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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

One-pot Tough Hydrogels for Injection and 3D Printing

Arthur Helsen
Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Bioelectronics and Nanotechnology

SUPERVISOR :
Prof. dr. Louis PITET

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One-pot Tough Hydrogels for Injection and 3D Printing

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ABSTRACT

Double-network (DN) hydrogels are considered viable systems for synthetic tissue development due to their excellent mechanical properties and similarities to native tissue such as cartilage. Generally involving tedious synthesis routes, the injection thereof is considered challenging. Employing the highly selective and robust thiol-yne chemistry, a one-pot method can be developed. Through combination of a thiol-yne crosslinked poly(ethylene) oxide (PEO) network with an alginate network, a processable, tough DN hydrogel can be obtained *via* a one-pot reaction. PEO was end-functionalized *via* esterification (conversion > 99 %) and crosslinked *via* thiol-yne chemistry to yield single-network (SN) hydrogels. End-group stochiometric ratios, polymer weight percent and PEO molar mass were systematically varied. SN hydrogels exhibited 670 kPa compressive strength at 85 % strain. Deviating from the stoichiometric balance of end-groups consistently led to decreased mechanical performance. Alginate was combined with the modified PEO in a one-pot reaction for the final DN hydrogel formulations. DN hydrogels were observed to have a compressive strength of 198 kPa at 73 % strain. Injection of both SN and DN hydrogel led to film formation. These findings are considered a promising step toward the translation of this system to 3D printing, and its potential application in the biomedical field.

INTRODUCTION

Soft tissue, e.g. cartilage, tendons, ligaments, and muscle, are prevalent throughout the human body, acting as load-bearing tissue. Since native tissue is prone to injury and disease, the development of artificial soft tissue is one of great importance. Osteoarthritis is known to be a leading cause of disability in the global population, instigated by injured cartilage and bone (1). Currently, the majority of treatments are ineffective due to its complexity and multimorbidity, and require a more tailored approach (1, 2). Being a disease involving the whole joint, researchers are attempting to develop artificial materials that can be used as a native tissue mimic containing adequate mechanical strength and tissue supporting functions (3). However, despite significant progress being made throughout the years, some gaps in the development of artificial tissue remain present to date. For example, articular cartilage experiences a daily continuous mechanical load of ~10 megapascals (MPa) in its native environment (4). Hence, materials used in artificial cartilage development should have mechanical properties fulfilling the requirements of native tissue. Moreover, these materials should also demonstrate adequate biocompatibility and processability to ensure translation to an *in vivo* environment. Thus, it is important to have a material capable of balancing i) the required rheological properties, ii) adequate supporting biological properties and iii) processability (5).

An example of a material capable of fulfilling these goals is the hydrogel. First described in 1960 by Wichterle and Lim, hydrogels are defined as three-dimensional crosslinked polymer networks that can absorb a substantial amount of water (6, 7). Due to their innate porosity, soft consistency and tendency to absorb water, hydrogels are valuable assets in the development of state-of-art biological and biomedical applications (8). Currently, hydrogels are being used in a variety of applications ranging from contact lenses to drug delivery devices, wound dressings, hygiene products etc. (6,

8). Hydrogels can also be utilized as an effective extracellular matrix (ECM) mimic, showing increased cell proliferation, differentiation and good cell viability (9, 10). Hydrogels have also been exploited in additive manufacturing techniques such as 3D printing, showing good processability and subsequent development of complex shapes and structures (11). These features, along with excellent mechanical properties, make hydrogels a relevant and promising candidate for bridging the current gaps and the development of synthetic tissue. In this work, the development of tough, processable hydrogels and their relevance in tissue engineering were investigated.

Hydrogels can be described based on their polymeric network structure(s). Single-network (SN) hydrogels consist of a single hydrophilic polymeric network, and have been developed as model systems for medicine, photochemistry, tissue engineering etc. (12) Generally, these hydrogels exhibit a soft, ductile and brittle structure (13). These characteristics are owed to the SN's heterogeneity and low polymer chain density, causing the hydrogel to fracture due to accumulation of stress at the shortest chain (13). These properties limit their use in applications where mechanical properties are of high concern, such as substitutes for soft tissue (e.g. cartilage). Hence, researchers focused on the development of novel, tough hydrogel systems capable of overcoming these shortcomings in existing SN hydrogels. Here, double-network (DN) hydrogels have proven to be a valuable asset in the development of tough synthetic tissue constructs.

Since their first description in 2003 by Gong and co-workers, DN hydrogels have proven to be a major contributor in biomimetic material development (4, 14). The researchers were able to synthesize a tough, double-network hydrogel system (PAMPS-PAAm), consisting of poly(2-acrylamido-2-methylpropanesulfonic acid) (PAMPS) and polyacrylamide (PAAm) as a first- and second polymeric network respectively, exhibiting extremely high mechanical strength (14). Gong et al. reported that an optimized double-network structure was able to withstand a compressive force greater than several tens of MPa. For reference, articular cartilage experiences a normal load of ~10 MPa in its native environment (15). When compared to SN hydrogels based on PAAm, the PAMPS-PAAm DN hydrogel outperformed the SN hydrogel in virtually every measurement, e.g. showing a fracture stress 21 times higher compared to the SN hydrogel. Given that a mechanically tough hydrogel is required for artificial cartilage development, DN hydrogels thus are considered a go-to material.

The drastic enhancement in mechanical strength seen in DN hydrogels is largely accountable to two factors: i) the combination of two independent networks containing different structures & crosslinking densities, and ii) the ratio of these two networks (14). In this work, the DN structure will be limited to a physically- and chemically-crosslinked network. Here, the first physically crosslinked network is considered a tightly crosslinked and brittle network. Conversely, the second network is soft and ductile, due to the low crosslinking density of the covalent network. The second network is capable of interpenetrating and filling the voids of the first network, thereby creating a more homogeneous double-network system (4). The hydrogel obtains its mechanical properties due to the synergistic effect between the two networks. As described by Sun et al., the brittle alginate network dissipates the energy due to breaking of the ionic-crosslinks when stress is applied to the hydrogel (16). Increasing stress causes further unzipping of the alginate chains, while after a period of time the ionic crosslinks can reform (16). This induces healing of the internal damage. On the other hand, the PAAm network remains intact and allows the hydrogel to recover to a certain extent (16). As an efficient energy dissipation mechanism is of great interest for the development of tough materials, the use of a DN formulation containing physical- and chemical-crosslinking thus brings many benefits.

To this point, we discussed the viability of DN hydrogels as synthetic tissue, due to their excellent mechanical properties. However, another important attribute for the hydrogel to have is good processability. The processability of a hydrogel is largely attributed to its rheological properties, such as shear thinning characteristics, and synthesis procedures (5). In this research, we will mainly focus on the latter. Traditionally, DN hydrogel formulations involve a laborious two-step polymerization method where the first high swelling, highly crosslinked polymeric network is synthesized and immersed into a solution containing the second network's precursor

monomers (12). The monomers will then diffuse into the first network and polymerize to form a second, loosely crosslinked network. (12, 17). Yet, not only is this procedure considered time consuming and tedious, it also limits the use of DN hydrogels for complex structure development *via* additive manufacturing (e.g. 3D printing) (12, 17). Additive manufacturing (AM) involves building a 3D solid structure, usually based on layer-by-layer assembly of material (18). Due to its excellent accuracy, reproducibility and high degree of automation, 3D printing can be utilized to develop tissue constructs with predefined geometries under meticulous control (5, 18). This makes AM a viable research area to combine with the development of synthetic tissue. Although hydrogels have been used increasingly in 3D printing over the last 5 years, the tedious preparation methods (and thus processability) have complicated their translation to develop complex structures for tissue engineering (19). A novel approach involves the development of a one-pot procedure, thereby overcoming the shortcomings seen in synthesis procedures.

Chen et al. have described one-pot mechanisms to develop DN hydrogels, thereby eliminating the uncontrollable swelling and need for diffusion of precursor molecules into the first network seen in conventional preparation methods (20). By transferring all reactants into a single pot, the researchers were able to create agar-PAAm hydrogels with excellent mechanical properties comparable to the chemically crosslinked PAMPS-PAAm hydrogels designed by Gong et al. Chen et al. managed to synthesize a tough PEG/PAA hydrogel *via* this one-pot procedure, reaching mechanical strengths up to 10 MPa (21). Employing this strategy, both networks can be prepared in parallel, thus drastically reducing the synthesis time, providing a relatively straightforward, fast and controllable route for DN hydrogel synthesis (12). However, the possible interference of the multiple crosslinking chemistries applied in DN hydrogel synthesis has to be kept in mind when designing a one-pot procedure. Therefore, it is important to incorporate highly selective and robust crosslinking mechanisms that can be easily controlled and integrated in a one-pot method procedure.

So far, it has become evident that DN hydrogels are a viable candidate as synthetic tissue, but are traditionally developed *via* tedious routes. To overcome this hurdle, a one-pot method has been proposed capable of reducing synthesis time while also allowing the development of complex structures. Metal-free bio-orthogonal reactions, such as the strain-promoted azide-alkyne cycloaddition (SPAAC) and Diels-Alder addition, have long been employed to crosslink polymers and functionalize hydrogels due to their robustness and ability to proceed under physiological conditions (pH 7.4, saline, 37 °C) (22). However, due to the reagents used in SPAAC chemistry being costly and the Diels-Alder addition having slow gelation times, other chemistries were explored for hydrogel development (23, 24). Another chemistry utilized to synthesize hydrogels is called click chemistry, first highlighted by Sharpless et al. in 2001 (25). The researchers described click chemistry as being selective, high yielding, rapid, and able to proceed under mild reaction conditions along with minimal side reactions. Due to these characteristics, it has been widely exploited for synthetic material development and is therefore an interesting candidate to incorporate in a one-pot procedure (26). Specifically, thiol-ene click chemistry, carried out via either the radical or nucleophilic pathway, has received a lot of attention for the development of biomaterials (27). Although the radical pathway is considered suitable for hydrogel synthesis, the presence of radicals and initiators can affect cell viability, thereby limiting its use in tissue engineering (28). On the other hand, the nucleophilic addition of thiols occurs over a double bond without the need of an initiator or radicals, causing bond formation between the thiol and alkene (29).

