Structure, morphology and mechanical properties of supramolecular hydrogels

Citation for published version (APA):

DOI:
10.6100/IR760623

Document status and date:
Published: 01/01/2013

Document Version:
Publisher’s PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher’s website.
• The final author version and the galley proof are versions of the publication after peer review.
• The final published version features the final layout of the paper including the volume, issue and page numbers.

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license above, please follow below link for the End User Agreement:
www.tue.nl/taverne

Take down policy
If you believe that this document breaches copyright please contact us at:
openaccess@tue.nl
providing details and we will investigate your claim.

Download date: 13. Aug. 2019
Structure, morphology and mechanical properties of supramolecular hydrogels

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Technische Universiteit Eindhoven, op gezag van de rector magnificus prof.dr.ir. C.J. van Duijn, voor een commissie aangewezen door het College voor Promoties, in het openbaar te verdedigen op maandag 18 november 2013 om 16:00 uur

door

Marcel Maria Elisabeth Koenigs

goingen te Tegelen
Dit proefschrift is goedgekeurd door de promotoren en de samenstelling van de promotiecommissie is als volgt:

voorzitter: prof.dr.ir. J.C. Schouten
1e promotor: prof.dr. R.P. Sijbesma
2e promotor: prof.dr. E.W. Meijer
leden: prof.dr. J. van Esch (TU Delft)
        prof. dr. ir. L. Brunsveld
        dr. R. Oda (Chimie et Biologie des Membranes et des Nanoobjets)
        dr. C. Storm
        dr. H.M. Wyss
# Table of contents

## Chapter 1 .................................................................................................................. 1

*Introduction* .................................................................................................................. 1

1.1 Definition of a gel .................................................................................................. 2
1.2 Supramolecular gels ............................................................................................. 2
1.3 Hydrogel characterization ....................................................................................... 5
  1.3.1 Rheology ........................................................................................................ 6
  1.3.2 CryoTEM ....................................................................................................... 8
1.4 Bis(urea) ............................................................................................................... 10
1.5 Hydrogels in tissue engineering .......................................................................... 12
1.6 Aim of this thesis ................................................................................................. 13
1.7 References ............................................................................................................ 16

## Chapter 2 .................................................................................................................. 21

*Tuning the crosslink density between semi-flexible rods using the self-sorting of the bis(urea) motif* ............................................................................................... 21

2.1 Introduction .......................................................................................................... 22
2.2 Synthesis ............................................................................................................... 24
2.3 Morphology .......................................................................................................... 25
2.4 Rheology ............................................................................................................... 29
2.5 Discussion ............................................................................................................. 31
2.6 Conclusion ............................................................................................................ 34
2.7 Experimental ....................................................................................................... 36
2.8 References ............................................................................................................ 40

## Chapter 3 .................................................................................................................. 43

*Crosslinking of semi-flexible rods by metal-carboxylic acid interactions* .......................................................................................................................... 43

3.1 Introduction .......................................................................................................... 44
3.2 Synthesis ............................................................................................................... 46
3.3 Solubility of AU4UA and AU6UA ........................................................................ 47
3.4 Network morphology characterization by CryoTEM ........................................... 48
3.5 Rheology ............................................................................................................... 50
  3.5.1 Addition of calcium to U6U and AU6UA ..................................................... 50
  3.5.2 Influence of pH on crosslinking efficiency .................................................. 51
  3.5.3 Gelation with other metals ........................................................................... 53
  3.5.4 Non-covalent interactions with carboxylic acid as crosslinking mechanism .................................................................................................................. 55
Chapter 1

Introduction
1.1 Definition of a gel
Scientists have documented hydrogels as materials that behave differently from solids or liquids already in the middle of the 19th century. Ever since, these materials have been the topic of research in a growing field. Giving a comprehensive definition of hydrogels, and gels in general, has always been a challenge. This was recognized by D. Jordan Lloyd, who stated: “The colloidal condition, the "gel," is one which it is easier to recognize than to define, and even recognition is confused by the fact that the limits between gel and sol, on the one hand, and gel (...) on the other, are not precise, but consist of a gradual change.” Despite an elusive definition of the class of materials, some characteristics are easily distinguished. The most important feature of gels is that they consist of at least two components, one of those being the solvent that is present in a large quantity, the other forming a network. The current IUPAC definition of a gel is “A non-fluid colloidal network or polymer network that is expanded throughout its whole volume by a fluid.” The IUPAC definition furthermore uses a categorization similar to the one introduced earlier by Flory: The network can contain a covalent polymer network, a polymer network formed through the physical aggregation of polymer chains, a polymer network formed through glassy junction points, lamellar structures including mesophases and/or particulate disordered structures. This categorization is useful, because it addresses the structural criteria of gels whilst recognizing their solid-like mechanical behavior. Although the categorization by Flory focuses on organic polymers, inorganic gels, such as silica gel, have similar characteristic viscoelastic properties. In this thesis gels based on organic polymers are considered, more specifically organic supramolecular polymer networks.

1.2 Supramolecular gels
Supramolecular gels are gel networks that consist of (supramolecular) polymers connected via physical aggregation of the polymer chains, either with ‘sticky’ groups in the main chain of the polymers or as supramolecular crosslinks between the polymers. Due to the presence of non-covalent bonds,
supramolecular gel networks are inherently dynamic and not permanent. The dynamic nature creates challenges in the characterization of supramolecular gels, since the timescale of the experiment can determine the investigated properties, especially if the timescale of the experiment is much larger or much shorter than the relaxation time of the supramolecular interaction. Recently, the design, structure, morphology, rheology, and applications of supramolecular gels have been reviewed. Gelating compounds have been prepared using a myriad of different functional groups and interactions. The gels can be classified based on the molecular weight of the gelators. One category consists of gels of supramolecular polymers (or supramacromolecular gels), i.e. covalent polymers with supramolecular functional groups. The other category concerns gels consisting of low molecular weight gelators (commonly abbreviated to LMWG). Despite the various designs and morphologies of supramolecular hydrogels, there are some common properties. The dynamic nature of the non-covalent bonds leads to a transient network, which has multiple timescales. One of them is the timescale of formation and breakage of the specific supramolecular interaction in the gel, another is the timescale of relaxation of the polymer chains or segments within the network.

The dynamic nature of supramolecular gels gives rise to desirable functional properties such as self-healing. Self-healing materials regain their original mechanical properties after macroscopic network failure, for instance when the yield point of a gel is exceeded but also when a material is cut in half and reconnected to form an ‘as new’ section of the material. Because of the reversible nature of the bonds self-healing is often encountered in supramolecular gels. The fundamental requirements for self-healing are the same as for thixotropy in fluid mechanics: the material should be viscoelastic and the network should be dynamic to enable changes in and recovery of mechanical properties. A distinction is made between the reversible bond formation of the supramolecular motifs and the slower reformation of crosslinks within the network that is determined by the relaxation (or diffusion) time of the polymer segments in the network. Since self-healing concerns recovery after macroscopic failure of the network, the crosslink sites are separated and thus no immediate reconnection of the
preexisting crosslinks can occur. The self-healing process thus requires a diffusion of supramolecular binding sites through the network. The diffusion of binding sites is an inherent property of supramolecular gels because of the high quantity of solvent present in which the mobility is much higher than in solid materials. Self-healing is further promoted by the timescale of the supramolecular interactions that is fast in comparison to the diffusion timescale. The diffusion of the supramolecular binding sites is slow compared to the timescale of the supramolecular binding interactions. Thus once binding sites come in close proximity, a non-covalent bond is formed. Therefore, self-healing is an intrinsic property of supramolecular gels. Other interesting properties of supramolecular hydrogels include the accessibility of guest-incorporation, thermoreversibility and tunability of the non-covalent binding energy.

**Fibrous supramolecular gels**

One particularly interesting group of supramolecular hydrogels is based on rods or fibers of supramolecular aggregates which, for the sake of simplicity, are aggregates with a high aspect ratio. These rods either form networks by themselves due to rod-rod interactions or are crosslinked to form a viscoelastic material. These networks are particularly relevant because of their analogy with biological materials that are built up from fibrous proteins. The analogy to biological structures is exploited in peptide amphiphiles, which use known peptide sequences for α-helices and β-sheets to self-assemble into supramolecular rod-like aggregates. Investigation of the aggregation behavior of peptide amphiphiles has shown for example that in β-sheet-forming peptides the chirality of the amino acids determines the helicity of the formed tape. Other small molecules have been used in the design of systems that assemble into long fibers. A common abbreviation is LMOG, or Low Molecular mass Organic Gelators, which form SAFINs or Self-Assembled Fibrous Networks. These systems are discussed in detail in the seminal work *Molecular Gels*. The small molecules are mostly amphiphilic and use a wide range of supramolecular interactions. When the molecules are designed with directional supramolecular interactions, a high tendency for the formation of rods with a fixed structure of high regularity is found.

