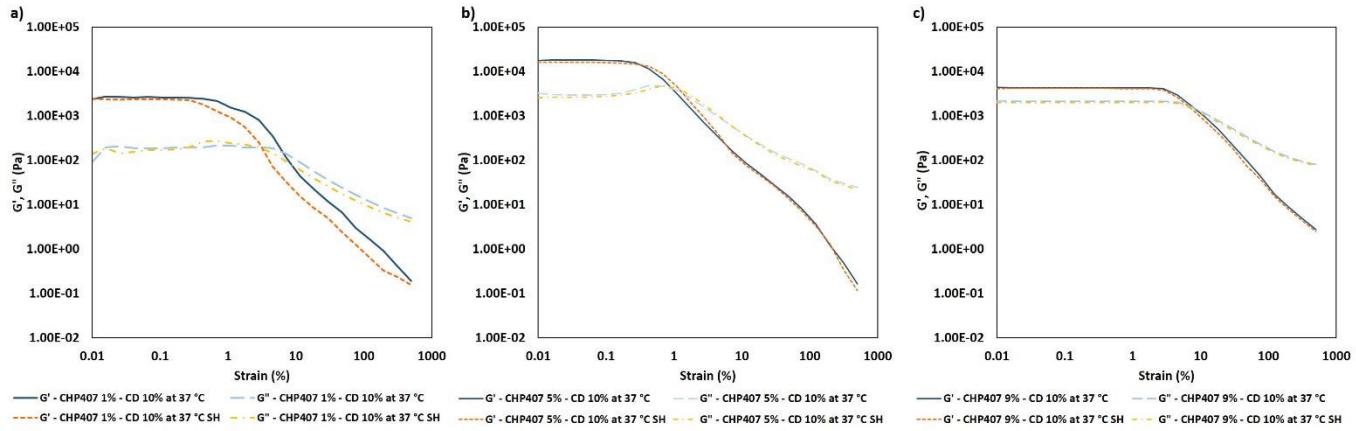

Figure 6: G' and G'' trends as a function of applied deformation for a) CHP407 1% - CD 10%, b) CHP407 5% - CD 10% and c) CHP407 9% - CD 10% measured before (blue continuous line and light blue long dashed line for G' and G'', respectively) and after recovery (i.e., quiescence at 37 °C for 15 min) (orange short dashed line and yellow dash-dotted line for G' and G'', respectively).

The complete gelation process of SM gels was also confirmed by frequency sweep tests. As an example, Figure 7 reports the entire characterization of CHP407-based hydrogels containing CDs at 10% w/v (i.e., CHP407 1% - CD 10%, CHP407 5% - CD 10% and CHP407 9% - CD 10%). All the formulations at PEU concentrations lower than 7% w/v were characterized by an elastic modulus (G') greater than loss modulus (G") within the whole analyzed frequency range, thus indicating that the systems were in a gel state at all the tested temperatures. Although a slight dependency of the mechanical properties of these hydrogels over frequency was observed, a more developed gel state was observed for formulations with lower PEU content, suggesting that the predominance of PPR crystal formation and assembly in these systems accounted for the achievement of more organized gel networks. In accordance with this observation, at 9% w/v PEU concentration, where the thermo-sensitive component played an important role in terms of gelation and mechanical properties, as previously stated, a strong dependence of both G' and G'' over frequency was observed and the systems behaved in a similar way as purely PEU-based thermosensitive sol-gel systems.[45] Indeed, cross-over points between G' and G" were observed at both 25 and 30 °C, highlighting a biphasic nature of the systems, with sol and gel phases coexisting. At 37 °C these formulations appeared to be in the gel phase, but both G' and G'' retained a strong dependence over frequency. Nevertheless, despite the similarity with pure PEU-based systems, an important contribution of SM structures in the definition of the overall mechanical properties of the gels can be identified. Indeed, in the present work formulations containing PEU at 9% w/v concentration exhibited a G' value at 100 rad/s of approx. 10^5 Pa, whereas this value was achieved in purely thermo-sensitive sol-gel systems by significantly increasing polymer concentration up to 15% w/v.[45,47] According with strain sweep test results, increasing CD content within the systems induced an increase in G' values at all the tested temperatures (e.g., at 37 °C, CHP407 1% - CD 9% and CHP407 1% - CD 10% exhibited G' values at 100 rad/s of 1290 and 3970 Pa, respectively). Similarly, at low PEU contents, CHP407-based systems exhibited better mechanical properties (i.e., higher G' values) with respect to NHP407-based ones, resulting from the previously hypothesized improved interactions occurring between CHP407