The thiol-yne click reaction, considered to be a sister reaction of the thiol-ene reaction since it shares many of its characteristics, is a click reaction less exploited. Here, a mono- or bis-hydrothiolation of a terminal alkyne bond can take place. The reaction can be radical-, metal- or main-group mediated, and can be used as a ligation tool for e.g. small molecules, polymerizations and protein modification (26). Having proven to be a versatile and robust reaction mechanism, thiol-yne chemistry can thus be employed to synthesize polymeric networks. Several research groups have reported hydrogel synthesis *via* thiol-yne chemistry, attempting to tackle the same problems described in this work. Macdougall et al. designed robust hydrogel materials utilizing the thiol-yne

click reaction to crosslink alkyne- and thiol-terminated PEO molecules (30, 31). Fan et al. developed injectable SN hydrogels based on a thiol-yne click reaction between alkyne end-functionalized poly(ethylene) oxide (PEO) chains and a thiol end-functionalized PEO crosslinker (32). Interestingly, both research groups used PEO as their polymer to end-functionalize and incorporate in a DN hydrogel.

Being one of the most commonly used commercially available polymers, PEO has become a 'blank slate' for researchers in different scientific fields (19). Furthermore, PEO has been FDA approved as a biocompatible molecule, and its flexibility as a polymer have pushed the boundaries of its use in hydrogel development (33). Its basic structure is capped with flexible pendant hydroxyl groups, enabling end-functionalization through a variety of different chemical reactions, such as the thiol-yne reaction (31, 34). Other chemical reactions have led to the incorporation of maleimides, norborenes, acrylamides, etc. (31, 34-36). Hence, these features make PEO an excellent candidate for hydrogel development in tissue engineering. Since PEO has shown to be a viable material to incorporate in hydrogels *via* thiol-yne chemistry, an interesting approach would be combining the PEO polymeric network containing thiol-yne crosslinks with a second polymeric network, essentially creating a DN system. Here, the PEO network would form the covalently crosslinked, soft and ductile network of the DN system.

As mentioned previously, the combination of a chemically- and physically-crosslinked network can be translated to tough DN hydrogel formulations. Combining a covalently crosslinked PEO network with a physically crosslinked network, such as alginate, it's expected that a processable, tough DN hydrogel can be prepared. Here, alginate has proven to be a viable polymer for use in DN hydrogels, exhibiting a zip-like crosslinking mechanism using divalent cations (Ca^{2+}, Ba^{2+} etc.) (37). The divalent cations can re-form ionic bonds after breaking, thus (partially) recovering the hydrogel's strength (38). Being a natural polysaccharide, mainly obtained from brown algae, alginate has good inherent biocompatibility and versality, being used frequently in DN hydrogel development (11, 39). For example, Ooi et al. were able to develop alginate- and PEO-based hydrogels crosslinked *via* thiol-ene chemistry capable of being 3D printed, showing a wide range of mechanical properties and the incorporation of adhesive peptides (40). The researchers highlighted the modularity of both the material's properties and biofunctionality, further stressing its feasibility to be used as 3D cell scaffolds for tissue engineering.

In summary, DN hydrogels have played a major role in the development of synthetic tissue due to their excellent mechanical properties and similarities to soft tissue such as cartilage. However, the tedious synthetic preparation commonly employed limit their use as injectable materials. It's posed that by using the highly selective thiol-yne chemistry, a one-pot synthesis method can be developed. This one-pot method will prove useful for hydrogel injection and complex structure development. Thus, it's expected that by combining two polymeric networks with selective crosslinking chemistries, PEO and alginate respectively, processable DN hydrogels can be obtained.

In this project, it was investigated if a novel, injectable DN hydrogel can be made *via* a one-pot method. It's hypothesized that a processable DN hydrogel can be prepared from PEO and alginate in a one-step procedure suitable for injection and 3D printing. To test this hypothesis, the first step involved end-functionalizing PEO with propiolic acid, thereby rendering it compatible with thiol-yne click chemistry. Next, PEO was end-functionalized with 3-mercaptopropionic acid yielding a thiol end-functionalized crosslinking molecule, capable of forming a SN hydrogel when combined with PEO-alkynone *via* thiol-yne crosslinking chemistry. Then, the PEO SN was combined with alginate in a one-pot reaction, resulting in DN hydrogel formation. **Figure 1** gives an overview of the steps involved in designing the DN hydrogel system, adapted from Macdougall et al. (41). Both SN- and DN-hydrogels were subject to mechanical testing to observe their mechanical properties. Finally, an attempt was made to inject both SN- and DN hydrogels, thereby yielding processable hydrogel formulations. Combining these materials with selective crosslinking chemistries, its posed that novel DN hydrogel formulations can be developed with potential of being applied in the biomedical field, further expanding the arsenal of synthetic tissue.

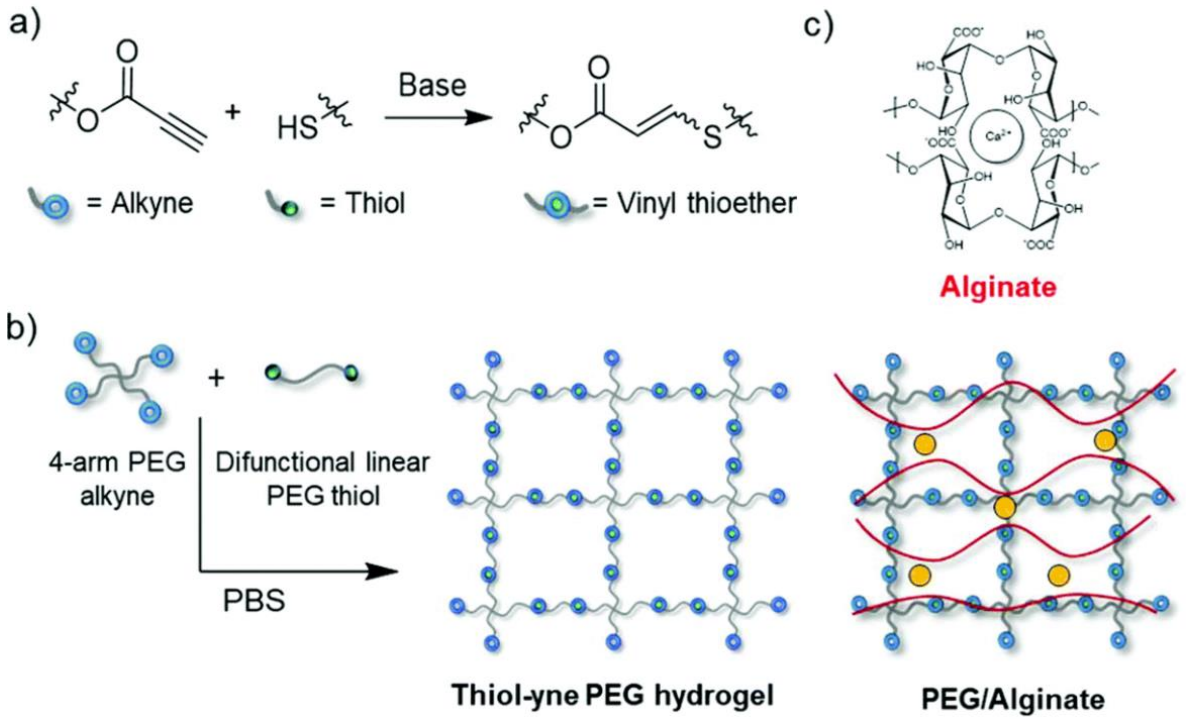

Figure 1: Overview of the proposed system to develop SN- and DN-hydrogel formulations. a) End-functionalization of PEO with propiolic acid and 3-mercaptopropionic acid, respectively; b) PEO-SN hydrogel synthesis by crosslinking of modified PEO; c) Design of a PEO/alginate DN hydrogel system. Image adapted from (41).

EXPERIMENTAL PROCEDURES

Materials – Propiolic acid (Sigma-Aldrich, 95 %) was purified *via* distillation to yield a transparent product before end-functionalization. Chloroform ($CHCl_3$, > 99 %), Dichloromethane (DCM, > 99 %), Diethyl ether ($C_4H_{10}O$, > 99.5 %), Phosphate Buffered Saline (PBS) tablets (0.01 M) and Toluene (C_7H_8, > 99.8 %) were obtained from Fischer Scientific (Belgium). Sulphuric Acid (H_2SO_4, 95 %) Triethylamine (TEA, 99 %), Magnesium sulfate ($MgSO_4$, 99 %), Calcium chloride ($CaCl_2$, 96 %), Sodium bicarbonate ($NaHCO_3$, 99.8 %) were obtained from Arcos Organics (Belgium). Pentaerythritol ethoxylate (15/4 EO/OH), 2,2'-(Ethylenedioxy) diethanethiol ($C_6H_{14}O_2S_2$, 95 %), 3-Mercaptopropionic acid ($C_3H_6O_2S$, > 99 %), pentaerythritol tetrakis(3-mercaptopropionic acid (PETMP), 1,5,7-Triazabicyclo[4.4.0]dec-5-ene (TBD), Sodium alginate and Glycerol ethoxylate (molar mass = 1.0 kg mol^{-1}) were obtained from Sigma-Aldrich (Belgium). 4-arm Poly(ethylene glycol) (pentaerythritol) (molar mass = 2.0 kg mol^{-1}) was obtained from JenKem Technology (U.S.A.). Polyethylene glycol 600, 1000 and 6000 (molar mass = 600, 1000 and 6000 g mol^{-1}, respectively) were obtained from Merck KGaA (Germany). Polyethylene glycol 1500 (molar mass = 1500 g mol^{-1}) was obtained from Alfa Aesar. Dimethyl sulfoxide-d_6 (DMSO-d_6, 99.9 %) was obtained from Eurisotop (France). Chloroform-d_1 ($CDCl_3$, 99.8 %) was obtained from Deutero (Germany).

^{1}H-NMR characterization - ^{1}H-NMR samples were prepared in $CDCl_3$ unless stated otherwise. Measurements were performed at room temperature using a Agilent/Varian 400 MHz Inova spectrometer. The chemical shift scale (δ) in ppm was calibrated relative to TMS (0 ppm). Free induction decays were collected with a 90° pulse of 5.0 µs, a spectral width of 6 kHz, an acquisition time of 4 s, a preparation delay of 12 s and 64 accumulations. A line-broadening factor of 0.2 Hz was applied before Fourier transformation to the frequency domain. Spectra were analyzed in Mestrenova software.