This structure can in many cases be related to the crystal structure of the compounds, however evidence that the
exact structure is retained in aqueous solution is often difficult to obtain.\textsuperscript{25} Adding water-solubilizing chains to known stack-forming supramolecular motifs is a specific form of this structure-based design, which is often successful provided that the balance between solubility and binding strength is maintained.\textsuperscript{26} Gel formation of rod-like supramolecular aggregates is regularly ascribed to the network of rods, without describing the specific mode of interactions between the rods.\textsuperscript{20} This is an important element of the network properties since high aspect ratio rods are usually stiffer than polymer chains. A network of stiff fibers without crosslinks or entanglements would not yield a strong gel.\textsuperscript{15,20} Introducing interactions between the aggregates has been shown to yield networks with mechanical properties that are not an inherent property of the rod-like aggregates themselves.\textsuperscript{27} A network can be formed by direct interaction between rods acting as entanglements or crosslinks, but the rods may also be crosslinked by the addition of specific crosslinkers. The crosslinkers can be supramolecular telechelic polymers, with supramolecular motifs at the chain ends that are incorporated into the rods.\textsuperscript{28–31} Crosslinking supramolecular rods creates a doubly transient network, comparable to the network properties of segmented polymers with supramolecular crosslink sites in the main chain.\textsuperscript{32} However, systems based on supramolecular rods offer more tunability of the interactions and flexibility of the main chain of the (supramolecular) polymer. Systems based on the crosslinking of supramolecular rods also prove an interesting platform for the investigation of fundamental network properties, such as crosslink density versus crosslinker concentration and crosslink efficiency in terms of loops and bridges formed in the network.\textsuperscript{33}

1.3 Hydrogel characterization
Characterization of hydrogels has been performed with a wide range of techniques.\textsuperscript{7} With any of those techniques, care has to be taken to prevent drying because it may change the properties of the gel. It is obvious that gel properties that are influenced by solvent content, such as the mechanical properties, change upon loss of water. For other aspects of gels, such as the network morphology, the effects of drying are also important, but less commonly appreciated. Network morphology can change dramatically upon removal of
solvent due to collapse, especially with weak fibers or high solvent fraction gels. Furthermore, fiber formation is often concentration dependent and improper sample preparation may lead to erroneous conclusions about the fiber properties, even when lyophilization is used to minimize artifacts. Lyophilization has been shown to cause morphological changes to fibers and complex fibrous structures. Therefore, the morphological characterization of the hydrogels in this thesis has been performed with CryoTEM whenever possible, because it characterizes the gels in their solvated state.

1.3.1 Rheology

Gels are viscoelastic materials, with mechanical properties that are intermediate between elastic solids and viscous fluids and their mechanical behavior is neither described by Newton’s law (for linear viscous fluids) nor Hooke’s law (for pure elastic solids). Oscillatory rheology simultaneously measures viscous and elastic properties and is therefore a valuable technique in the characterization of gels. In these measurements, the linear elastic response of the material is given as the storage modulus $G'$ and the linear viscous response is the loss modulus $G''$.

Figure 1: a) Plate-plate geometry in an oscillatory rheometer. b) Elastic, viscous and viscoelastic response to an applied strain.

These terms originate from the energy stored in the sample upon deformation and the energy that is dissipated. In general, gels that have $G' > G''$ are interpreted as solid-like materials, whereas gels with $G'' > G'$ are considered liquid-like. However, when interpreting oscillatory shear measurements, care has to be taken to perform the measurements in the linear regime, where there is no (local) change of the moduli with changing strain. Outside of this regime, the measured stress is no longer proportional to the applied strain.
and this means the material behaves in a non-linear fashion. This has consequences for the interpretation of the storage and loss moduli in the strain softening and strain stiffening regime, since in the non-linear regime the interpretation of the storage modulus as a measure for the elastic response (and the loss modulus as a measure for the viscous response) is not valid anymore. Thus, because this interpretation of the modulus is not valid more designated experiments should be used to get more information about the material, such as representation of the measurement in a Lissajous plot.\textsuperscript{42} This will also give more insight in the reversibility of the yielding of the material.

**Strain stiffening**

In contrast to the above-mentioned strain softening, some materials stiffen above a certain strain. These materials are strain stiffening and show an increase of the moduli upon deformation. Strain stiffening has an important biological function in rupture prevention of soft lung tissue and blood vessels.\textsuperscript{43} In biological tissues strain stiffening originates from the mechanical behavior of filamentous proteins, such as collagen and actin.\textsuperscript{44} These proteins form fibers that have an intermediate flexibility and thus have a persistence length that is comparable to their contour length.\textsuperscript{45–47} The persistence length ($L_p$) of semi-flexible proteins (and any polymer) is given as the length over which correlation between segments in the direction of the chain is lost, i.e. the length over which the motion of one segment does not correlate with the motion of another segment.\textsuperscript{48} The contour length ($L_c$) is in this context given as the length of the chain in an extended form, equal to the length of the extended polymer backbone. Regarding only the flexibility of these proteins is not sufficient to explain their behavior as a network. In order to explain the properties of the network, a model was developed that describes the strain stiffening of biological tissue as a network of semi-flexible rods crosslinked by short flexible linkers.\textsuperscript{47}
Besides protein networks, other materials display strain stiffening of which rubbers are the most commonly known. However, both the morphology and mechanical behavior of rubbers are very different from gel networks because the latter contains solvent. In synthetic supramolecular systems, non-linear mechanical behavior has been studied but the origin varies across the different systems. These systems show strain stiffening behavior caused by biomimetic rigid filaments, reorganization of reversible bonds and strain-induced entanglement constraints. Ultimately, strain stiffening in these systems is based on either a structural reorganization upon deformation or on the non-linear extension of a chain. In Chapter 5 the shared properties of systems in these categories will be discussed in more detail.

1.3.2 CryoTEM

Cryogenic transmission electron microscopy is a specialized electron microscopy method in which the samples are prepared in their liquid state and cryogenically frozen instead evaporating the liquid. This is especially useful for the imaging of supramolecular assemblies which often display concentration-dependent morphologies. Furthermore, cryogenically frozen samples remain solvated and thus any change in morphology by changing the local environment is prevented.
The power of CryoTEM is illustrated by the fact that the whole aggregation pathway of micelles has been imaged with this technique, showing both the geometrical change from spheres to rods and the growth of the rods (Figure 3). By increasing the concentration in small increments, the transition from a population completely consisting of spheres to an infinitely long network of interconnected rod-like micelles was observed for the gemini surfactant dimethylene-1,2-bis(dodecyl dimethylammonium bromide (12-2-12). CryoTEM can be used to determine the size of aggregates, but the analysis of a statistically significant number of aggregates often requires a high number of images, especially when fibers are analyzed which are longer than a single image at high magnification, while the diameter of the fibers is too small to obtain sufficient resolution at a lower magnification.

In the characterization of hydrogels, CryoTEM gives valuable information about the morphology of the network. However, the standard sample preparation method is not designed for highly viscous solutions. The samples studied in this thesis were prepared with a VitroBot instrument, which automates the blotting and
injection into the cryogenic medium. During the preparation process the liquid sample is blotted using filter paper. This leaves a very thin layer of sample that is subsequently frozen in liquid ethane. Blotting of viscous or solid-like samples has to be carefully tuned to give a sample thickness around 200 nm. This ensures that enough sample is present while it remains transparent to the electron beam without too much scattering. Furthermore, thicker samples more slowly transport heat from the sample to the liquid ethane, which hinders the vitrification process and results in crystallized water.

1.4 Bis(urea)
The urea group has been known since the early days of organic chemistry. Its preparation in 1828 is considered as the starting point of organic synthesis. Besides the use of urea in protein chemistry for denaturation, urea and its derivatives have had relatively little use in the history of organic chemistry. However, the bifurcated hydrogen bonding structure of urea has found a place in supramolecular chemistry (Figure 4).

A specific urea-based motif is the bis(urea), which is a term used to describe molecular structures that have two ureas in close proximity. The array of two double bifurcated hydrogen bonding motifs has been utilized in various systems including gels, surfaces, supramolecular polymers and thermoplastic elastomers. The distance between the urea groups in the solid state is 0.46 nm. For aggregates in solution it is assumed that the distance is similar. The majority of bis(urea) systems reported in literature have an aliphatic spacer between the two urea groups, but other variations have been synthesized, including those with cyclohexylene, tolylene and phenylene spacers. Aliphatic spacers have been used as indiscriminate separation between the two urea groups, but have recently shown to be self-sorting (see following section).
Semi-flexible rods of bis(urea) bolaamphiphiles

Poly(ethylene glycol) (PEO) based bolaamphiphiles with a central hydrophobic segment with the bis(urea) motif have been studied in our group previously. The general structure of the bolaamphiphiles is shown in Figure 5.

![Figure 5: Molecular structure of self-sorting bolaamphiphiles with variable aliphatic spacer length m.](image)

The bis(urea) motif forms hydrogen bonds in aqueous solution because it is shielded from the environment by the hydrophobic spacer between the PEO and the bis(urea) segments. Combined with the geometry of the molecular structure, this results in a rod-like assembly of the bolaamphiphiles.