chains and CDs. Despite the relevant contribution of SM crosslinks in determining gel mechanical properties and network development, all the tested formulations were characterized by a thermo-responsive behaviour. Indeed, all SM hydrogels increased their moduli when the temperature was increased from 25 to 37 °C (e.g., G' at 100 rad/s of CHP407 1% - CD 9% increased from 985 to 1290 Pa with increasing temperature from 25 to 37 °C). This behaviour is ascribable to the thermo-sensitivity of PEUs, which in their free domains still formed hydrophobic interactions with increasing temperature.

To better clarify this aspect, Table 3 reports the storage modulus value measured at 100 rad/s for all the investigated formulations at 25, 30 and 37 °C. In accordance with strain sweep test results, irrespective of CD content, intermediate PEU concentrations (i.e., 5% and 7% w/v) represented the best conditions in terms of gel mechanical properties, probably as a consequence of the synergistic and balanced effects of the physical crosslinks given by crystallized PPRs and the hydrophobic interactions of free PEU domains.

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Table 3: Storage modulus value measured at 100 rad /s through frequency sweep tests carried out at 25, 30 and 37 °C on NHP407- and CHP407- based SM hydrogels.

	G' (Pa) at 100 rad/s - 25 °C	G' (Pa) at 100 rad/s - 30 °C	G' (Pa) at 100 rad/s - 37 °C		G' (Pa) at 100 rad/s - 25 °C	G' (Pa) at 100 rad/s - 30 °C	G' (Pa) at 100 rad/s - 37 °C
NHP407 1% - CD 9%	760	900	1150	**CHP407 1% - CD 9%**	985	985	1290
NHP407 1% - CD 10%	2080	2740	3260	**CHP407 1% - CD 10%**	1920	3430	3970
NHP407 5% - CD 9%	10240	12500	12500	**CHP407 5% - CD 9%**	8080	11590	11980
NHP407 5% - CD 10%	15230	18000	18000	**CHP407 5% - CD 10%**	14500	25260	26260
NHP407 7% - CD 9%	6080	11500	12370	**CHP407 7% - CD 9%**	11850	19940	21040
NHP407 7% - CD 10%	6900	11870	12620	**CHP407 7% - CD 10%**	18440	26300	25770
NHP407 9% - CD 9%	5040	11600	13820	**CHP407 9% - CD 9%**	1890	5970	7410
NHP407 9% - CD 10%	3110	8930	11100	**CHP407 9% - CD 10%**	2860	8900	11400