Synthesis of alkynone- and thiol-functionalized PEO – To a solution of linear PEO (M_n = 600 g mol^{-1}, 10 g, 16.6 mmol) in toluene (150 mL), propiolic acid (M_n = 70.05 g mol^{-1}, 4.65 g, 66.4 mmol) was added. After heating the

solution to 80 °C and stirring until clear, 4 drops of H_2SO_4 were added. The solution was heated to reflux under Dean-Stark conditions (125 °C) overnight. The solvent was removed *in vacuo*, and dissolved in 100 mL CH_2Cl_2. After washing with saturated $NaHCO_3$ solution (20 mL) and brine (20 mL), the organic phase was dried ($MgSO_4$) and filtered (Macherey-Nagel, MN 615 ¼, 150 mm). The solvent was evaporated *in vacuo*, leaving a dark-amber colored viscous oil (yield: 68.2 %, conversion > 99 %). Higher molar mass PEO (was precipitated in ice-cold diethyl ether and dried *in vacuo* overnight, yielding a white powder. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 4.27-4.30 (t, -C*H*$_2$OCO-), 3.56-3.69 (m, -OC*H*$_2$C*H*$_2$O-), 3.00 (s, C*H*C≡CCOO-).

2-arm PEO$_{2K}$-SH Synthesis – In a typical esterification reaction, as described above, 2-arm PEO_{2K} (M_n = 2000 g mol^{-1}, 5 g, 2.5 mmol) was esterified with 3-mercaptopropionic acid (M_n = 106.14 g mol^{-1}, 1.061 g, 10 mmol). After washing, the product was precipitated in ice-cold diethyl ether (400 mL) and dried *in vacuo* overnight, yielding a white powder (yield: 72 %, conversion: 50-90 %). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 4.24-4.27 (t, -C*H*$_2$OCO-), 3.59-3.82 (m, -OC*H*$_2$C*H*$_2$O-), 2.73-2.79 (m, -OCCH$_2$C*H*$_2$SH), 2.73-2.79 (m, -OCC*H*$_2$CH$_2$SH), 1.66-1.68 (t, -SH).

3-arm PEO$_{1K}$-SH Synthesis – In a typical esterification reaction, as described above, 3-arm PEO_{1K} (M_n ~ 1000 g mol^{-1}, 5 g, 5 mmol) was esterified with 3-mercaptopropionic acid (M_n =106.14 g mol^{-1}, 2.119 g, 20 mmol) yielding an amber colored viscous liquid (yield: 70.2 %, conversion: >99 %). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 4.22-4.24 (t, -C*H*$_2$OCO-), 3.46-3.71 (m, -OC*H*$_2$C*H*$_2$O-), 2.69-2.75 (m, -OCCH$_2$C*H*$_2$SH), 2.61-2.65 (m, -OCC*H*$_2$CH$_2$SH), 1.62-1.66 (t, -SH).

4-arm PEO$_{2K}$-SH synthesis – In a typical esterification reaction, as described above, 4-arm PEO_{2K} (M_n ~ 2000 g mol^{-1}, 5 g, 2.5 mmol) was esterified with 3-mercaptopropionic acid (M_n =106.14 g mol^{-1}, 1.06 g, 10 mmol) yielding an amber colored viscous liquid (yield: 65 %, conversion: 90 %). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 4.22-4.24 (t, -C*H*$_2$OCO-), 3.50-3.68 (m, -OC*H*$_2$C*H*$_2$O-), 2.71-2.76 (m, -OCCH$_2$C*H*$_2$SH), 2.60-2.66 (m, -OCC*H*$_2$CH$_2$SH), 1.63-1.67 (t, -SH).

Single-Network Hydrogel Synthesis – Polymer content was varied between 10 and 25 w/v%. The stochiometric ratio of alkynone:thiol was varied systematically. Hydrogels were synthesized by dissolving PEO_{600}-alkynone (0.0903 g,1.30 mmol) in 400 µL $PBS_{7.4}$ solution. The 3-arm PEO_{1K}-SH (0.1097 g, 0.9 mmol) was dissolved in 600 µL $PBS_{7.4}$ solution. The two solutions were mixed and transferred into molds for further analysis.

Double-Network Hydrogel Synthesis – DN hydrogel formulations were developed containing 1, 1.5, 2 and 3 w/v% alginate and 19, 18.5, 18 and 17 w/v% PEO respectively, keeping total solid content at 20 w/v%. The stochiometric ratio of alkynone:thiol was kept at 1:1. DN hydrogels were prepared by dissolving PEO_{600}-alkynone (0.0858 g, 120 µmol) in 300 µL $PBS_{7.4}$. 3-arm PEO_{1K}-SH (0.1402 g, 80 µmol) was dissolved in 600 µL $PBS_{7.4}$. 0.1 mL of alginate (10 w/v%) was added to the 3-arm PEO-SH solution and mixed with the linear PEO-alkynone. Solutions were transferred into molds and submerged in a $CaCl_2$ solution (0.1 – 0.5 M). Samples were prepared for mechanical testing.

Uniaxial Compressive Testing - Uniaxial compressive testing was performed on a Shimadzu Autograph AGS-X universal tester containing a load cell of 500 N. Hydrogel samples were circular (diameter: 12 mm, thickness: 5 mm). Samples were left to cure for 24 h after forming to ensure complete crosslinking. The preload force was set at 0.05 N and tests were carried out at a compression velocity of 1 mm/min. Each gel was subject to 85% strain unless stated differently. Data was analyzed using OriginPro 8.5.1 software.

Tensile Testing – Tensile testing was performed on a Shimadzu Autograph AGS-X containing a load cell of 500 N. Hydrogel samples were dog-bone shaped (width: 3 mm, thickness: 3 mm). Samples were left to cure for 24 h after forming to ensure complete crosslinking. The preload force was set at 0.05 N and tests were carried out at a compression velocity of 100 mm/min. Each gel was subject to strain until breaking unless stated otherwise. Data was analyzed using OriginPro 8.5.1 software.

Single- and Double-Network Hydrogel Injection – Hydrogel formulations (20 wt%, 1:1) were prepared as described above. Samples were onto filter paper soaked in 0.1 M TBD solution (+ 0.5 M $CaCl_2$ solution for DN formulations) using a 1 mL syringe.

RESULTS

PEO-Alkynone Synthesis – As previously mentioned, the first step of this project was to end-functionalize PEO with propiolic acid, yielding PEO-alkynone. This polymer was used later on to crosslink with PEO-SH *via* thiol-yne chemistry, thereby forming SN hydrogels. Since this polymer was also used in DN hydrogel formulations, its successful synthesis is considered an essential part of the project. PEO-alkynone was synthesized according to methods described in '*Synthesis of alkynone- and thiol-functionalized PEO'*. The reaction scheme is given in **S1, Scheme 1**.

Figure 2 shows the stacked ^{1}H-NMR of PEO-alkynone of different molar mass (M_n = 600 to 6000 g mol^{-1}). An attempt was made to end-functionalize a range of different M_n PEOs and observe the properties of their respective hydrogel formulations. A first observation is the difference in intensity of ^{1}H-NMR signals when increasing PEO molar mass from 600 to 1500, 2000 and 6000 g mol^{-1}). This is accounted to the lower hydroxyl end-group concentration present in higher molar mass PEO chains. End-functionalization was confirmed in all samples by the triplet at 4.24 ppm, as reported in literature (31). The integration-value was set at 4 for all samples due to the presence of 4 protons. The terminal RC≡CH bond was seen at 3.0 ppm, with an observed integration value ranging from 1.8 – 2.0 for all samples. The slight deviation in integration-values between samples can be due to incomplete end-functionalization of PEO chains, as confirmed by Electrospray Ionization (ESI) (**S2, Figure 1**).

Thus, the integration-value represents a mean of modified PEO chains containing only one- and two alkynone end-groups. This also resulted in a broader distribution of molar mass. Furthermore, the incomplete end-functionalization severely impacted the PEO-alkynone's crosslinking capabilities in hydrogel formulations. Satellites of the PEO repeating units were seen between 3.6 and 3.8 ppm. The polymer peaks seen in ^{1}H-NMR spectra were in accordance with published literature, thus confirming successful product synthesis (31). PEO_{600}-alkynone molar mass was

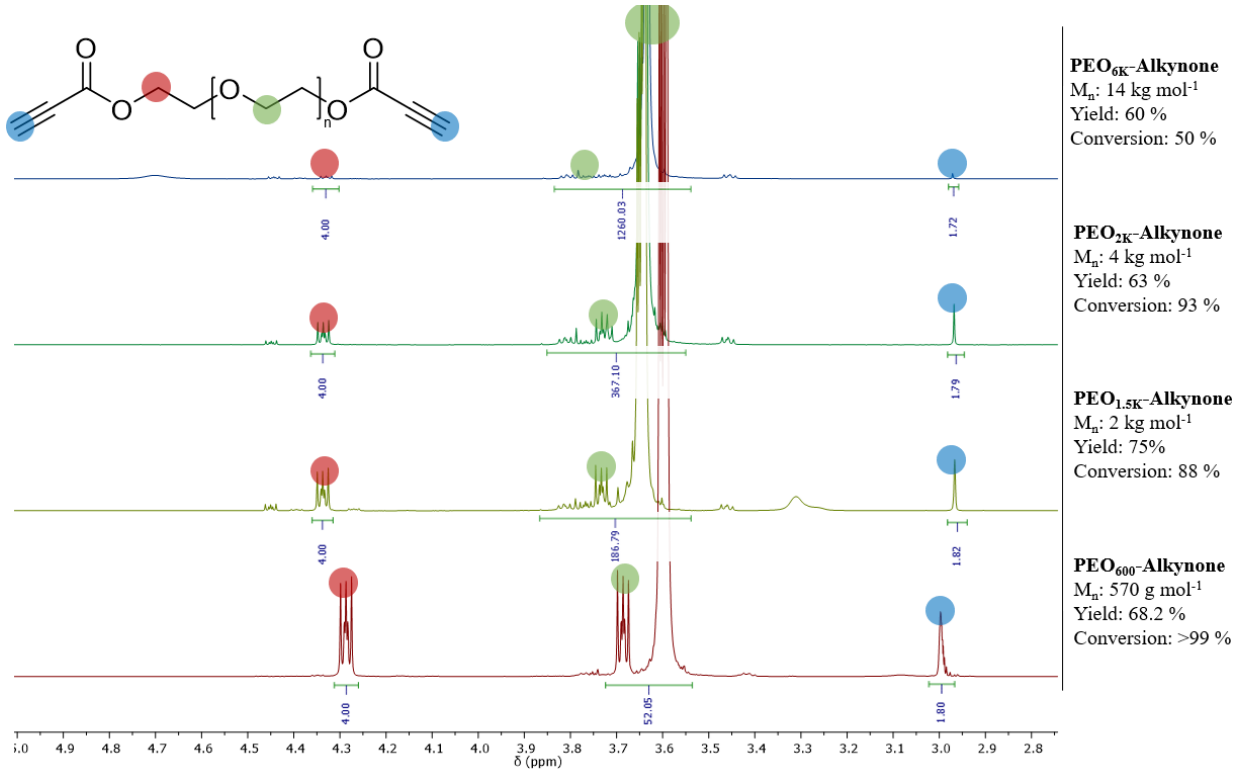

Figure 2: Stacked ^{1}H-NMR spectra of PEO_n-Alkynone in $CDCl_3$. Product peaks are highlighted in their respective colors. $PEO_{1.5K}$-alkynone ^{1}H-NMR: broad peak at 3.30 ppm is H_2O. PEO-alkynone (1.5 kg mol^{-1} and higher) displayed satellites at 4.46 ppm.

determined to be 580 g mol^{-1} *via* ^{1}H-NMR analysis based on the integration-values of the repeating units seen at 3.65 ppm (multiplet). Conversion of hydroxyl- to alkynone end-groups was determined *via* ^{1}H-NMR analysis of the crude product, and found to be decreased (ranging between 50 - 90%) in samples of higher M_n. PEO_{600}-alkynone was found to have consistent conversion (> 99%) after multiple synthesis procedures.