![Figure 6: Semi-flexible rods formed by the assembly of the bis(urea) bolaamphiphiles (U3U at 1 mg/mL).](image)

CryoTEM has shown that bolaamphiphiles with $3 < m < 7$ form semi-flexible rods as shown in Figure 6. The diameter of the rods is 3-5 nm, which corresponds approximately with the contour length of the bolaamphiphiles (Figure 6). Although the morphology of rods of different bolaamphiphiles is similar, it has been shown that bolaamphiphiles with non-matching bis(urea) motif self-sort in solution (Figure 7).
In the rods, probes that can form an exciplex upon molecular contact can be incorporated when the probes have a bis(urea) motif that has the same CH$_2$ spacer. Upon mixing the intensity of the exciplex emission is followed and the equilibrium value is determined. When using rods with different bis(urea) motifs and their respective matching probes, a much lower intensity of the exciplex emission is observed. This shows that there is self-sorting, but the effect is not 100%, which means some non-matching bolaamphiphiles are present in rods.

1.5 Hydrogels in tissue engineering

The application of hydrogels in tissue engineering is a very active field that focuses on the biocompatibility, biomimicry, bioactivity and injectability of hydrogels. Injectable hydrogels are being designed to function inside of the body and are not administered by surgery, but by using less invasive methods, e.g. via a syringe or catheter. This requires that the gels are formed in the body by means of a trigger, or that they show shear thinning and set quickly after extrusion into the body. Other requirements, such as biodegradability, mechanical properties and biological functionality depend on the application and preferably, can be tuned in a modular fashion.

Biomimetic hydrogels aim to imitate the properties of biological tissue, with a strong interest in mimicking the properties of the extracellular matrix. The interest in biomimetic properties is both fundamental as well as application oriented. The fundamental aspect concerns the imitation and understanding of mechanical properties of biological tissue, such as the strain stiffening of...
biopolymers that is discussed in paragraph 1.3.1. Ultimately, this should lead to a better insight in the mechanisms that govern biological processes and the behavior of cells in their native environment.\(^77\) In tissue engineering, such biomimetic materials can serve as scaffolds for cell growth and stem cell cultures. In their pioneering paper Discher et al. showed that the differentiation of stem cells is influenced by the elasticity of the cell culture scaffold.\(^79\)

![Figure 8: Influence of scaffold elasticity on the differentiation of stem cells.\(^79\)](image)

A scaffold with a low Young’s modulus was shown to favor differentiation of brain cells, whereas a stiff scaffold will favor differentiation into bone cells (Figure 8). Further research has shown that it is not the bulk (macroscopic) mechanical properties of the scaffold but the mechanical properties of the environment directly surrounding the cells that is key in influencing the differentiation.\(^80\) This is especially relevant when the distance between crosslinks is of the same order of magnitude as the dimensions of the cell (i.e. the distance between the relevant receptors).

### 1.6 Aim of this thesis

The aim of this thesis is to develop supramolecular hydrogels with biomimetic mechanical properties, focusing on the strain stiffening that is characteristic for fibrous biopolymers in their native environment. The design of the hydrogelators used in this thesis is generally based on the use of the directional bis(urea) motif to enhance specificity of aggregation of amphiphilic molecules with PEO hydrophilic segments. In the consecutive chapters, several hydrogel systems are developed, and the complex relation between
molecular structure, morphology and mechanical properties of the
supramolecular hydrogels is described.
Chapters 2, 3 and 4 follow a bottom-up approach, adopting the
design shown in Figure 2. Semi-flexible rods are crosslinked with
flexible linkers to obtain an experimental counterpart to the
theoretical model that was developed to describe the behavior of
fibrous networks of biopolymers. In Chapter 5 a top-down approach
is followed that shows the characterization of a strain stiffening
hydrogel. Combined, these chapters give a detailed insight into the
relations between the characteristics on different length scales of
supramolecular hydrogels. In the final chapter a more application-
minded approach is taken, because an affordable and synthetically
accessible supramolecular hydrogel is designed and characterized.
Chapter 2 describes the crosslinking of semi-flexible rod-like micelles
formed by PEO and bis(urea) based bolaamphiphiles. The
crosslinkers are long, flexible poly(ethylene glycol) chains (M_w= 8
kDa), functionalized at both ends with two of the same hydrophobic
bis(urea) segments as in the bolaamphiphiles. When the two ends
become incorporated in different rods, a crosslinked viscoelastic
network is formed. The efficiency of crosslinking is improved by
making use of heterocrosslinkers with two different hydrophobic
ends. These heterocrosslinkers have less tendency to form
mechanically inactive loops within the same rod and thus form more
bridges between rods at the same crosslinker concentration.
Although the gels have tunable mechanical properties, they show no
strain stiffening and possible solutions for this problem are
addressed in Chapters 3 and 4.
Chapter 3 proposes a solution for the absence of strain stiffening in
the gels reported in Chapter 2, by eliminating the long PEO linker in
the crosslinks. The system of rod-like micelles is crosslinked by the
interaction between carboxylic acids and calcium ions. This creates
much shorter crosslinks than the PEO linker and thus eliminates
crosslinker flexibility as a possible cause for the absence of strain
stiffening. The carboxylic acid crosslinking sites are introduced with
a dicarboxylic acid bis(urea) bolaamphiphile that is incorporated into
the rods.
Chapter 4 addresses another potential origin of the lack of strain
stiffening in Chapter 2, namely the weakness of the non-covalent
bonds in the semi-flexible rods. When straining the network, these
will fail before the covalent bonds in the crosslinker. Therefore, a diacetylene group is introduced between the two urea groups, to polymerize the rods with the crosslinker in the axial direction after self-assembly.

Chapter 5 presents the characterization of a strain stiffening hydrogel based on segmented polymers of PEO and the bis(urea) motif. Below the critical gelation concentration these polymers form nanoparticles, which upon increasing concentration form fibers. With the formation of fibers, a change from strain yielding to strain stiffening is observed in the rheological characterization of the hydrogel. Using descriptive theoretical models from literature, the cause for strain stiffening is attributed to a structural reorganization of the fibrous network.

In Chapter 6, an isomeric mixture of dimerized fatty acid is functionalized with two PEO chains of varying length in order to obtain hydrogels from affordable components with a relatively low molecular weight. The influence of the PEO length on the mechanical properties is studied with basic qualitative methods as well as oscillatory rheology.
1.7 References

(10) Steed, J. W.; Atwood, J. L. In Supramolecular Chemistry; John Wiley & Sons, Ltd, 2009
(38) De Vicente, J. Viscoelasticity - From Theory to Biological Applications; InTech, 2012.
(40) Barnes, H. A. A handbook of elementary rheology; University of Wales, Institute of Non-Newtonian Fluid Mechanics, 2000.
(41) D. Weitz; H. Wyss; R. Larsen GLT Lab. J. 2007, 3-4, 68–70.
(49) Flory, P. J. Chem. Rev. 1944, 35, 51–75.


(75) Place, E. S.; Evans, N. D.; Stevens, M. M. Nat. Mater. 2009, 8, 457–470.


Chapter 2

*Tuning the crosslink density between semi-flexible rods using the self-sorting of the bis(urea) motif*
2.1 Introduction
Hydrogels are crosslinked materials that absorb a substantial amount of water. They are of enormous economical importance due to their use as food additives, in the oil industry and for biomedical applications. In all applications their mechanical behavior is of paramount importance, and is determined to a large extent by crosslink density. In physical hydrogels, the crosslinks are reversible, and control over mechanical behavior can be obtained by tuning chemical structure to create well-defined and specific crosslinking interactions. Crosslinks are formed by specific parts of the components through aggregation by non-covalent interactions, such as ion complexation and hydrophobic interactions. Specificity and additional strength of aggregation may be obtained by additional physical interactions such as hydrogen bonding. The combination of multiple non-covalent interactions gives highly desirable mechanical properties to natural hydrogelators such as collagen or actin. The recent realization that mechanical properties and forces play an important role in the behavior of cells has opened up new markets for materials with tunable mechanical properties, with considerable potential for use in, for instance, tissue engineering. Despite their attractive mechanical properties, the use of natural hydrogelators in biomedical applications is limited by biocompatibility issues. With the aim of gaining full control over properties of biocompatible hydrogels, several synthetic approaches to physical hydrogels have been reported. Amino acids are popular building blocks in synthetic hydrogelators, both in engineered polypeptides and synthetic peptide amphiphiles because the chemical diversity of amino acids allows tuning of hydrophobicity and creates the possibility to engineer specific recognition motifs. Alternatively, hydrogelators have been developed with biocompatible poly(ethylene oxide) (PEO) as hydrophilic component and peptide or with fully synthetic motifs as aggregating parts. Gels composed of fully synthetic components give maximum freedom in designing specific interactions and can therefore lead to precisely controlled structures and functions. It has been shown that combined hydrophobic interactions with the hydrogen bonding recognition motif of the urea functional group in amphiphilic bis(urea) molecules that aggregate in water form rod-like micelles and tubular structures.
Tuning the crosslink density between semi-flexible rods using the self-sorting of the bis(urea) motif

Figure 1: Structures of the bolaamphiphiles and crosslinkers. The numbers m and n designate the number of methylenes between the ureas.