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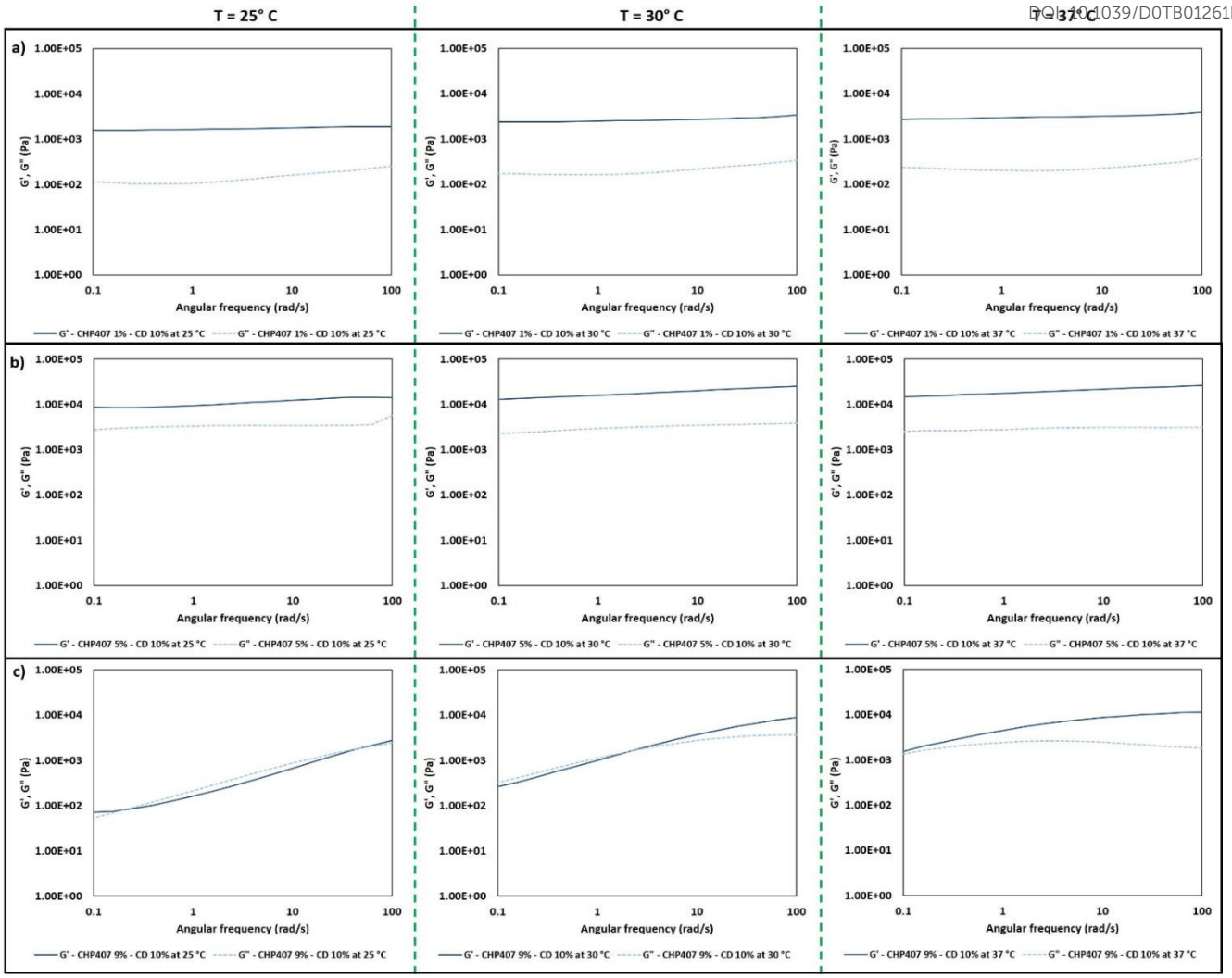

Figure 7: G' (blue continuous line) and G'' (light blue dashed line) trends as a function of angular frequency at 25, 30 and 37 °C for a) CHP407 1% - CD 10%, b) CHP407 5% - CD 10% and c) CHP407 9% - CD 10%.

Self-healing evaluation conducted through strain tests on SM gels highlighted the remarkable recovery capability of the starting mechanical properties of the here-developed systems. Indeed, at least the 80% of G' was recovered at every strain cycle within 30 seconds of restoration (at 0.1%) after network breakage through application of 100% strain. Table 4 summarizes the percentage of G' recovery after 3 rupture cycles for all the formulations, meanwhile Figure 8 reports the trends of G' and G'' as a function of time measured during strain tests of SM hydrogels based on CHP407 and CDs at 10% w/v concentration, as representative of the typical registered behaviour. Interestingly, formulations based on PEU at 9% w/v concentration showed a hardening effect during the recovery phase of each rupture cycle, which could be correlated to a progressive rearrangement of PEU-based micellar domains in response to applied deformation, in agreement with reports on purely thermo-sensitive PEU-based sol-gel systems.[45,47]

Table 4: Percentage of storage modulus recovery ($G'_{recovery}$) at the third recovery phase with respect to the starting G' value (i.e., G' value at 600 seconds).