The lower conversion rates for higher M_n PEO could be due to a decreased hydroxyl end-group concentration, as the total polymer mass and solvent volume were kept the same in all reactions. Therefore, less PEO chains of higher M_n are present in the same amount compared to PEO_{600}. As a result, less polymer will be end-functionalized, confirmed by ^{1}H-NMR analysis. An attempt was made to increase the conversion for higher M_n PEO by increasing the reaction time. This, however, showed to have little to no effect on conversion. The solvent volume was also lowered to 100 mL to increase total polymer concentration for reactions of higher M_n PEO. This led to a brown liquid which did not precipitate in diethyl ether and was thus rendered unusable. Due to these reasons, $PEO_{1.5K-6K}$-alkyone polymers were not used in hydrogel formulations, as discussed later in this work.

Reaction conditions were optimized by changing catalysts and tweaking reaction temperature. Reactions were first carried out using p-toluenesulfonic acid as catalyst, due to its ease of use compared to H_2SO_4. However, higher conversion was obtained using H_2SO_4 and was therefore the preferred catalyst. A possible explanation for this increase in conversion is that the p-toluenesulfonic acid has aggregated and taken up water, thereby decreasing its reactivity. Temperature also impacted the reaction, mainly since it controls the amount of solvent reflux. After tweaking reaction temperature, 125 °C was found to be ideal for the solvent to reflux and reaction to proceed overnight.

In all PEO_{600}-alkynone synthesis reactions, the yield was determined to be ~70 %. This amount is in accordance with literature published by Macdougall et al., also indicating a yield of 70 % (31). The 30 % loss of product can be explained by the washing steps involved in polymer work-up since PEO is soluble in both water and organic phase (DCM). However, adequate washing is necessary since the sample's pH ranged between 5 and 6 after work-up. Since the thiol-yne reaction is base-catalyzed, an acidic environment could worsen the reaction in SN-& DN-hydrogel formulations, as discussed later on.

Another interesting observation is the difference in color seen between different batches of PEO_{600}-alkynone. The first batch was an amber colored oil, while the second batch was a dark orange colored oil. Interestingly, the color of the second batch changed over time, becoming more amber after several days. A third batch was prepared, showing an amber color before solvent evaporation overnight. After several hours, the sample turned dark orange. A possible explanation for this is that the first batch contained leftover solvent, since this batch was not left overnight *in vacuo*. Another explanation could be its exposure to air after repetitive use of the polymer. Storing the products under argon or vacuum could be a solution to overcome this coloration. An attempt was made to decolorize the samples by filtration through Celite 545 using charcoal, but failed to yield a transparent product. Propiolic acid also showed signs of coloration after distillation. The reagent, which was dark brown upon opening the bottle and transparent after the first distillation, turned pink after repeated exposure to air. This can be directly linked to the PEO-alkynone of which the coloration can be caused by small amounts of propiolic acid having degraded overtime. Distilling the pink propiolic acid, yielded a transparent reagent.

In summary, the end-functionalization of PEO_{600} with propiolic acid proceeded in accordance with literature, showing adequate conversion (> 99 %) and yield (~70 %) as confirmed by ^{1}H-NMR analysis (31). Therefore, this polymer was determined fit for use in SN- and DN hydrogel formulations. Higher M_n PEO was found to be inadequately end-functionalized, while also showing significantly lower yields.

PEO-SH synthesis – The second step of this project involved end-functionalization of PEO with 3-mercaptopropionic acid (3-MPA) yielding PEO-SH, acting as crosslinker of PEO-alkynone in hydrogel formulations. PEO-SH was synthesized according to methods described in '*Synthesis of alkynone- and thiol-functionalized PEO'*. Since the reaction of PEO and 3-MPA follows the same protocol as PEO-alkynone, the same factors were

found to contribute to the overall reaction. The reaction scheme is given in **S1, Scheme 2**.

Figure 3 shows the stacked ^{1}H-NMR spectra of linear- , 3-arm- and 4-arm PEO-SH, respectively. These different PEO architectures were end-functionalized with 3-MPA in an attempt to analyze their influence on the properties of hydrogel formulations. End-functionalization with 3-MPA was confirmed in all samples by the presence of a triplet at 4.21 ppm, as reported in literature (31). The integration-value was set at 4, 6 and 8 for the linear, 3- and 4-arm PEO-SH, respectively. The triplet of the terminal R-SH bond was seen at 1.64 ppm. For the linear-, 3- and 4-arm PEO-SH polymers, integration-values of 2.11, 2.10 and 2.74 were observed. Interestingly, all values deviate from the expected and reported values (2, 3 and 4 respectively) (31). This can be attributed to incomplete end-functionalization of PEO with 3-MPA, resulting in a broad distribution of end-functionalized polymers. These findings were confirmed *via* ESI analysis (**S2, Figure 2**).

Furthermore, as is the case with the PEO-alkynone, the incomplete end-functionalization could impact the PEO-SH's function as crosslinker in the hydrogels. Only the 3-arm PEO-SH was used in hydrogel formulations due to the late delivery of 4-arm PEO and difficulties end-functionalizing it. As for the linear PEO-SH, it was found to be not capable of forming SN hydrogels. PEO repeating unit molar mass was determined *via* ^{1}H-NMR spectroscopy based on the integration-values of the repeating units seen at 3.59 ppm (multiplet). Satellites of PEO repeating units were seen between 3.46 and 3.71 ppm. Conversion of hydroxyl- to thiol end-groups in linear- and 3-arm PEO-SH was determined to be >99 % *via* ^{1}H-NMR analysis of the crude product. 3-arm PEO-SH yield was determined to be ~70 %. The obtained product was observed to be a yellow colored viscous liquid. All peaks seen in ^{1}H-NMR spectra, end-group conversion and yield were in accordance with published literature, thus further indicating successful product synthesis (31). To sum up, 3-arm PEO-SH was successfully synthesized

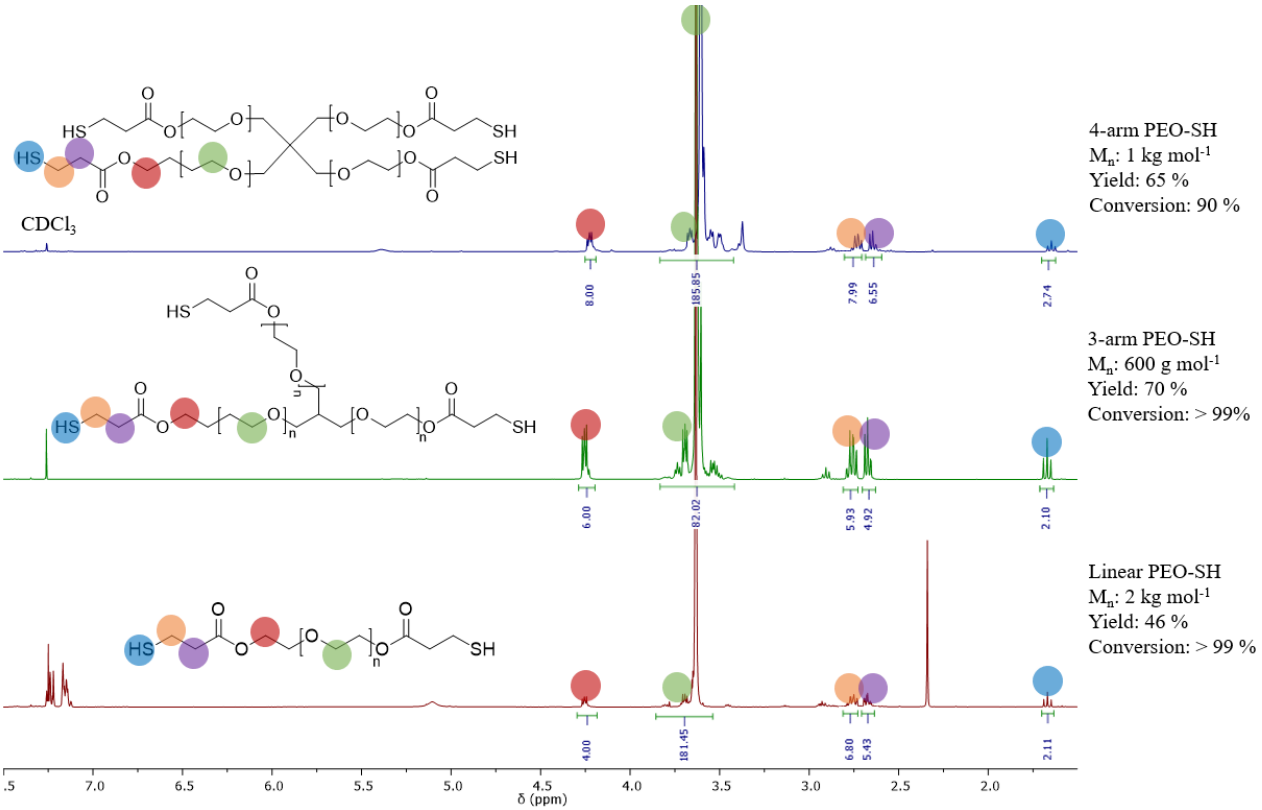

Figure 3: Stacked ^{1}H-NMR spectra of linear-, 3- and 4-arm PEO-SH in $CDCl_3$. Product peaks are highlighted in their respective colors. Multiplet & singlet peaks at 7.2 ppm and 2.3 correspond to toluene.

showing a conversion > 99% and yield of ~70 %, as confirmed by ^{1}H-NMR analysis. The next step involves combining the modified PEO precursors to achieve SN hydrogel formulations.