The reversible nature of the bonds within the rod-like micelles makes them suitable as platforms for reversible crosslinking. Moreover, the bolaamphiphiles reported by us do not gelate in the absence of crosslinkers, making them ideal building blocks to control crosslinking with specifically designed crosslinkers. Recent work from our group showed that segmented polymers of PEO and bis(urea) motifs have the potential to form injectable scaffolds for biomedical applications. The introduction of the bis(urea) recognition unit also was shown to give rise to the phenomenon of self-sorting. Bolaamphiphiles with different chemical structures form separate micellar populations in water, based on molecular self-recognition of the bis(urea) motif in mixtures of enantiomeric bolaamphiphiles or bolaamphiphiles with different spacing between urea groups.

The aim of the work described here is to gain maximum control over the mechanical properties of hydrogels by controlling the network topology with specific supramolecular interactions. In theoretical work, Storm et al. showed that any network created by suitable flexible crosslinking of semi-flexible filamentous proteins in solution creates a viscoelastic network. Their theoretical model predicts that crosslinking bis(urea) based rod-like micelles with a flexible linker provides similar viscoelastic properties.

Hence, a system was designed to crosslink rods via a flexible linker. The crosslinkers are composed of two of the bis(urea) hydrophobic motifs also found in the bolaamphiphiles, connected by a flexible polyethylene glycol linker (Figure 1). A crosslinker with two bis(urea)
blocks can connect two micellar rods by incorporation of the bis(urea) into two separate rods.

![Image]

**Figure 2**: Limitation of crosslinking by formation of loops (left); suppression of loop formation in a system of mixed bolaamphiphiles and heterocrosslinker (right).

However, such a crosslinker may also form intramicellar loops, thereby reducing crosslinking efficiency (Figure 2). Intramicellar loops will not contribute to the crosslink density and will therefore decrease crosslinker efficiency. By making use of self sorting, intramicellar loop formation may be suppressed and crosslinking efficiency may be increased by using crosslinkers that preferentially connect different rods. Crosslinking by heterobifunctional molecules has been reported in literature, where the heterocrosslinker is used to influence the mechanical properties of the material. Here, we introduce heterobifunctional crosslinkers with self-sorting bis(urea) motifs and compare their crosslinking efficiency with homobifunctional crosslinkers by determining mechanical properties of the gels. The effects on gel modulus are compared to the predicted values using a standard statistical-mechanical approximation of the network topology, combined with the theoretical predictions from the semiflexible network model.

### 2.2 Synthesis

A synthetic strategy was developed to prepare crosslinker molecules that combine two terminal hydrophobic bis(urea) blocks with a central hydrophilic PEO block ($M_w = 8000$). The synthetic strategy aims at crosslinkers with a segment sequence hydrophilic–hydrophobic–hydrophilic–hydrophobic–hydrophilic and provides crosslinkers with a single type of hydrophobic block ($m=n$). However, with this strategy heterocrosslinkers cannot be obtained in a straightforward manner, and therefore we decided to prepare
crosslinkers with a statistical mixture of U4U and U6U hydrophobic segments. The resulting mixture of crosslinker molecules is expected to contain 50% of the 4X6 heterocrosslinker and 25% of each homocrosslinkers, 4X4 and 6X6. This mixture is denoted as \( 4X6 \) to indicate the difference with pure 4X6.

The multistep synthesis (Scheme 1) was performed through reaction of the PEO derivative 1 with amine functionality with one of the isocyanato groups. The excess diisocyanate was separated from the product by precipitation of the reaction mixture with hexane. Intermediate 2 with a single isocyanate group was reacted with PEO derivative 3 to give the desired crosslinker.

2.3 Morphology
As shown before, the UnU bolaamphiphiles form long rod-like micelles that can be imaged with CryoTEM.\(^{13,18}\) The samples for TEM and rheology were prepared by mixing the respective dry compounds and dissolving them in deionized water under sonication at 40°C.
As a reference the rod-like aggregation of U6U, used in this chapter, is shown in Figure 3. The length of the rods lies above 1 micrometer and the diameter is 3-5 nm. In the absence of bolaamphiphiles, the crosslinkers form ill-defined aggregates in solution.

When a matching crosslinker was added to a solution of bolaamphiphiles, the length of the rods significantly decreased from an average of over 1 micrometer to 30 - 300 nm (Figure 4). A
CryoTEM image of the effect of 6X6 on a solution of U6U rods is shown in Figure 4. This is shown at a decreased concentration of 0.2 mg/mL of U6U and 0.1 mg/mL of 6X6 compared to Figure 3, since at higher concentrations the identification of crosslinking becomes difficult due to the close packing of the rods. Furthermore, Figure 4 shows pair formation of the rods, which is caused by the addition of the crosslinker.

In Figure 5 close packing is observed for the rods at 4 mg/mL of U6U with 2 mg/mL of 6X6 and therefore no difference is observed between crosslinked and non-crosslinked rods. Compared to Figure 4, the concentration is one order of magnitude higher, but is still one magnitude lower than the concentration for the gels in section 2.4. Therefore, besides sample preparation issues due to the viscosity, CryoTEM images at higher concentrations will not give further information on the crosslink density.
A decrease in rod length of U6U upon addition of crosslinker 6X6, as shown in Figure 4, was also deduced from small angle X-ray scattering data (Figure 6 and Figure 7). The rod dimensions were approximated by fitting the scattering profiles to the Kholodenko model for worm-like micelles. The length of uncrosslinked rods exceeded 140 nm, the highest length that could be determined within the range of q values of the scattering wave vector. In the presence of 1.25 mg/mL of 6X6 crosslinker, a decrease in the length
of the rods, to an average length of 72.4 ± 0.4 nm was determined. This length is longer than the average distance between crosslinks and will therefore have a limited influence on the mechanical properties. In the Kholodenko model, a length scale that is a measure for the stiffness is included that is defined as the part of the worm-like micelle that can be seen as a straight cylinder. The fitted value of this parameter increases from 3.6 to 6.5 nm (± 0.09 and ± 0.05 respectively) upon addition of the crosslinker, indicating that coupling of the rods also has an effect on their stiffness. This can be explained by seeing the rods coming closer to each other and therefore having more resistance to bending.

2.4 Rheology

Linear and non-linear rheological behavior of the crosslinked semi-flexible rods was determined under oscillatory shear. All measurements were performed at a frequency where the modulus is constant over a broad frequency range. To ensure an even comparison the weight percentage of the gelators was kept constant. Typical concentrations were 1.0 wt% for the amphiphilic rods and 0.5 wt% for the crosslinker.

![Figure 8: Strain-dependent storage and loss modulus, showing the effect of crosslinking at constant concentration of U6U (1 wt%) and 6x6 (0.5 wt%) respectively.](image)

Figure 8 shows a significant increase of the moduli upon adding crosslinker to the bolaamphiphiles. The storage modulus of the solution of bolaamphiphiles was around 0.08 Pa, where the torque is close to the detection limit of the instrument. Therefore, not all data
points are reliable, but an approximate value of the modulus can still be given. Mixing the rods and the matching crosslinker creates a viscoelastic network in water with a modulus that exceeds the values of the individual components. The network resulting from a solution of the crosslinker 6X6, which is an associative polymer, has a $G'= 4 \text{ Pa}$, giving a viscous liquid.

![Graph](image)

**Figure 9:** Strain dependent storage and loss modulus, showing the effect of matching and non-matching crosslinking at constant concentration of U4U or U6U (1 wt%) and 6x6 (0.5 wt%) respectively.

In order to investigate the effect of molecular recognition on the mechanical properties, 0.5 wt% of the 6X6 crosslinker was mixed with separate, 1 wt% solutions of non-matching U4U bolaamphiphile rods and with matching U6U rods. Figure 9 shows the strain dependent measurement of the storage and loss moduli of these systems. The modulus of the system with matching crosslinkers (U6U + 6X6) increased approximately 75 fold compared to the solution of the crosslinker alone. Remarkably, upon addition of the non-matching U4U bolaamphiphiles, both moduli decreased by a factor of almost 7.
Tuning the crosslink density between semi-flexible rods using the self-sorting of the bis(urea) motif

![Graph showing strain-dependent storage and loss modulus](image)

Figure 10: Strain-dependent storage and loss modulus, showing the effect of homocrosslinking or heterocrosslinking at constant concentrations of U4U+U6U (1 wt%) and crosslinker (0.5 wt%) respectively.

Half of the molecules of statistical heterocrosslinker 4X6 contain two different bis(urea) motifs, which have been shown to self-sort in solution. Thus, incorporation of 4X6 in a system containing both bolaamphiphiles may be expected to preferentially crosslink between U4U and U6U micelles, and to have a decreased fraction of mechanically inactive loops as depicted in Figure 2. As a reference 6X6 is given that has similar moduli as 4X6, which is not shown.

When 0.5 wt% of the heterocrosslinker was dissolved together with 1 wt% of a 1:1 mixture of bolaamphiphiles U4U and U6U, a gel with a storage modulus of 6000 Pa was obtained (Figure 10). This value is approximately 15 times higher than the modulus (390 Pa) obtained with homocrosslinker 6x6 in the same mixture of bolaamphiphiles.