	$G'_{recovery}$ (%)		$G'_{recovery}$ (%)
NHP407 1% - CD 9%	91	**CHP407 1% - CD 9%**	98
NHP407 1% - CD 10%	91	**CHP407 1% - CD 10%**	97
NHP407 5% - CD 9%	88	**CHP407 5% - CD 9%**	80
NHP407 5% - CD 10%	89	**CHP407 5% - CD 10%**	82
NHP407 9% - CD 9%	96	**CHP407 9% - CD 9%**	100
NHP407 9% - CD 10%	86	**CHP407 9% - CD 10%**	100

Based on the thermal and mechanical characterization of the here-developed SM gels, three main classes of PEU/CD-based SM gels could be distinguished, namely low, high and medium PEU containing systems, in which the behaviour is predominantly determined by SM complexes, PEU thermo-

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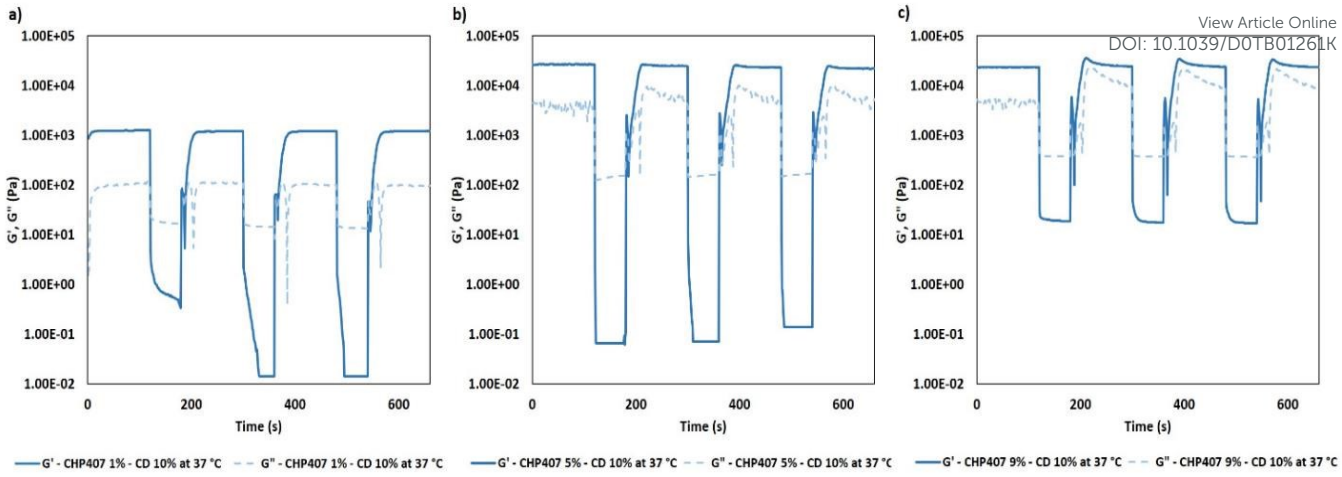

Figure 8: Strain test curves (i.e., trends of G' G' (blue continuous line) and G" (light blue long dashed line) as a function of time) representing the self-healing behaviour at 37° C of a) CHP407 1% - CD 10%, b) CHP407 5% - CD 10%, c) CHP407 9% - CD 10%.

sensitivity or both of them, respectively. Based on these considerations, 1, 5 and 9% w/v PEU concentrated formulations were selected as representative of low, medium and high PEU-containing SM gels and further characterized in terms of swelling and dissolution behaviour in aqueous medium.