SN Hydrogel Synthesis – The first step in creating a SN hydrogel involves solubilizing the polymers in the required solvent, in this case PBS or H_2O. Here, PBS was chosen due to its similarities to a physiological environment. Since PEO is a hydrophilic polymer, no problems were expected solubilizing it in PBS. Dissolving PEO_{600}-alkynone was successful, showing a homogeneous, but slightly opaque solution. When attempting to dissolve the 3-arm PEO-SH, especially in higher concentrations, small amber colored bubbles had formed resulting in a heterogeneous solution. The same solution did not form a gel when combined with the dissolved PEO_{600}-alkynone. It was assumed that the thiol caused the modified PEO to be insoluble, possibly due to its acidic nature. Measuring the 3-arm PEO-SH's pH, it became apparent that the sample was acidic (pH = 5). After additional washing of the polymer, a pH neutral polymer was obtained. Repeating the same solubility test and at lowered concentration, the 3-arm PEO-SH was able to dissolve in PBS. The pH of PEO-alkynone was found to be neutral.

After overcoming the solubility issues seen with the 3-arm PEO-SH, the following step involved combining the modified PEO chains to create SN hydrogel formulations. SN hydrogels were made according to methods described in '*Single-Network Hydrogel Synthesis'*. Several SN hydrogel formulations were prepared, varying between 15 and 20 w/v% polymer respectively. A second variable, namely the stoichiometric ratio of alkynone to thiol end-groups, was systematically varied. All hydrogel formulations were prepared in molds for curing. Hydrogels were characterized by compression and tensile testing. **Table 1** shows the composition of all relevant SN hydrogel formulations. First, the influence of polymer w/v% on gelation was investigated. All formulations containing 15 w/v% and 20 w/v% polymer content, with stochiometric ratios of 1:1, 1.2:1 and 1:1.2 respectively, formed SN hydrogels in PBS. For the second batch of SN hydrogels, gelation time (t_{gel}) was determined to be < 1 minute *via* the vial tilt method. It was expected that an increased polymer w/v% would cause faster gelation, due to the increased concentration of reactive end-groups present in the solution (42). However, no significant difference in t_{gel} was observed between different formulations.

A first impression of the hydrogels indicated that the 20 w/v% gel formulations were more firm compared to the 15 w/v% formulations, as is expected with higher polymer content (42). Here, the 1:1 (alk:thiol) formulations were observed to be the most firm gels. Varying the end-group ratios led to significant differences in physical properties between formulations. An increased alkynone ratio (1.2:1) led to a more sticky and fragile formulation.

Table 1: An overview of PEO_{600}-alkynone and 3-arm PEO-SH SN hydrogel formulations.

Polymer w/v%	PEO-Alkynone	PEO-SH	Ratio Alkynone:SH end-groups	Gel formation	t_{gel}
15 w/v%	PEO_{600}-alkynone	3-arm PEO-SH	1:1	Yes	30 min[1], <1 min[2]
			1:1.2	Yes	30 min[1], <1 min[2]
			1.2:1	Yes	30 min[1], <1 min[2]
			1:1.5	No	/
			1:2	No	/
			2:1	No	/
20 w/v%	PEO_{600}-alkynone	3-arm PEO-SH	1:1	Yes	30 min[1], <1 min[2]
			1:1.2	Yes	30 min[1], <1 min[2]
			1.2:1	Yes	30 min[1], <1 min[2]
			1:2	No	/
			2:1	No	/

[1]The first batch PEO-SH was used in this formulation. [2]The second batch PEO-SH was used in this formulation. t_{gel} = gelation time

Increasing the thiol ratio (1:1.2), resulted in a more firm gel that was less sticky compared to the 1:1.2 gels. Increasing the end-group ratios to 1:1.5 in 15 w/v% formulations resulted in amorphous materials unable to retain their shape when taken out of the mold. Doubling the stoichiometric ratio of alkynone:thiol end-groups did not result in hydrogel formation. Other attempted hydrogel formulations are described in **S3, Table 1**.

It was observed that all formulations with a stoichiometric imbalance of end-groups were unable to form hydrogels. It was thought that by increasing the concentration of end-groups, polymer chains would be saturated and thus prevented from crosslinking. For example, if each 3-arm PEO-SH molecule reacts with three PEO_{600}-alkynone molecules, no crosslinking can occur due to their excess. Another explanation is that the polymers prefer to create a linear chain since this could be an entropically more favorable conformation. Interestingly, gels containing excess alkynone end-groups were observed to be more sticky compared to the other formulations. This is presumably due to the presence of excess free end-groups in the hydrogel. In general, these experiments confirmed the importance of maintaining a balanced stoichiometry when designing SN hydrogel formulations. Altering the w/v% of the hydrogel appeared to have little effect on t_{gel}. However, the 20 w/v% gels were observed to be significantly more firm than 15 w/v% hydrogels due to the increase in polymer concentration. These results were obtained using a second batch of PEO_{600}-alkynone and 3-arm PEO-SH.

To see if these results were reproducible, a comparison was made between two different batches of modified PEO polymers, the first and second batch respectively. Interestingly, as seen in **Table 1**, the t_{gel} of the first batch was determined to be 30 minutes. This significant increase in t_{gel} over the second batch was likely due to sample impurities (e.g. remaining solvent or incomplete end-functionalization). However, it had to be determined which modified polymer was causing the longer t_{gel}. The two batches of alkynone-modified PEO_{600} were crosslinked with the 3-arm PEO-SH used in formulations that gelled within 1 minute. Here, it was observed that both formulations also gelled within 1 minute. This directly confirms that the first batch of thiol-modified PEO was responsible for the longer t_{gel}. Therefore, it is important to gain a better understanding of this batch-to-batch variability. Ideally, it should be eliminated since the t_{gel} is crucial when using the system for injection and/or 3D printing. Having successfully synthesized different SN hydrogel formulations, compression and tensile tests were performed to determine their mechanical properties.

SN Hydrogel Compression and Tensile Testing – Generally, when designing a covalently crosslinked single-network for DN hydrogels, this network is loosely crosslinked and responsible for the soft and flexible characteristics of the DN hydrogel (13). Therefore, higher molar mass PEO polymers are preferred due to the longer distance between crosslinks, making the network more loosely crosslinked (13). However, despite many attempts, no high M_n PEO-alkynone (1.5 kg mol^{-1} to 6 kg mol^{-1}) formed a SN hydrogel. Therefore, compression and tensile testing was only performed on PEO_{600}-alkynone hydrogel formulations.

Figure 4 shows the tensile and compression curves obtained from 15 w/v% and 20 w/v% hydrogel formulations described in **Table 1**. In **Figure 4A** and **4C**, the 20 w/v%, 1:1 SN formulation was observed to reach 35 kPa force at 45 % strain before breaking. A significant increase in tensile strength compared to the 15 w/v%, 1:1 formulation, reaching a tensile strength of 9 kPa at 42 % strain. The increase in tensile strength could be owed to the increase in polymer concentration in hydrogel formulations (13). Interestingly, both the 15 and 20 w/v% 1.2:1 formulations showed a detrimental loss of tensile strength but reached up to 90% and 70 % strain, respectively, before breaking. The 15 w/v% 1.2:1 formulation was very sticky and broke almost instantly when removed from the mold. The samples were damaged before measurement, but data was included for completeness. Due to an excess in thiol stoichiometric ratio, both 15 and 20 w/v% formulations were found to have worsened mechanical properties compared to the stoichiometrically balanced formulations.

In compression testing, as seen in **Figure 4B** and **4D**, the 20 w/v% 1:1 formulation was able to reach 670 kPa of compressive strength at 80 % strain. A difference of ~600 kPa in compressive strength was seen between the 15 w/v% and

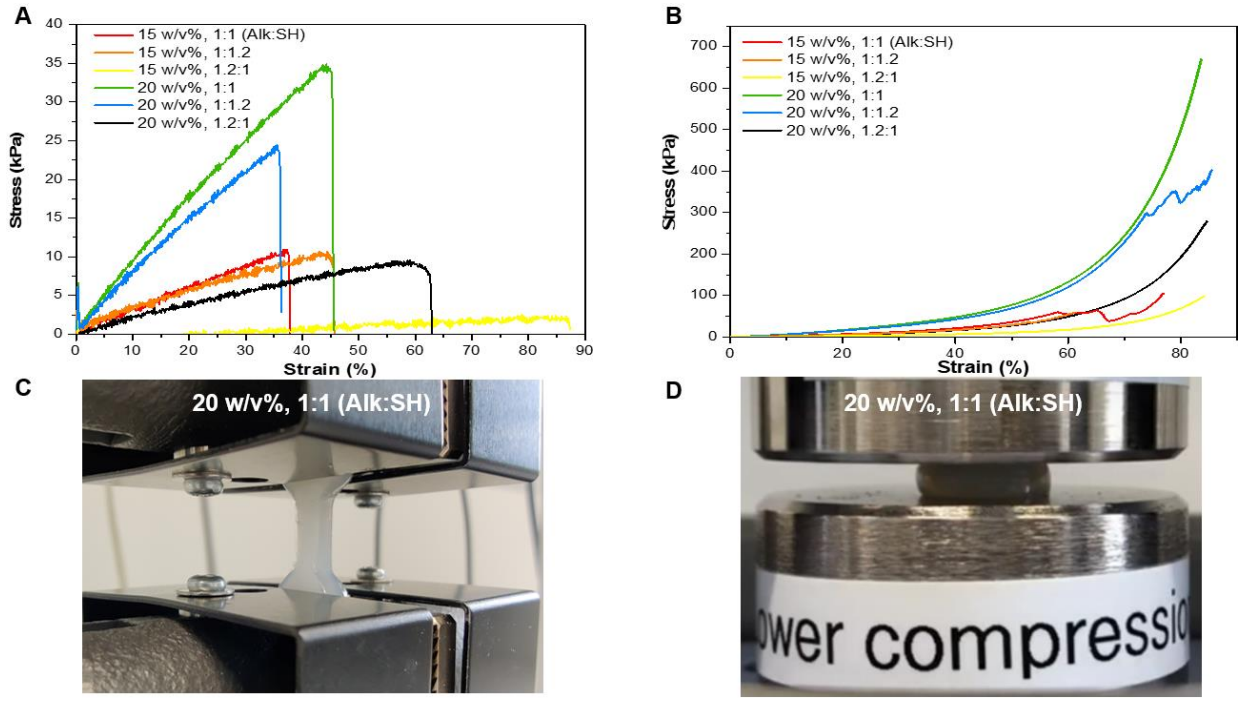

Figure 4: Representative stress-strain curves of 15 and 20 w/v% PEO-SN hydrogels in a, c) tensile and b, d) compression testing. Figure C and D show 20 w/v%, 1:1 (alk:SH) SN hydrogel formulations during testing. All formulations were tested *in triplicate*, except 15 w/v% 1:1.2 (alk:SH) (n = 1).