2.5 Discussion

Crosslink density is often only moderately controlled in viscoelastic networks. The addition of the homocrosslinker to the bolaamphiphiles shows this phenomenon as well. Even though the molar ratio of the rods and the crosslinker can be determined, one of the possible drawbacks of the system with the homocrosslinker is the possibility of looping of the flexible linker. In equilibrium and at equal composition, the probabilities of looping or crosslinking are determined statistically by the binding affinities. For homocrosslinkers, this affinity must be identical regardless of whether the linker loops back, or bridges to a neighboring rod. While the energetics are the same, the effect on mechanics is not: In
the case of both ends of a linker connecting the same rod (i.e. a loop) it provides no contribution to the modulus of the system. When the connection is made between two different rods, the network structure is reinforced and the crosslink does contribute to the macroscopic properties, i.e., the modulus of the network. Since we want maximal control over the effective linking in a network, we seek to decrease the amount of loops formed in the network. We achieve this by exploiting the self-sorting effect in a solution of mixed semi-flexible rods. The design of the heterocrosslinker is such that the two bis(urea) motifs have different methylene spacers. Mixing this heterocrosslinker with a mixture of two different self-sorting bolaamphiphiles creates a system where the crosslinker will preferentially bind between two rods. The driving force for this behavior is the difference in binding energy for the matching and non-matching bis(urea) motifs. This difference can be determined by reviewing the previous self-sorting results of this system,\textsuperscript{18} according to Equation 1

\[
\Delta G = \ln K_{eq} = \ln \frac{k_{non-match}}{k_{match}} = \ln \left( \frac{U6U^{\prime}inU4U_r}{U6U_r \times U4U_r} \right) = \ln \left( \frac{U6U^{\prime}inU4U_r}{U6U^{\prime}inU6U_r} \right).
\]

**Equation 1:** Calculating the difference in binding energy between matching and non-matching U6U\textsuperscript{\prime} is the number of molecules in the rods, U6U\textsubscript{f} is the number of free monomers, U6U\textsubscript{r} is the number of rods.

From this it can be determined that the difference in binding energy between the U4U and U6U motifs is -6.48 kJ/mol, which equals -2.6 k_BT at room temperature. The net effect, therefore, is that the energy of the bridge configuration is lowered thereby rendering it more likely to occur. In what follows, this value is used as a mismatch penalty, which is defined as the energetic cost of non-matched insertion of a linker bis(urea) domain. Each of these states is characterized by an energy which equals the number of mismatches (i.e., 0, -2.6 k_BT or -5.2 k_BT). Using standard statistical-mechanical techniques, we compute the probabilities of each of these states as

\[
P(state) = \left( \frac{1}{Z} \right) e^{-\frac{\Delta G(state)}{k_BT}}
\]

with P(state) being the probability for loops or bridges and Z being the partition function describing the possible configurations. This is
subsequently summed over all states that yield bridges, and this procedure is repeated for the homo- and the heterosystem to determine the probability ratio of bridges between the hetero and homo systems which also gives the ratio of the number of bridges:

\[
\frac{P_{\text{bridge}}(\text{hetero})}{P_{\text{bridge}}(\text{homo})} = 1.45 = \frac{N_{\text{bridge}}(\text{hetero})}{N_{\text{bridge}}(\text{homo})}
\]

In other words, at the exact same polymer concentration, the heterocrosslinker system is 45% more effectively linked by the same amount of crosslinker – a powerful way to create densely connected gels without increasing their mass. To relate this figure to an increase in mechanical modulus, the relation between \(G'\) and gel architecture for semiflexible systems is needed, appropriate for the fairly rigid rods in the system. MacKintosh and coworkers\textsuperscript{27} derived that

\[
G' \propto \frac{k_B T L_p^2}{\xi^{-1} \zeta_p^2}
\]

where \(L_p\) is the persistence length of the rods, \(\xi\) is the mesh size of the gel, and \(L_x\) is the length between two linker molecules along the backbone of a polymer. We compare the homo and the hetero system, which will have approximately similar persistence lengths. The crosslinking length, however, will scale as the inverse number of crosslinkers and as such the mesh size is a function of the polymer concentration, and scales as [crosslinker]\textsuperscript{-2}.\textsuperscript{28} While it might appear that this concentration remains constant, there is a subtlety here: only those rods that are actually part of the meshwork contribute to the low-strain, low frequency modulus. This is where the hetero-network receives another boost in modulus; because of the enhanced bridging, more rods are recruited into the network and the effective concentration rises as a result. With this recruitment effect, the concentration ratio between the homo- and hetero systems also becomes 1.45, and this is summarized as the predicted relative increase in modulus

\[
\frac{G'(\text{hetero})}{G'(\text{homo})} = \left(\frac{c(\text{hetero})}{c(\text{homo})}\right)^4 \left(\frac{N_{\text{bridge}}(\text{hetero})}{N_{\text{bridge}}(\text{homo})}\right)^3 = \left(\frac{N_{\text{bridge}}(\text{hetero})}{N_{\text{bridge}}(\text{homo})}\right)^7 \approx 14
\]

This number agrees well with what is found experimentally in Figure 10, an increase of a factor of about 15 in storage modulus upon switching from the homo- to the hetero system. This illustrates the
feasibility of mechanical control through designer crosslinks: the system is highly dependent on even small changes in the energetics. Shen et al.\textsuperscript{24} have reported on the pronounced effect of heterobifunctional crosslinkers on the erosion resistance of physically crosslinked hydrogels, but the difference in modulus between systems with homo and heterocrosslinkers is about 20%. In recent work by Mes et al., an increase in modulus, of 10-30% compared to homocrosslinkers was reported.\textsuperscript{23} However, direct comparison between homocrosslinkers and heterocrosslinkers is difficult, because additional parameters, such as solubility are effected by the change in structure.

The 15-fold increase in storage modulus as a result of the increased efficiency of the heterocrosslinker in this chapter shows that small molecular differences can have a great impact on the macroscopic properties of hydrogels, especially when important parameters such as the crosslinking density are targeted. The current work shows quantitatively that the increase can be attributed to the difference of 2.6 $k_B T$ in binding energy and using standard statistical-mechanical calculations that this would give an increase of a factor 14 in the storage modulus. This closely matches our experimental result demonstrating that indeed, due to the effects of self sorting of the different bis(urea) motifs and therefore the heterocrosslinking, considerable increases in mechanical functionality may be obtained.

### 2.6 Conclusion

The crosslink density is an important parameter for the macroscopic mechanical properties of a viscoelastic network. However, in addition to the concentration of crosslinkers, the efficiency of the crosslinker has to be regarded. In the system with the homocrosslinker it was shown that the semi-flexible rods could be linked together into a viscoelastic network. The heterocrosslinker suppresses looping, which is an intrinsic problem in physically crosslinked systems: the system is driven to an equilibrium state in which the probability of forming a loop is statistically larger when there is no preference for binding other rods.

The efficiency of the heterocrosslinker in forming bridges between the rods, is shown by a modulus that is a factor of 15 higher than the modulus of the system with the homocrosslinker. This shows that by using self-sorting and the resulting difference in binding energy of -
6.5 kJ/mol, a highly efficient crosslinked system is obtained. Standard statistical-mechanical calculations show that the difference in binding energy would result in a network topology whose $G'$ is increased by a factor of 14, very close to the experimental results. The difference in the crosslinking efficiency of the homocrosslinker and heterocrosslinker shows supramolecular control over the mechanical properties.
2.7 Experimental

**Materials**: Solvents used in synthesis were reagent grade. CH$_2$Cl$_2$, CHCl$_3$, Et$_3$N and pyridine were distilled from CaH$_2$. All PEO derivatives were dried in vacuum over P$_2$O$_5$ during at least 12 h. The reagents 11-aminoundecanoic acid, poly(ethylene glycol)-monomethyl ether ($M_n = 350$), 1,4-diisocyanatobutane, 1,6-diisocyanatohexane and polyethylene oxide ($M_w = 8000$) were purchased from Aldrich, Fluka, or Acros and were used without additional purification. 11-Aminoundecanoyl-(poly(ethylene glycol)-monomethylether)-ester$^1$ was prepared according to literature procedures.

**General Methods**: NMR spectra were acquired on a 400 MHz Varian Mercury Vx (400 MHz for $^1$H-NMR, 100 MHz for $^{13}$C-NMR). Proton and carbon chemical shifts are reported in ppm downfield of tetramethylsilane using the resonance of the deuterated solvent as internal standard. Splitting patterns are designated as singlet (s), doublet (d), triplet (t) and multiplet (m). Infrared spectra were measured on a Perkin Elmer 1600FT-IR. Matrix assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF) was performed on a Perceptive DE PRO Voyager MALDI-TOF mass spectrometer using $\alpha$-cyano-4-hydroxycinnamic acid as the calibration matrix.

**Rheology**: Mechanical properties of these hydrogels were tested by using rheology. Dynamic viscoelastic measurements were determined using a stress-controlled rheometer (Anton Paar, Physicia MCR501) equipped with a sand-blasted plate-plate geometry to prevent slippage. Measurement temperature was fixed at 20°C.