3.3.3 Swelling and stability in aqueous medium

The evaluation of swelling and dissolution potential of hydrogel systems is fundamental when a device is designed for drug/gene delivery applications. In fact, the characterization of the dynamic exchange occurring between the gels and the external environment is a key point to define the potential release kinetics of an encapsulated payload in an aqueous milieu. To this aim, swelling and dissolution behaviour of the here-developed PEU-based SM gels were evaluated in physiological-like conditions, i.e., in phosphate buffered saline at pH 7.4 and 37 °C. SM gels generally showed good stability in the testing conditions, with composition-dependent swelling and dissolution trends. As shown in Figure 9, even the hydrogels at the lowest PEU concentration (1% w/v) maintained their shape and wet weight up to 5 days of incubation, meanwhile they were completely dissolved at the 7 days step. This behaviour could be explained by observing the hydrogel dissolution trend: although the wet weight was not characterized by significant variations (maximum -8%), the dry weight of the hydrogels showed an increasing weight loss (>50%) over time. Hence, an important release of mostly non-crystalline and more unstable components (i.e., free CDs and PEU molecules and/or aggregates) took place (as highlighted by dissolution data) during gel incubation in aqueous media, which was concomitant with a relevant fluid absorption from the external PBS aqueous solution. A similar behaviour was observed also for formulations containing PEU at 5% w/v concentration, but an overall lower stability in aqueous environment was assessed. Indeed, systems with this composition were characterized by a significantly higher weight loss in wet conditions (de-swelling behaviour suggesting an evident prevalence of dissolution/erosion phenomena over swelling) compared to gels at 1% w/v PEU concentration. This suggested that samples with lower PEU/CD ratios could probably form more stable domains due to a higher SM self-assembly yield. Formulations at 9% w/v PEU content exhibited a behaviour more similar to that typical of purely thermo-

sensitive gels,[45,47] in accordance with our previous considerations. In terms of stability in aqueous environment, these formulations showed the longest residence time, with approx. 50% of their dry weight lost after 7 days incubation in PBS. However, considering differences among the systems in terms of overall concentration, in the short term (i.e., at 24 and 72 h incubation time) the dry weight lost by 9% w/v PEU concentrated samples was approximately the same as that of systems with PEU at 1 or 5% w/v concentration, thus suggesting a higher dissolution rate for systems containing PEU at higher concentration. While a PEU-concentration dependence was observed in terms of dissolution trend, the behaviour of the systems in aqueous media did not exhibit a clear composition-related trend. Indeed, formulations at 1 and 9% w/v PEU concentration exhibited higher stability in the wet state which could be ascribed to their predominant PPR or micellar hydrogel nature. Differently, 5% w/v PEU concentrated systems showed more pronounced de-swelling which can be correlated to their PEU/CD ratio that did not allow a clear predominance of PPR structures over micellar ones or vice versa. Increasing CD content for 9 to 10% w/v induced a further stabilization of the systems as a consequence of the achievement of better assembled supramolecular networks, with more evident effects of 1 and 5% w/v PEU concentrated formulations.

3.3.4 Cytotoxicity tests

Cytotoxicity tests indicated that CD content within the systems did not play a significant role in determining the biocompatibility of the developed formulations. Differently, a PEU-concentration dependent cytotoxicity was observed, with hydrogel systems based on both PEUs and characterized by concentrations ranging between 1 % and 5 % w/v being non-cytotoxic (around 100% viability). Concerning systems with PEU concentration of 9% w/v, a slight cytotoxicity was observed (55-60% cell viability for both NHP407 9% - CD 9% and CHP407 9% - CD 9%). This behaviour could be explained considering the nature of the supramolecular network characterizing these hydrogels, which was not completely developed owing to an unbalanced PEU/CD mass ratio. Indeed, as we previously commented, SM hydrogels containing PEU at 9% w/v concentration were composed of a predominant thermo-responsive network, which allowed a low amount of CDs to form PPR-based crystals. This aspect could be an incisive variable in terms of the dynamic response of these systems in conditions highly enhancing mass exchange with the external