20 w/v% 1:1 formulations, owed to the increased polymer content. Besides outperforming the other hydrogel formulations, the 20 w/v% 1:1 gel remained intact after the first compression test. The Young's moduli of the SN hydrogel formulation are given in **S3, Figure 1**. It was observed that the 20 w/v% 1:1 (alk:SH) formulation had the highest Young's moduli ($E_{tensile}$ = 0.6 kPa; $E_{compression}$ = 0.85 kPa), while still remaining low compared to e.g. cartilage ($E_{compression}$ = 950 kPa) (43). A significant difference was seen between the 15 and 20 w/v% SN formulations (0.3 kPa vs 0.6 kPa respectively), highlighting the increased softness of the formulations with lower polymer content. In both 15 and 20 w/v% formulations, it was seen that increasing the alkynone end-group concentration led to significantly lower Young's moduli.

To observe its swelling behavior, the 20 w/v% 1:1 SN hydrogel was swollen in water until equilibrium and dried for 7 days. Hence, the equilibrium water content (EWC) could be determined *via* the EWC equation (**Equation 1.1**). The 20 w/v% 1:1 formulation displayed a high EWC percentage (90 %). This is indicative that the SN hydrogel has a porous structure capable of holding large amounts of water (31). As was seen in SN hydrogel synthesis, the stoichiometric ratio was an important characteristic in hydrogel formulations. Besides determining the gelation time, a change in mechanical properties is seen between different formulations. This indicates that apt optimization is required to obtain hydrogels with superior mechanical properties. Thus far, based on the mechanical properties, gelation time and EWC, the 20 wt% 1:1 formulation proved to be the best performing hydrogel sample obtained in this work. Therefore, this formulation was chosen to incorporate into DN hydrogels.

$$EWC\ (\%) = \frac{W_s - W_d}{W_s}\ x\ 100\ \%$$

Equation 1.1. Equilibrium Water Content (EWC), where W_s is the equilibrium swollen mass and W_d is the dry mass of the hydrogel.

Alginate Single-Network Hydrogel Synthesis – As mentioned previously, the modified PEO polymers were combined with alginate to form a DN hydrogel in a one-pot reaction. Thus, after successfully developing the PEO SN hydrogels, an alginate SN hydrogel had to be developed and put to mechanical testing. This way, the hypothesis that

combining two distinct networks in a DN hydrogel formulation synergistically increases its mechanical strength could be tested. Alginate SN hydrogels were developed by dissolving sodium alginate in PBS. A 0.2 M $CaCl_2$ solution was used to induce crosslinking, yielding alginate SN hydrogels.

Tensile and compression tests are seen in **S4, Figure 1**. 7.5 w/v% Alginate SN hydrogels displayed a compression strength of 1.5 MPa at 87 % strain. However, the gels were unable to recover to their original shape, thus showing plastic behavior (44). Tensile testing confirmed that alginate SN hydrogels are less elastic, reaching 30 % strain due to the increased crosslinking density of the network. These findings are in accordance with published literature (38). Alginate 1.5 w/v% hydrogels were also prepared varying the $CaCl_2$ concentration to observe its influence on t_{gel}. This led to better insight in t_{gel} of the alginate when synthesizing DN hydrogels and ultimately their use in injection and 3D printing. In a 0.5 M $CaCl_2$ solution, alginate gels formed within 45 minutes, as compared to 3 hours for a 0.1 M $CaCl_2$ solution. Thus, by increasing the concentration $CaCl_2$, gelation times decrease significantly. However, due to the increased amount of Ca^{2+}-ions in the solution, a more brittle alginate network could be formed, thereby modifying the mechanical properties of the network. This has to be kept in mind when designing DN hydrogels. However, optimization of the alginate SN hydrogel is beyond the scope of this project.

Double-Network Hydrogel Synthesis – So far, the modified PEO polymers were able to form SN hydrogels with distinct mechanical properties based on their stoichiometric ratio and w/v%. Having observed that the 20 w/v% 1:1 SN hydrogel formulation displayed superior mechanical properties, it was the preferred formulation for DN hydrogel development. The alginate SN hydrogel's w/v% was varied from 1 to 3 w/v% to determine the DN formulation exhibiting the best mechanical properties. DN hydrogel synthesis was carried out as described in the methods '*Double-Network Hydrogel Synthesis*'. All DN hydrogel formulations are described in **Table 2** and shown in **Figure 5C-D**. A first observation was the incompletely formed DN 1 formulation. The gel didn't form completely due to an unexpected fast gelation time (< 1 min).

Table 2: Polymer content of DN hydrogel formulations (20 w/v%, 1:1 (alk:SH))

	DN 1	**DN 2**	**DN 3**	**DN 4**
Total w/v%			20	
PEO SN w/v%	19	18	17	18.5
Alginate w/v %	1	2	3	1.5

This was then taken into account when preparing the other DN formulations. Another observation of the DN hydrogels shows an inhomogeneous distribution of alginate in all formulations. This can be owed to inadequate mixing of alginate with the PEO chains caused by phase separation of the two polymers (41). This in turn is expected to affect their mechanical properties. DN 3 was observed to be the most brittle formulation, presumably due to its increased alginate content. DN 4 was the most homogeneous formulation being completely opaque (**Figure 5D**), showing good distribution of alginate. Therefore, it is assumed that the ratio of PEO to alginate could be an important factor in gel formation and determining the mechanical properties.

The mechanical performance of the four DN hydrogel formulations is given in **Figure 5A-B**. Interestingly, a significant difference in tensile stress and strain (17 kPa, 20% strain) between DN 1 and 2 was observed. Due to lack of material, only one DN hydrogel was prepared for each formulation except DN 4. Therefore, comparing the differences between mechanical properties is considered misleading. However, they do indicate that the alginate and modified PEO w/v% play a significant role in determining the tensile strength of this DN hydrogel system. The compression testing data of DN 4 indicated a compressive strength of 198 kPa and 191 kPa at 70 and 73 % strain respectively. Compared to the SN hydrogel formulations in **Figure 4**, the DN hydrogels are less elastic but reach similar tensile strength. Nonetheless, the 20 w/v% 1:1 SN hydrogel showed superior mechanical properties to all DN hydrogel formulations. The DN formulation's decrease in mechanical properties is likely due to the introduction of a densely crosslinked alginate network to the already densely crosslinked PEO SN. Hereby, its solid content and crosslinking

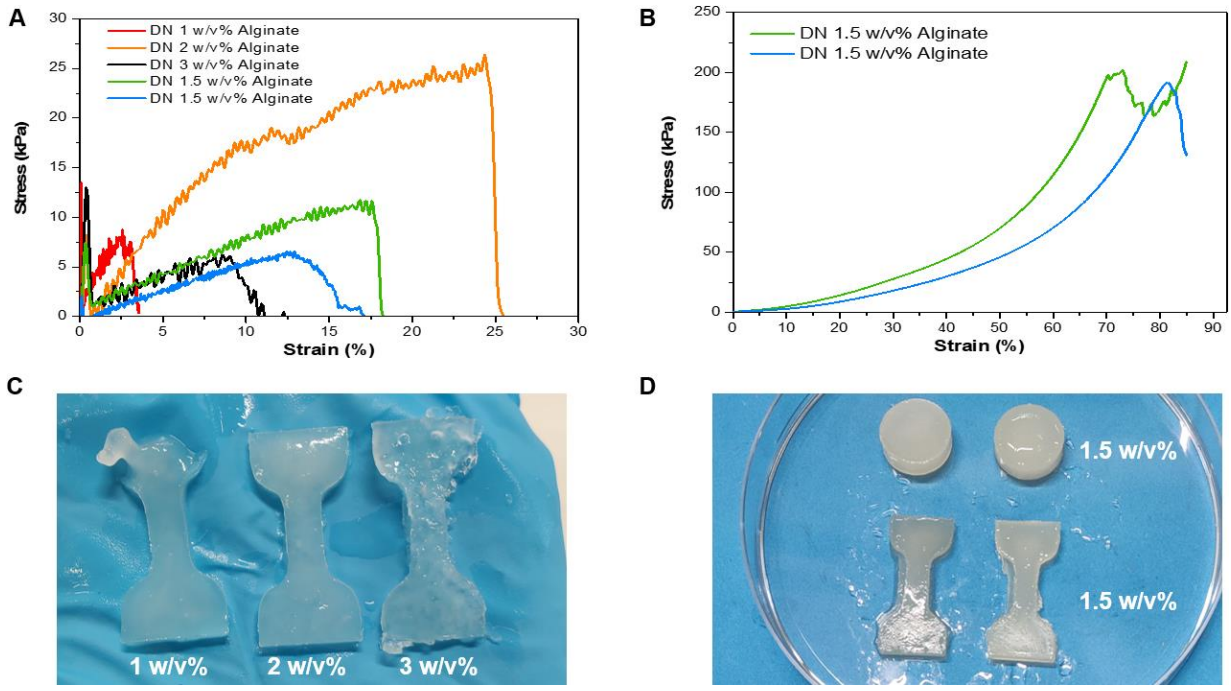

Figure 5: Representative stress-strain curves of 20 w/v% PEO-alginate DN hydrogels in a, c and d) tensile and b, d) compression testing. Total polymer content was kept at 20 w/v% for all DN hydrogels.

density increase, thus lowering its elastic properties.

Comparing the Young's moduli of the 20 w/v% 1:1 SN hydrogel and DN 2 & 4 hydrogel formulations, higher Young's moduli were seen for both DN formulations (**Figure 6**). This indicates that the DN formulations are more stiff compared to the SN hydrogel formulations, which is in accordance with observations. However, as mentioned earlier, the DN hydrogel results are based on a single measurement per formulation and thus have to be interpreted with care. Also, no compression Young's modulus was obtained for DN 2 due to a lack of material. More conclusive results could be obtained by increasing the amount of hydrogel samples, thereby gaining a better understanding of intra- and inter hydrogel formulation variability. Furthermore, due to the aforementioned reasons, the synergistic effect of the two networks on the mechanical properties could not be confirmed in these experiments.

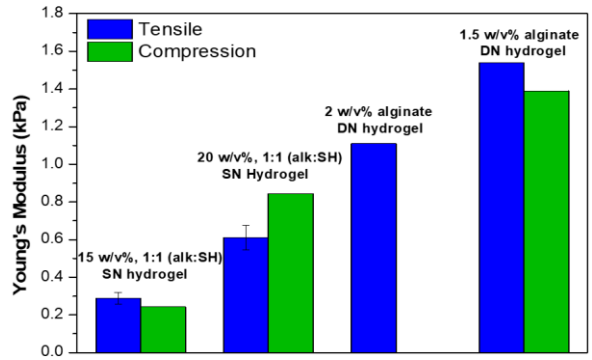

Figure 6: Representative Young's Moduli of SN- and DN hydrogels. 2 w/v% alginate DN hydrogel compression Young's Modulus was not measured.