**Cryogenic transmission electron microscopy**
Samples for cryogenic transmission electron microscopy (cryo-TEM) were prepared in a ‘Vitrobot’ instrument (PC controlled vitrification robot, patent applied, Frederik et al 2002, patent licensed to FEI) at room temperature and a relative humidity >95%. In the preparation chamber of the ‘Vitrobot’ a 3 μL sample was applied on a Quantifoil grid (R 2/2, Quantifoil Micro Tools GmbH; freshly glow discharged just prior to use), excess liquid was blotted away and the thin film thus formed was shot (acceleration about 3 g) into liquid ethane. The vitrified film was transferred to a cryoholder (Gatan 626) and
Tuning the crosslink density between semi-flexible rods using the self-sorting of the bis(urea) motif

observed at -170 °C in a Tecnai microscope operating at 120 kV. Micrographs were taken at low dose conditions.

Small-angle X-ray scattering (SAXS)
The Small-angle X-ray scattering (SAXS) measurements were performed at the Dutch-Belgian BM26B beamline at the ESRF in Grenoble (France). A sample-to-detector distance of 4.53 m was used together with an X-ray photon energy of 12 keV. The observed \( q \) range was \( 0.04 \text{ nm}^{-1} \leq q \leq 2.07 \text{ nm}^{-1} \), where \( q \) is the magnitude of the scattering vector \( q = (4\pi / \lambda ) \sin \theta \), and where \( \lambda \) is the X-ray wavelength and \( \theta \) is half of the scattering angle.

SAXS images were recorded using a 2D Pilatus 1M detector with 748×748; pixel dimension and with 260 μm2 pixel size. The 2D images were radially averaged in order to obtain the intensity \( I(q) \) vs. \( q \) profiles. The beam centre and the \( q \) range calibrations were achieved by using the position of the diffraction peaks of a silver behenate.

The liquid samples were contained in 2 mm borosilicate capillaries. Standard data reduction procedures, i.e. subtraction of the empty capillary contribution, correction for the sample absorption, were applied. Water has been used as secondary standard calibrants in order to perform intensity calibration on an absolute scale in cm\(^{-1}\).

The SAXS intensity \( I(q) \) scattered by an ensemble of monodisperse objects can be written as:

\[
I(q) = N_p (\Delta \rho)^2 V^2 P(q) S(q)
\]

where \( N_p \) is the number density of scattering objects, \( \Delta \rho \) is the electron densities difference between the object and the surrounding media (i.e. solvent), \( V \) is the object volume, \( P(q) \) is the object form factor and \( S(q) \) is the inter-particle structure factor which takes into account the correlation between the objects in solutions.

Synthesis of tetra(urea) based crosslinkers:
The multistep synthesis (Scheme 1) was performed through reaction of the PEO derivative 1 with amine functionality with one of the isocyanate groups of 1,6-diisocyanatoalkane. The excess 1,6-diisocyanatoalkane was separated from the product by precipitation of the reaction mixture with hexane. Intermediate 2 with one isocyanate group was reacted with PEO derivative 3 to give segmented copolymer, 6X6.

General procedure:
A mixture of 11-aminoundecanoyl-(poly(ethylene glycol)-monomethylether)-ester hydrochloride salt (71.6 mg 0.119 mmol)
and triethylamine (20 mg, 0.2 mmol) in dry dichloromethane was added very slowly dropwise to an excess of 1,6-diisocyanatoalkane (80 mg, 0.47 mmol) over 30 min at 0°C and was allowed to stir for 3 h. Then the solvent was evaporated in vacuum and was washed with dry hexane 3-4 times to remove excess of 1,6-diisocyanatoalkane. Then the monosubstituted isocyanate was reacted with a mixture bis(11-Aminoundecanoyl-(poly(ethylene glycol)-ester hydrochloride salt) (4) (500 mg, 0.059 mmol) and triethylamine (20 mg, 0.2 mmol) in dichloromethane and stirred at room temperature for 6 h. Then the reaction mixture diluted with chloroform and extracted with brine. The organic layer was then dried over anhydrous sodium sulphate and evaporated to give white solid which was recrystallized from diethylether and dichloromethane to yield final products.

**U6U-PEO8000-U6U (6X6)**

Yield: 70%

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta = 5.12, 4.95$ (bs, 8H, NH), 4.22 (t, 4H, $^3J$(H,H) = 4.0 Hz, CH$_2$OCO), 3.71-3.46 (m, 376H, OCH$_2$), 3.38 (s, 6H, OCH$_3$), 3.20-3.09 (m, 16H, CH$_2$N), 2.32 (t, 8H, $^3J$(H,H) = 8 Hz, CH$_2$CO), 1.65-1.58 (m, 8H, CH$_2$CH$_2$CH$_2$NH), 1.51-1.40 (m, 16H, CH$_2$CH$_2$CH$_2$NH), 1.27 (bs, 32H, CH$_2$).

$^{13}$C-NMR (100 MHz, CDCl$_3$, T=295K): $\delta = 173.82, 160.97, 72.54, 71.91, 70.82, 70.59, 70.55, 70.54, 70.30, 69.18, 63.35, 61.67, 59.01, 45.83, 40.38, 34.18, 30.39, 29.82, 29.48, 29.38, 29.34, 29.21, 29.08, 26.94, 25.51, 24.87.

FT-IR (cm$^{-1}$): 3333, 2879, 1733, 1615, 1579, 1466.

GPC (THF; PS standards): $M_n = 10129$ g/mol, PDI = 1.01.

MALDI-TOF [M+Na$^+$] = 10651 ± n*44

**U4U-PEO8000-U4U (4X4)**

Yield: 75%

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta = 4.97, 4.75$ (bs, 8H, NH), 4.22 (t, 4H, $^3J$(H,H) = 4.0 Hz, CH$_2$OCO), 3.71-3.46 (m, 376H, OCH$_2$), 3.38 (s, 6H, OCH$_3$), 3.20-3.09 (m, 16H, CH$_2$N), 2.32 (t, 8H, $^3J$(H,H) = 8 Hz, CH$_2$CO), 1.65-1.58 (m, 8H, CH$_2$CH$_2$CH$_2$NH), 1.51-1.40 (m, 16H, CH$_2$CH$_2$CH$_2$NH), 1.27 (bs, 32H, CH$_2$).

$^{13}$C-NMR (100 MHz, CDCl$_3$, T=295K): $\delta = 173.82, 158.85, 71.91, 70.60, 70.55, 70.49, 70.34, 69.18, 63.35, 59.01, 40.39, 39.74, 34.18, 30.40, 29.46, 29.36, 29.32, 29.22, 29.18, 29.06, 27.54, 26.92, 24.87.

FT-IR (cm$^{-1}$): 3333, 2879, 1733, 1615, 1579, 1466.
Tuning the crosslink density between semi-flexible rods using the self-sorting of the bis(urea) motif

GPC (DMF; PS standards): $M_n = 9591$ g/mol, PDI = 1.027.
MALDI-TOF [M+Na$^+$] = 10498 ± n*44.

U6U-PEO8000-U4U (50% 4X6 + 50% 6X6 & 4X4)
Yield: 65%
$^1$H-NMR (400 MHz, CDCl$_3$): $\delta = 5.12$, 4.95 (bs, 8H, NH), 4.22 (t, 4H, $^3J$(H,H) = 4.0 Hz, CH$_2$OCO), 3.71-3.47 (m, 332H, OCH$_2$), 3.38 (s, 6H, OCH$_3$), 3.20-3.09 (m, 16H, CH$_2$N), 2.33 (t, 8H, $^3J$(H,H) = 8 Hz, CH$_2$CO), 1.65-1.58 (m, 8H, CH$_2$CH$_2$CH$_2$NH), 1.51-1.40 (m, 14H, CH$_2$CH$_2$CH$_2$NH), 1.28 (bs, 32H, CH$_2$). $^{13}$C-NMR (100 MHz, CDCl$_3$, T= 295 K): $\delta =$ 173.85, 158.76, 72.56, 71.92, 70.56, 70.44, 69.20, 63.37, 61.71, 59.03, 40.47, 39.76, 34.20, 30.35, 29.45, 29.31, 29.06, 27.46, 26.90, 24.88.
FT-IR (cm$^{-1}$): 3333, 2879, 1732, 1614, 1578, 1466.

GPC (THF; PS standards): $M_n = 9904$ g/mol, PDI = 1.021.
MALDI-TOF [M+Na$^+$] = 10315 ± n*44
2.8 References

Tuning the crosslink density between semi-flexible rods using the self-sorting of the bis(urea) motif


(22) Testard, V.; Oberdisse, J.; Ligoure, C. Macromolecules **2008**, *41*, 7219–7226.


Chapter 3

Crosslinking of semi-flexible rods by metal-carboxylic acid interactions
3.1 Introduction
Chapter 2 showed that crosslinking of U6U bolaamphiphiles using tetra(urea) crosslinkers yields solid hydrogels with storage moduli up to 6 kPa. However, contrary to what was expected, the materials displayed no strain stiffening at high strains. One of the possible causes for this is the length of the poly(ethylene glycol) (PEO) linker between the hydrophobic bis(urea) segments. One of the possible solutions is to use a method of crosslinking without a long flexible polymer as the linker. In this chapter, the possibility of introducing crosslinking sites in the U6U rods is investigated using metal-carboxylic acid interactions.