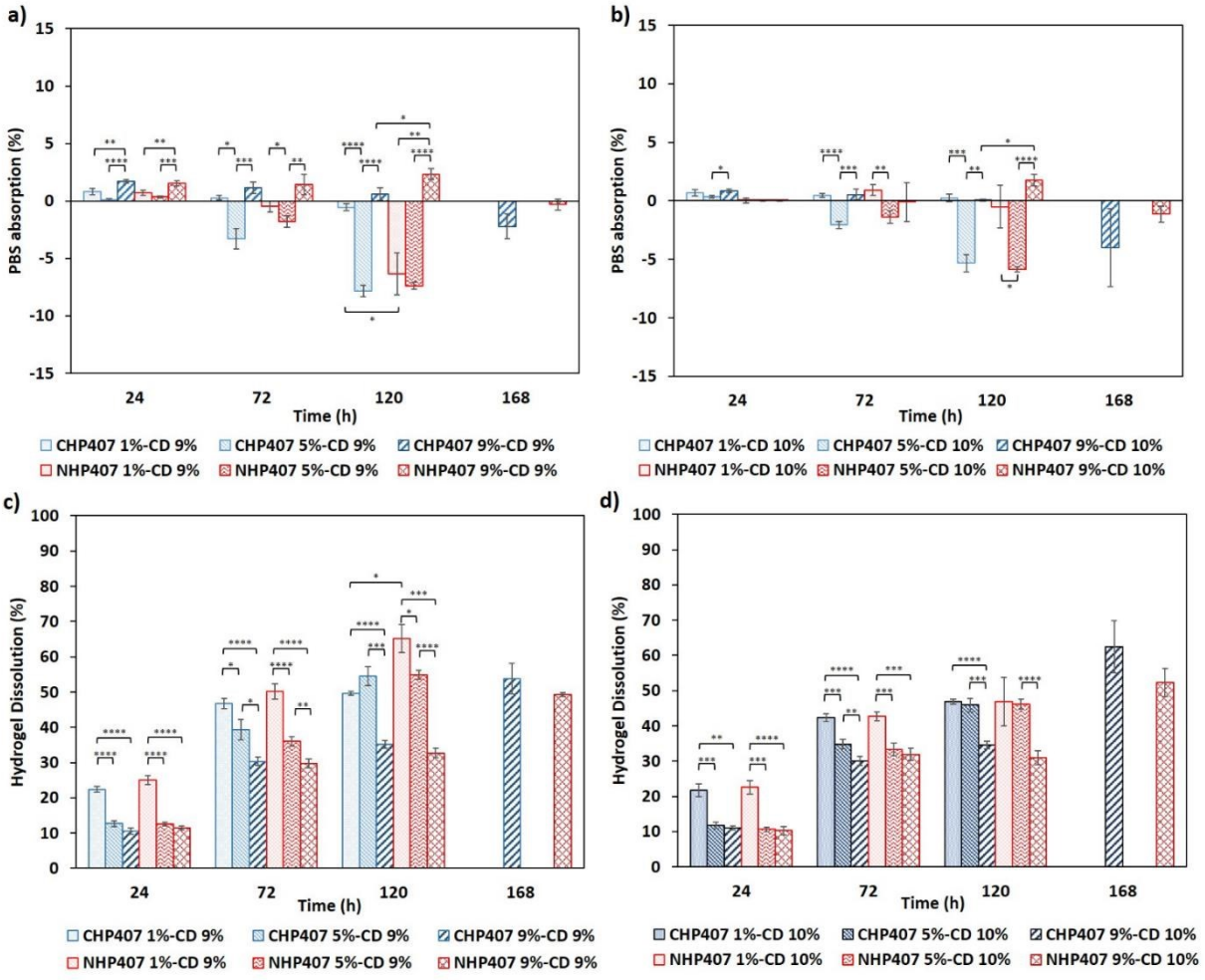

Figure 9: Bar diagrams of the percentage of swelling (a and b for systems with CD at 9 and 10% w/v concentration, respectively) and dissolution (c and d for systems with CD at 9 and 10% w/v concentration, respectively) of CHP407- (blue) and NHP407- (red) based SM gels.