Nevertheless, the development of PEO/alginate DN hydrogels was deemed successful, but showed to have relatively poor mechanical properties compared to the SN hydrogel formulations. This can be overcome by further optimizing the polymer content of both networks, thereby overcoming problems such as phase separation and brittleness. Crosslinking density of both networks can be tuned to obtain gels with different mechanical properties, swelling behavior, as well as using higher M_n PEO chains to create a more elastic network.

It still had to be determined if these formulations were capable of being injected. To do so, 20 w/v% 1:1 SN hydrogel and 1.5 w/v% alginate DN hydrogel formulations were prepared in a 1 mL syringe as a one-pot system and injected onto a filter paper containing 0.1 M TBD solution

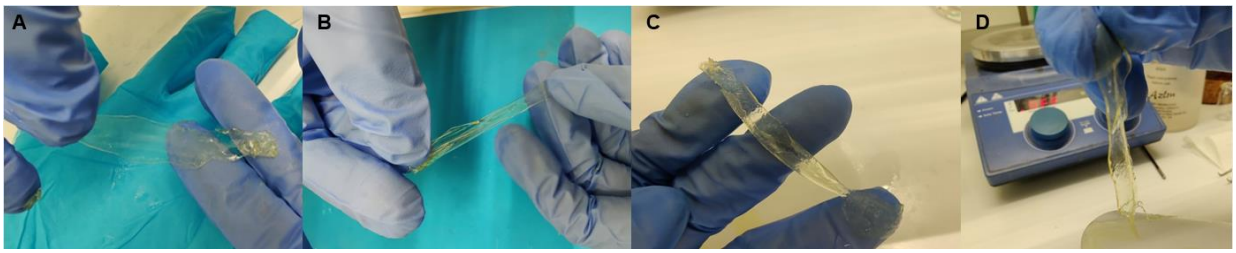

Figure 7: Injection of a, b) SN hydrogel and c, d) DN hydrogel formulations. a, b) 20 w/v% 1:1 SN hydrogels after injection onto filter paper containing 0.1 M TBD solution. c, d) 1.5 w/v% alginate DN hydrogels after injection onto filter paper containing 0.1 M TBD + 0.5 M $CaCl_2$ solution. Both SN- and DN formulations formed a film on the filter paper.

to induce instantaneous hydrogel formation. For DN injection, 0.5 M $CaCl_2$ solution was added to the 0.1 M TBD solution to induce alginate crosslinking. An attempt was made at strand formation by injecting the solution in a straight line, but instead led to the formation of a film as seen in **Figure 7**. All films were brittle and unable to retain their shape after being exposed to air and touch. SN hydrogels were also injected into a beaker containing 0.1 M TBD, but did not result in strand or film formation. Instead, the SN system clogged together forming a lump of material. Further investigation and optimization of the injection procedure has to be conducted to yield strand formation. Increasing the viscosity of the system could help the hydrogel maintain a cylindrical shape after being extruded. However, due to time restrictions, these tests could not be executed. Nevertheless, film formation with these hydrogel formations can be considered the first step toward injection of these materials into complex shapes and structures, and eventually their use in 3D printing applications.

CONCLUSION

In summary, it was posed that PEO/alginate DN hydrogel formulations could be developed *via* a one-pot method, rendering it suitable for injection and 3D printing. This was achieved *via* end-functionalization of PEO with propiolic acid and 3-MPA *via* esterification, yielding PEO-alkynone and PEO-SH, respectively. The conversion of hydroxyl- to alkynone- and thiol end-groups was found to be >99 % for PEO_{600}, thus indicating successful synthesis. High M_n PEO (1.5-6 kg mol^{-1}) end-functionalization proved to be challenging, reaching up to 93 % conversion ($PEO_{1.5K}$-alkynone). Next, thiol-yne click chemistry was successfully employed to create PEO SN hydrogels. Here, the 20 w/v% 1:1 (alk:SH) SN hydrogel formulation was able to withstand up to 670 kPa compressive force at 85 % strain. An imbalance in the stoichiometric ratio of alkynone to thiol end-groups proved to be detrimental to the hydrogel's mechanical performance. Combining the modified PEO SN with alginate in a one-pot reaction, yielded DN hydrogels reaching a compressive stress of 198 kPa at 73 % strain. Finally, the 20 w/v% 1:1 SN- and DN formulations were subject to injection, yielding a hydrogel film. Despite these systems not forming strands, a promising step toward the development of complex shapes and structures has been made. Hereby, the main goal of this project was achieved, namely using thiol-yne click chemistry to design processable DN hydrogel formulations *via* a one-pot method.

In future work, unreacted PEO-alkynone end-groups could be exploited to introduce novel ligands (e.g. antimicrobial molecules, cell-coupling domains, etc.) into the system *via* thiol-yne chemistry. These features could also be introduced *via* a second addition onto the alkyne, further highlighting the potential of the system. The PEO/alginate DN system can be further explored by incorporating higher M_n PEO, thereby creating DN systems with a range of mechanical properties for different applications. Tuning the hydrogel's crosslink density could lead to control over hydrogel swelling, which can ultimately lead to the encapsulation of cells. After *in vitro* testing, the hydrogel's *in vivo* performance could be determined to observe its viability as tissue construct. Further investigation to improve hydrogel injectability is a crucial part for translation to 3D printing. Using 3D printing, tissue constructs based on the proposed system can be developed. The combination 3D printing and medical imaging

techniques could lead to a patient-tailored approach for the development of tissue constructs. Ultimately, this system could lead to biomaterials with enhanced properties capable of being applied in the biomedical field, further expanding the arsenal of synthetic tissue.

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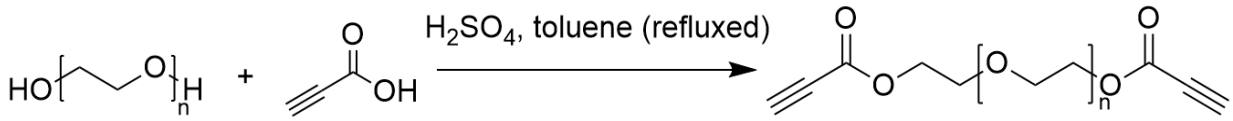
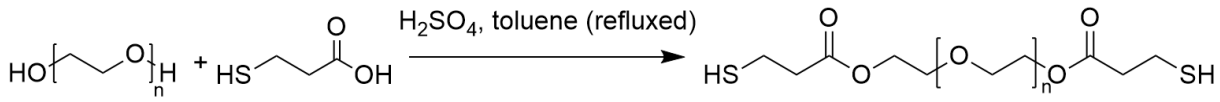
Author contributions – MAC and LP conceived and designed the research. AH and MAC performed experiments and data analysis. AH wrote the paper. All authors carefully edited the manuscript.

SUPPORTING INFORMATION

S1. Reaction Schemes



Scheme 1: Esterification of PEO and propiolic acid yielding PEO-alkynone.



Scheme 2: Esterification of PEO and 3-mercaptopropionic acid yielding PEO-SH.

S2. Electrospray Ionization

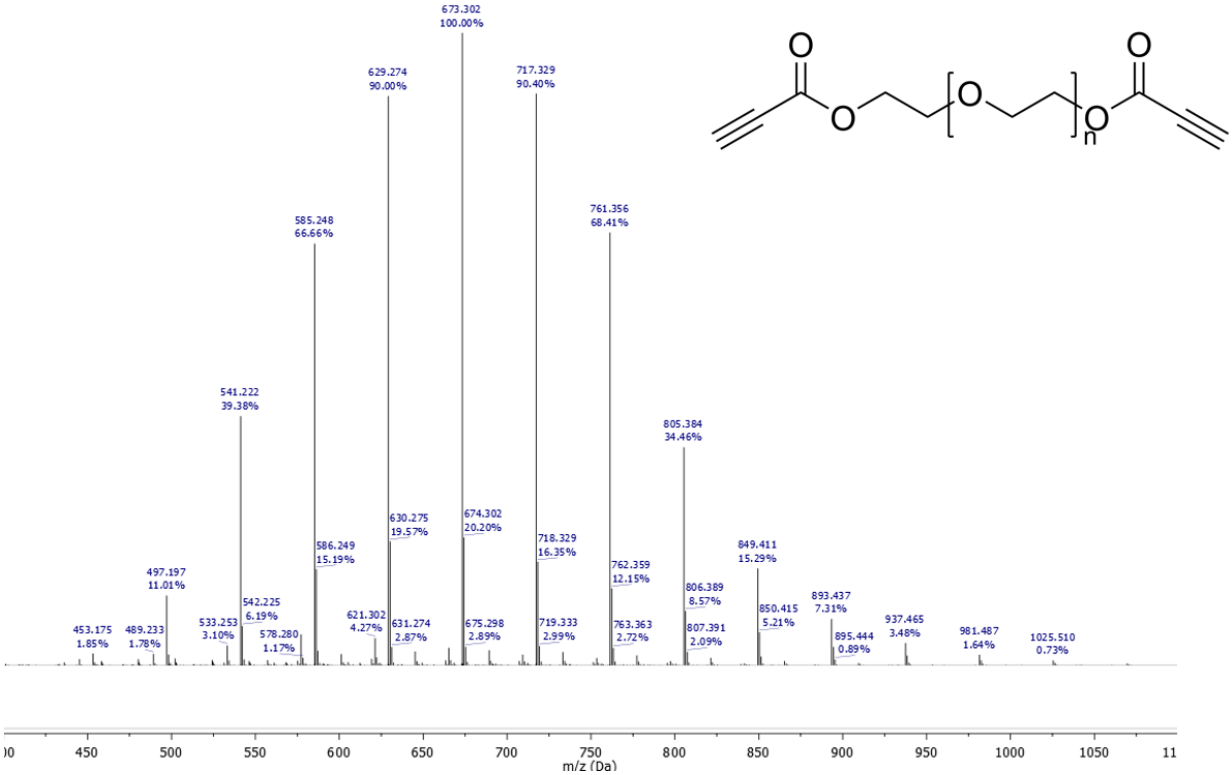


Figure 1: Representative ESI spectrum of PEO-alkynone (M_n = 650 g mol^{-1}). Distribution of M_m indicating a difference in amount of ethylene oxide repeating units (M_n = 44 g mol^{-1}). Polymer is charged with a Na^+-ion (M_n = 23 g mol^{-1}).