This method of crosslinking is designed in analogy with the crosslinks in the well-known alginate polymers. In alginites, the metal-carboxylic acid interaction results in a high crosslink density with a maximum of 1 crosslink per 2 monomer units. The monomers of alginate are β-D-mannuronic acid and α-L-guluronic acid. The polymer consists of isolated monomeric units of β-D-mannuronic acid and α-L-guluronic acid, distributed randomly in homopolymeric blocks of the other monomer, and of blocks with an alternating sequence. The ratio of the two acids and distribution of the blocks varies and depends on the source of the alginate polymers. The carboxylic acids in alginates interact most often with divalent cations, such as Mg\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\) and Ba\(^{2+}\), along with Ca\(^{2+}\) which is found most.

The interaction of metals with carboxylic acids is well-known and has been exploited in synthetic systems, but it also has biological functions such as in nerve excitation. The calcium-carboxylic acid binding is especially efficient; the calcium ion forms a tetrahedral complex with two acid groups and the diacetate configuration has a very high binding energy. The strong bond formation of calcium with carboxylic acids is explained by the strongly ionic nature of Ca\(^{2+}\) because it has a closed shell electron configuration.

The interaction of carboxylic acids with divalent metal ions has been used before in bolaamphiphiles to increase interaction between aggregates, but it is not common to have the carboxylic acid group as a modular component that can be added in tunable ratios.
The system discussed in this chapter is based on PEO bis(urea) bolaamphiphile U6U and a dicarboxylic acid crosslinker AU6UA (Figure 1).

![Figure 1: Bolaamphiphile U6U and the two carboxylic acid crosslinkers AU6UA and AU4UA.](image)

It is described how the AU6UA crosslinker is incorporated in U6U stacks and upon addition of calcium ions forms non-covalent metal-carboxylic acid bonds. These interactions create crosslinks between the U6U rods and thus form a viscoelastic network (Figure 2).

![Figure 2: Schematic representation of the crosslinking of U6U rods with AU6UA incorporated due to the addition of calcium.](image)

The tetra(urea) crosslinkers in Chapter 2 were designed to introduce strain stiffening in the viscoelastic network, but addition to solutions
of rod-like micelles resulted in strain softening instead. One of the possible reasons is that the PEO linker is too long and extends upon deformation of a sample instead of transferring shear force. Removal of the linker unit (i.e. replacing PEO with $M_w = 8$ kDa with acid-calcium-acid bonds), addresses this potential cause for the lack of strain stiffening.

The morphological and mechanical properties of the viscoelastic materials that are obtained from mixtures of U6U, AU6UA and metal ions in water are discussed, and the dependence of these properties on the concentration of the individual components, pH and type of metal is investigated.

3.2 Synthesis

The synthesis of bolaamphiphiles U6U and various derivatives has been reported before.\textsuperscript{16,17} For the purpose of this chapter, an alternative synthetic route towards the bis(PEO) bolaamphiphiles was designed. The original literature procedure was developed with a modular approach in mind that enables the incorporation of various spacer groups in the centre of the hydrophobic block.\textsuperscript{17} The alternative synthetic pathway depicted in Scheme 1 shows an approach that first yields a dicarboxylic acid bis(urea). Subsequently PEO is introduced to obtain bolaamphiphile UnU. This route has the advantage that it produces the proposed crosslinker as an intermediate product. Another potential advantage of this route is that it avoids two difficult purification steps in the literature procedure where bis(ureas) with no, one or two PEO chains are formed. In the alternative route, relatively cheap PEO can be used in large excess to drive the reaction to completion, and only a separation of bolaamphiphiles UnU and unreacted PEO would be required.

![Scheme 1: Alternative synthesis of UnU with the diacid derivative as an intermediate.](image-url)
However, the pathway from Scheme 1 did not proceed as intended, since the solubility of the dicarboxylic acids formed in the first step is very low in common organic solvents and water. Strong non-covalent interactions in the diacid compounds cause the low solubility.\textsuperscript{18} In apolar solvents the bis(urea) and carboxylic acid hydrogen bonding is very strong, while in polar organic solvents and water, aggregation of the aliphatic segments is the more dominant interaction. This was confirmed by adding hydrogen bonding cosolvents such as hexafluoroisopropanol (HFIP) and trifluoroacetic acid (TFA). These cosolvents improved the solubility of the diacids significantly. In a 1:1 mixture of deuterated chloroform and TFA, solubility of the reaction products was sufficient to show by NMR that the synthesis of AU4UA and AU6UA had been successful and had yielded products of high purity. However, the insolubility of the diacids in solvents suitable for the esterification did prevent the completion of the synthetic route (Scheme 1), since no reaction conditions could be found that gave acceptable yield and purity of the bolaamphiphiles.\textsuperscript{19–22} Thus, the dicarboxylic acid crosslinkers were successfully synthesized but subsequent functionalization with PEO was unsuccessful and therefore U6U bolaamphiphiles used in this study were synthesized according to the literature procedure.\textsuperscript{17}

3.3 Solubility of AU4UA and AU6UA
The previous section showed that the dicarboxylic acids were insoluble in common solvents without strong hydrogen bonding cosolvents. However, another method of dissolving the diacids that employs the supramolecular interactions of the bolaamphiphiles was successful. When AU6UA was dissolved in a solution of U6U (e.g. 2 wt% solution of U6U with 2 mol% AU6UA) a clear solution was obtained, indicating that AU6UA is incorporated in the rods of bolaamphiphiles. Furthermore, when mixing AU4UA and U6U in the same concentrations, AU4UA did not dissolve. Taking the self-sorting of the bis(urea) motif into consideration,\textsuperscript{17} the solubility difference in solutions of U6U between AU4UA and AU6UA shows that AU6UA is effectively incorporated into rods of matching bis(urea) bolaamphiphiles.
3.4 Network morphology characterization by CryoTEM

CryoTEM was used to image the morphology of the rod-like micellar aggregates of U6U in solution, and the effect of adding AU6UA on the size and length of the rods. After that the crosslinking due to the addition of calcium was visualized.

![Figure 3: CryoTEM image of U6U in water at 1 mg/mL (left) and U6U (2 mg/mL) with 3 mol% AU6UA (right).](image)

At 1 mg/mL U6U forms rods (Figure 3, left) as has been shown elsewhere in this thesis and literature. The diameter of the rods is 5-8 nm and the length varies but has a minimum of 300 nm. The addition of 3 mol% of AU6UA to a 2 mg/mL solution of U6U has no apparent influence on the morphology of the micellar rods. Figure 3 (right) shows rods with diameters of 5-8 nm and again with lengths above 300 nm. Based only on the TEM images, no influence on the flexibility (persistence length) of the rods was observed. Since the incorporation of the diacid molecules does not interfere with the formation of rods, the mixed rods can be used to study crosslinking of rods through the interaction of the acids with metal salts.
Ca\textsuperscript{2+} (added as CaCl\textsubscript{2}) was the first metal ion used to crosslink the rods via an interaction with AU6UA. Crosslinks between the rods will lead to network formation or bundling of rods. In a solution of 0.88 wt% of U6U with 3 mol% AU6UA and a Ca\textsuperscript{2+} to carboxylic acid ratio of 1:6, broad fibers were present with a diameter of 15-30 nm. (Figure 4). Elsewhere in the sample thicker bundles with a diameter of approximately 60 nm were present that are micrometers long (Figure 4, left). Thus, the addition of calcium to the solution of U6U and AU6UA leads to an increased interaction between the rods.

The fibers observed at several locations in the sample consist of multiple rods as is shown in Figure 5. The fibers split into smaller segments that have the same diameter as U6U rods observed in the absence of crosslinks. On the basis of the CryoTEM images it is not
possible to see whether the single U6U rods are crosslinked but the bundling is certainly an effect of the addition of AU6UA and Ca$^{2+}$. Apparently, under the conditions of the experiment, bundling of U6U rods is favored over network formation. However, the presence of separate 5-8 nm wide rods within the bundles shows that also when they are crosslinked, the rods retain their structure.

### 3.5 Rheology

#### 3.5.1 Addition of calcium to U6U and AU6UA

Solutions of the uncrosslinked U6U rods have very low moduli. Therefore, changes in viscoelastic properties of solutions of U6U in the presence of AU6UA and Ca$^{2+}$ can be attributed to metal-carboxylic acid interactions.

![Figure 6](image.png)

**Figure 6:** Strain sweep of the storage modulus of 1 wt% U6U solutions containing 0 mol% (green), 3 mol% (red), 5 mol% (blue) and 10 mol% (black) AU6UA.

The storage modulus $G'$ of a U6U (1wt%) solution with 0, 3, 5 and 10 mol% of AU6UA is shown in Figure 6. Although the morphology of the rods does not change notably upon addition of AU6UA (Figure 3), the storage modulus of the solutions decreases in the presence of diacid (Figure 6). The value of $G'$ is low in the absence of AU6UA, and decreases further from 1 Pa to 0.1 Pa when 10 mol% of the diacid is added. This shows that the network is slightly altered and since interactions between deprotonated diacid molecules in the absence of calcium ions are repulsive, no crosslinks are being formed. Therefore, either a change in flexibility of the rods, or their
change in length must cause the decrease in $G'$. The CryoTEM data show no evidence for a decrease in length upon the addition of AU6UA. A small decrease in flexibility could therefore be responsible for the observed decrease in $G'$. It is relevant to note the decrease, because it means that the reference value of $G'$ for the addition of calcium is slightly lower than $G'$ for a U6U solution.