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environment, as those recommended by the ISO10993 guidelines. Hence, the slight cytotoxicity of these formulations could be correlated with their relatively high dissolution rate (the total amount of dissolved mass was higher with respect to samples at 1% w/v PEU content) and their non-fully developed gel state (G' strongly depended over angular frequency at 37 °C). All together these data suggested that the optimal approach to exploit PEU chemistry potential in the design of SM gels relies in keeping PEU/CD weight ratio unbalanced, minimizing PEU content which favours the development of SM structures based on PPRs over the self-assembly of PEU chains into a micellar network. Hence, the presence of CDs into PEU solutions at 9% w/v concentration did not sufficiently stabilize the polymeric network, because interactions among micelles were probably predominant. However, it has been demonstrated that purely thermosensitive PEU-based formulations with higher polymer concentration compared to the here-characterized systems (e.g., 15% w/v) did not show any cytotoxic effect.[45] Indeed, their remarkable thermo-sensitive behaviour due to their higher polymeric content led to gel systems with prolonged stability in aqueous environment and low dissolution rates.

Based on these considerations, formulations containing PEU at 9% w/v concentration were no longer characterized in this work. Similarly, concerning CD content within the systems, SM hydrogels with 10% w/v concentrated CD were selected for further investigations because of their faster gelation kinetics, better mechanical properties and higher stability. Moreover, a higher CD content opens to the potential possibility to design systems able to encapsulate a high amount of drugs, with no obstacles for SM network formation.

3.3.5 Study of the release profile of FD4 model molecule from SM gels

FD4 was selected as model molecule for payload release studies since it represents an intermediate between small drugs (e.g., penicillins, anti-inflammatory drugs) and big bio-macromolecules (e.g., peptide sequences and proteins). All the characterized systems showed the capability to release the 100% of the loaded FD4 within 3-4 days (Figure 10). The process of FD4 release from the gels was concomitant with their progressive dissolution, which was accelerated under release testing conditions as a consequence of the many complete medium refreshes that were performed. Moreover, the presence of FD4 could affect the formation of the network and then the general behaviour of the gels in terms of stability and dissolution kinetics. In addition, despite its high water solubility (50 mg/ml), FD4 was progressively released from SM gels with no burst release behaviour (approx. 20% of the payload was released with 6 hours). No significant differences in FD4 release profile were observed between NHP407- and CHP407-based systems. Samples at 1% w/v PEU concentration exhibited a faster FD4 release within the first 6 hours of observation compared to those containing PEU at 5% w/v concentration. Then the profiles tended to overlap and samples at 5% w/v PEU concentration sustained FD4 release longer, probably due to a more stable SM hydrogel network. The performed tests showed release kinetics and stability behaviour comparable to those reported in literature for similar systems based on relatively high polymeric content (>10% w/v).[31,33,38,57,58] Differently, the here-synthesized PEUs showed the possibility to develop stable SM networks based on low synthetic polymer content (i.e. 1% w/v), without affecting final properties negatively.

4. Conclusions

The need for smart drug delivery systems based on physical networks is a concrete and challenging demand in the biomedical field. Physical hydrogels can satisfy many requirements for tissue engineering and drug delivery applications, such as tuneability, feasibility and dynamic behaviour. The design of engineered supramolecular systems mimicking the spontaneous self-assembly that occurs among biopolymers (e.g., proteins) represents a credible strategy to obtain highly functional and promising devices for these purposes. In this work, we demonstrated that custom-made amphiphilic PEUs are suitable macromolecules to develop SM gelling systems based on CDs. In detail, a library of PEU-based SM hydrogels was designed and thoroughly characterized, highlighting their quick gelation, stability (i.e., absence of phase separation), remarkable mechanical properties and self-healing behaviour. The dynamic response of SM gels in aqueous environments was also assessed, demonstrating that even low PEU concentrations (1 and 5% w/v) were able to guarantee a