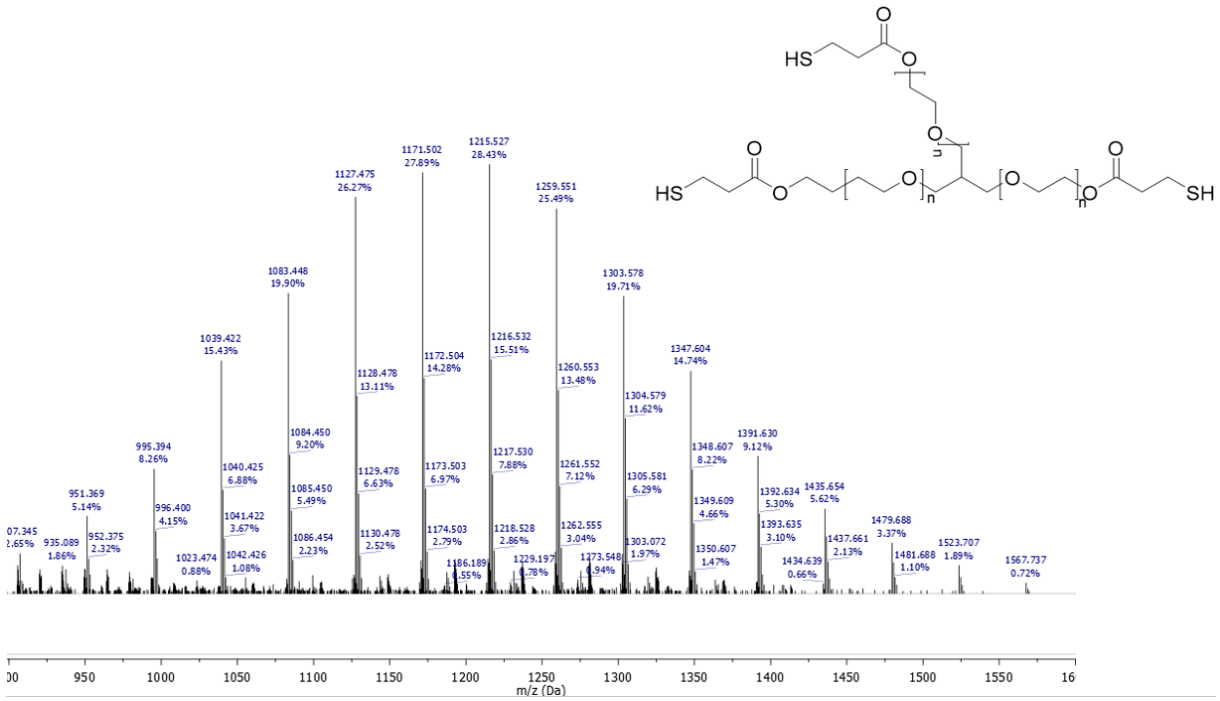


Figure 2: Representative ESI spectrum of 3-arm PEO-SH (M_n = 1192 g mol^{-1}). Distribution of M_n indicating either i) incomplete end-functionalization with 3-MPA (M_n = 88 g mol^{-1}) or ii) a difference in amount of ethylene oxide repeating units (M_n = 44 g mol^{-1}). Polymer is charged with a Na^+-ion (M_m = 23 g mol^{-1}). 3-MPA = 3-mercaptopropiolic acid

S3. SN Hydrogel Formulations

Table 1: An overview of PEO_{600}-alkynone and 3-arm PEO-SH SN hydrogel formulations.

Polymer w/v%	PEO-Alkynone	PEO-SH	Ratio Alkynone:SH end-groups	Gel formation	t_{gel}
10 w/v%	PEO_{600}-alkynone	3-arm PEO-SH	1:1	Yes	N.A.
15 w/v%	PEO_{2K}-alkynone	3-arm PEO-SH	1:1	No	/
20 w/v%	$PEO_{1.5K}$-alkynone	3-arm PEO-SH	1:1	No	/
20 w/v%	PEO_{2K}-alkynone	3-arm PEO-SH	1:1	No	/
25 w/v%	$PEO_{1.5K}$-alkynone	3-arm PEO-SH	1:1	No	/
25 w/v%	PEO_{2K}-alkynone	3-arm PEO-SH	1:1	No	/

w/v% = weight/volume %; PEO = polyethylene oxide; SN = single-network; SH: thiol; t_{gel} = gelation time

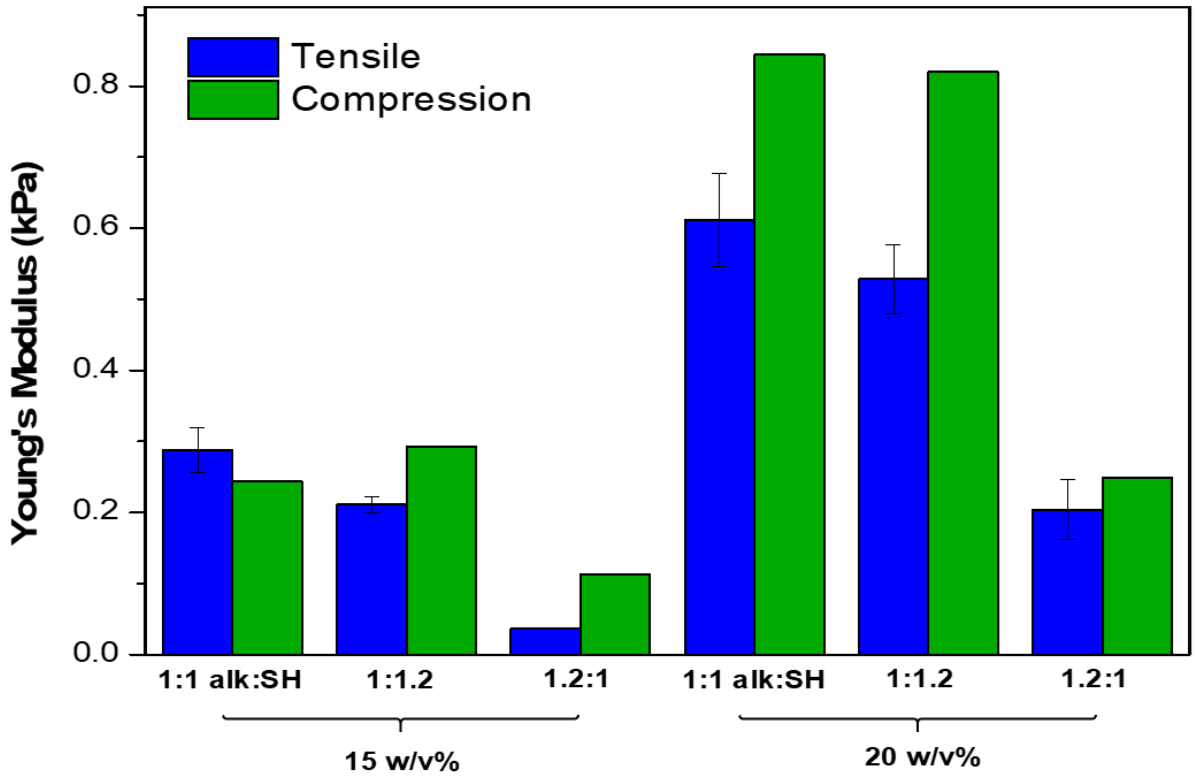


Figure 1: Representative Young's Moduli of 15 w/v% and 20 w/v% SN hydrogel formulations. A significant decrease in Young's modulus (both tensile and compression) is seen with increasing alkynone end-group concentration. alk = alkynone; SH = thiol; w/v% = weight/volume %.

S4. Alginate SN Hydrogels

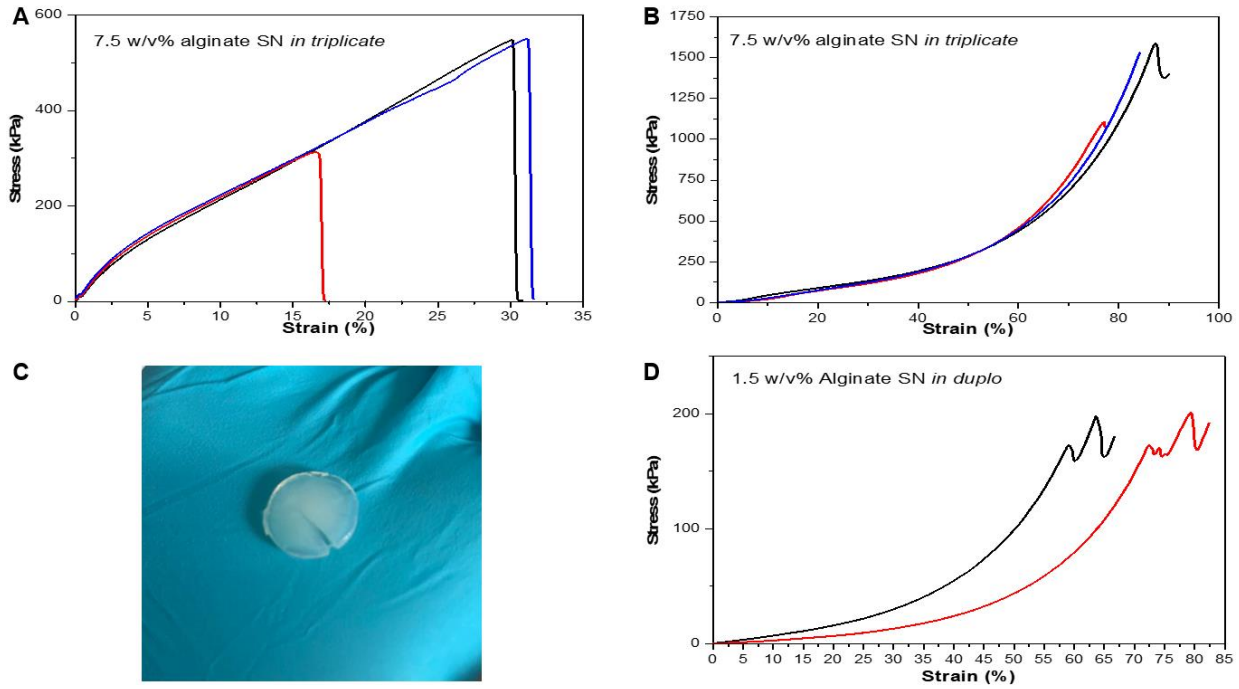


Figure 1: Representative stress-strain curves of a, b) 7.5 w/v% and d) 1.5 w/v% alginate SN hydrogels. c) 7.5 w/v% alginate SN hydrogel after compression testing.