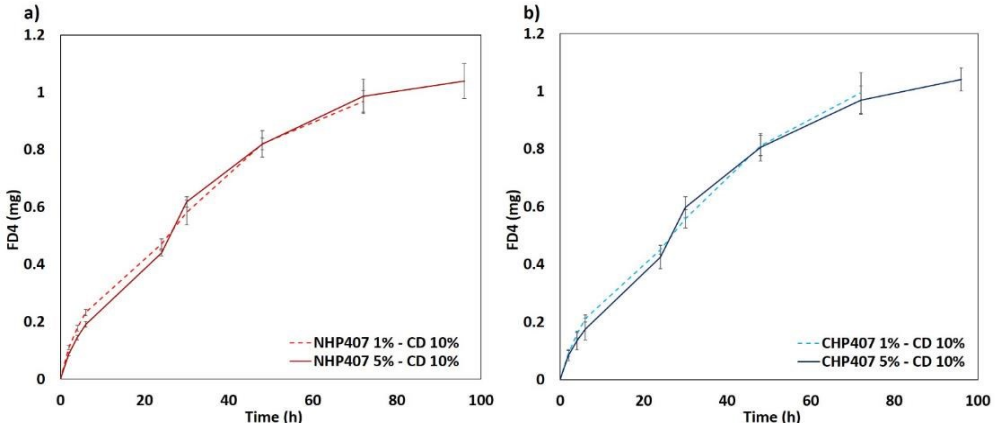

Figure 10: FD4 release profiles from SM hydrogels based on NHP407 (a, red) and CHP407 (b, blue) at 1 and 5% w/v concentration (dashed and continuous lines, respectively) (CD concentration fixed at 10% w/v).

continuous mass exchange with the external milieu and residence time in aqueous environment ranging between 5 and 7 days. The effect of PEU and CD content within the systems on the overall gel properties was investigated. In particular, a fine modulation of PEU concentration turned out to allow a proper balance between PPR and micelle contribution to the sol-to-gel transition. On the other hand, higher CD concentration provided gels with better mechanical properties and enhanced stability. Finally, focusing on the most promising hydrogel formulations (i.e., CD concentration at 10% w/v, PEU concentration of 1 and 5% w/v), the release kinetics of FD4 as model molecule was characterized showing a progressive and sustained delivery up to 4 days without any burst release phenomenon. Moreover, the presence of a potential amount of free CDs and PEU-based micelles make these injectable hydrogels particularly promising to release drugs encapsulated inside their domains. Indeed, drugs such as anti-inflammatory molecules (e.g., ibuprofen, curcumin) or antibiotics (e.g., ciprofloxacin) could be encapsulated into both the inner cavity of CDs and PEU-based micelles, thus permitting the design of dual-releasing drug carriers. The released structures based on CDs and PEU micelles or free chains could be thus exploited to deliver therapeutic agents to cells, relying on their capability to overcome many biological barriers and increase drug bioavailability in real physio-pathological environments.[59-62] In addition, their physico-mechanical properties make them particularly adaptable to tissue morphologies and deformations allowing a local release of therapeutic agents in well-defined target regions (e.g., antitumor treatment through intratumoral injection).

Conflicts of interest

There are no conflicts to declare

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Table of Content

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Supramolecular hydrogels based on custom-made poly(ether urethane)s and cyclodextrins as potential drug delivery vehicles: design and characterization

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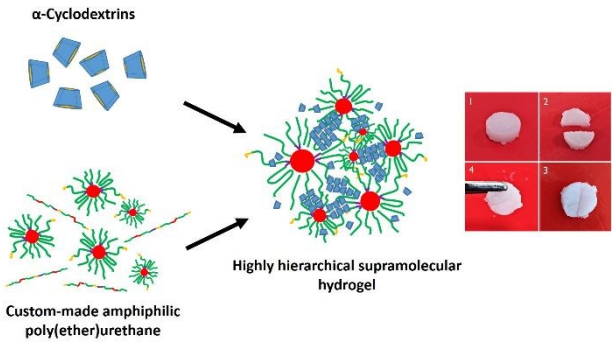

A library of poly(ether urethane)-based supramolecular hydrogels was designed, showing quick gelation, no phase separation, remarkable mechanical and self-healing properties.