![Figure 7: Effect on the modulus of an increasing calcium to carboxylic acid ratio. U6U (1 wt%) with 10 mol% AU6UA.](image)

When CaCl$_2$ is added to a solution of U6U (1 wt%) and AU6UA (10 mol%) in water, there is a small increase in the storage and loss moduli when the calcium to carboxylic acid ratio is increased from approximately 0.1 to 100 as is shown in Figure 7. The increase is modest and can be explained by the effects of fiber bundling shown in the CryoTEM images (Figure 4 and Figure 5). The CryoTEM images could not establish the formation of a network by crosslinking single rods, but the minor increase in modulus observed upon addition of calcium ions indicates that formation of such a network is not probable.

### 3.5.2 Influence of pH on crosslinking efficiency

The pK$_a$ of AU6UA, a dicarboxylic acid with a spacer of more than 4 CH$_2$ groups, is expected to be similar to the pK$_a$ of monocarboxylic acids of 4.8, when the diacid is freely dissolved in water.$^{23,24}$ Since the spacer in AU6UA is 10 CH$_2$ units long, the pK$_a$ is estimated to be 4.8.$^{24}$ However, when incorporated into the amphiphilic rods of
U6U, the acid groups of AU6UA are in a relatively apolar environment, where the pK\textsubscript{a} of carboxylic acids is generally higher. In an unbuffered aqueous solution of U6U (1 wt%) and AU6UA (10 mol%) the pH was determined to be pH ≈ 6, while pH = 3.8 was expected based on the pK\textsubscript{a} of 4.8. Thus full deprotonation of AU6UA carboxylic acid groups is not ascertained. Since deprotonation is required for efficient complexation of calcium ions, this could be an explanation for the modest increase in modulus shown in Figure 7.

On the other hand, the apparently more apolar environment of the carboxylic acids is caused by the PEO of the surrounding U6U bolaamphiphiles, which can also cause some steric hindrance. Therefore, the effect of the addition of calcium to a U6U and AU6UA solution at higher pH was investigated. The pH was increased using a Tris-HCl (2-amino-2-hydroxymethyl-propane-1,3-diol) buffer. Furthermore, to increase the possibility of COO\textsuperscript{−}–Ca\textsuperscript{2+} bond formation, the concentration of calcium ions was increased to obtain a large excess of Ca\textsuperscript{2+} over carboxylate.

At 2 wt% U6U and pH = 7.8 the effect of increasing both pH and [Ca\textsuperscript{2+}] are already visible during sample preparation. Above 250 equivalents of calcium per acid group, a self-supporting gel was obtained. This was further characterized by strain-dependent oscillatory rheology measurements that show an increase of G\textsuperscript{'} and G\textsuperscript{''} with increasing equivalents of calcium (Figure 8). The gel changes from a viscous liquid to a solid-like material with a G\textsuperscript{'} of 11 Pa. Another property that changes is the yield strain of the hydrogel, from 5 % strain without calcium to over 100 % strain at calcium concentrations above 250 equivalents.
The effects of further increasing the pH on the modulus are shown in Figure 9. At pH = 9 the modulus strongly increases to 13 Pa when 250 eq of Ca$^{2+}$ are added, however, only a small change is observed above 250 equivalents of Ca$^{2+}$. This indicates that there is either a saturation of the available crosslinking sites or that there is a geometric network constraint that more crosslinks do not lead to a higher modulus. Figure 9 also shows the influence of calcium-carboxylic acid interactions at pH = 13 in the same range of concentrations as the measurement for pH = 9. In contrast to the pH = 6 and pH = 9 measurements, a maximum of the modulus is observed for the modulus at 125 equivalents of Ca$^{2+}$ to carboxylic acid. This is probably due to precipitation of Ca(OH)$_2$, which has a solubility product of 5.02×10$^{-6}$ M$^3$. Hence, at pH 13 ([OH$^-$] = 0.1 M), the concentration of free Ca$^{2+}$ is 5 × 10$^{-4}$ M at most. Since the total amount of calcium in the system exceeds the amount that can be present as free calcium or calcium bound by carboxylates, solid calcium hydroxide particles will be formed. This leads to a complex multiphase system in which the contribution of the bis(urea)-based network on gel modulus cannot be evaluated separately.

### 3.5.3 Gelation with other metals

The binding of carboxylic acids with divalent cations is not exclusive for calcium. For example alginates are crosslinked by Mg$^{2+}$, Zn$^{2+}$, Cu$^{2+}$ and Ba$^{2+}$, indicating that interaction of these metal ions with a carboxylic acid is strong enough to induce changes in the mechanical properties. Especially zinc and copper proved to be effective crosslinkers for alginate. With alginates, there is no need for a high
pH to achieve sufficient crosslink density and therefore metal hydroxide formation does not create a problem. However, the hydroxides of the above mentioned metals have very low solubility, especially at high pH. This makes them less suited to increase the interaction between the U6U rods. Tabletop gelation tests with magnesium, zinc and copper salts showed that only zinc was suitable as a crosslinker in the U6U and AU6UA system. Critical gelation concentrations and pH were similar to the conditions required to induce gelation with calcium and comparable to optimal alginate crosslinking conditions.

As shown in Figure 10, the gels that were obtained with a Zn$^{2+}$ to carboxylic acid ratio of 500:1 and 2 wt% U6U with 5 mol% of AU6UA, had similar mechanical properties to the gels crosslinked with calcium at equal pH and ion concentration. The storage modulus was approximately 1 Pa at pH = 5.6 and $G' = 12$ Pa at pH = 7.8. The precipitation of Zn(OH)$_2$ is more pronounced at pH > 10 than for Ca(OH)$_2$, which is evident from their solubility product $K_{sp}$(Ca(OH)$_2$) = 5.02x10$^{-6}$ M$^3$ >> $K_{sp}$(Zn(OH)$_2$) = 3.0x10$^{-17}$ M$^3$. Therefore, zinc seems less suitable for crosslinking the U6U and AU6UA system than calcium.
3.5.4 Non-covalent interactions with carboxylic acid as crosslinking mechanism

Hydrogen bonding may provide an alternative to metal-carboxylate interactions for non-covalent crosslinking in the U6U and AU6UA system. The carboxylic acid group of AU6UA can also form hydrogen bonds with other acids or form salt bridges with suitable basic groups. To investigate these systems and circumvent potential problems of accessibility of the AU6UA, hidden in the core of the rod-like micelles, the use of telechelic PEO with either carboxylic acid or amine end groups was investigated.

![Figure 11: Telechelic bis(acid)-PEO or bis(amine)-PEO.](image)

The direct crosslinking by addition of bis(acid)-PEO or bis(amine)-PEO did not result in an increase in viscosity. However, when 5 mol% bis(amine)-PEO was added to an aqueous solution of U6U (2 wt%) and AU6UA (5 mol %), the opaqueness of the solution was reduced. A reference experiment with triethylamine instead of bis(amine)-PEO showed the same reduction in opaqueness. The effect may be caused by a reduction of the interaction of the carboxylic acid of AU6UA and the ethylene glycol repeat unit of the U6U bolaamphiphiles. It has been reported that carboxylic acids can interact with the oxygen of the PEO backbone. This decreases the solubility of the PEO and results in less dissolved rods. The interaction of a carboxylic acid with an organic base is much stronger and will compete with the acid-PEO interaction. This results in a better solubilized PEO and the opaqueness disappears. Further indication of an direct interaction of the carboxylic acid and triethylamine was a reference experiment with NaOH at pH = 13 that did not show a reduction in opaqueness.

Finally, crosslinking with a combination of bis(acid)-PEO and Ca$^{2+}$ was explored. It was anticipated that the bis(acid)-PEO would readily penetrate the PEO segment of the rods and interact with calcium bound to a AU6UA carboxylate group. This is schematically depicted in Figure 12.
Figure 12: Schematic representation of the crosslinking of bis(acid)-PEO in the presence of calcium in a U6U and AU6UA system.

Figure 13: Strain sweep (f = 1 rad/s) of 2 wt % U6U with 5 mol % AU6UA, [Ca\(^{2+}\)]/[COOH] = 500, pH=7.8 with various ratios of [bis(acid)-PEO]/[AU6UA] (left). Storage and loss moduli of 2 wt % U6U with 5 mol % AU6UA, [Ca\(^{2+}\)]/[COOH] = 500, pH=7.8 with optimal [bis(acid)-PEO]/[AU6UA] ratio of 0.1 and a reference experiment without bis(acid)-PEO (right).

The addition of the telechelic PEO to a 2 wt% U6U solution with 5 mol% of AU6UA and 500 equivalents of Ca\(^{2+}\) resulted in a small increase of the modulus, but a decrease in the yield strain of the material with a 1:10 ratio of [bis(acid)-PEO]/[AU6UA] (Figure 13). The deviation from the linear behavior of the storage modulus is taken as the yield point of the gel, since above this strain there is no immediate recovery of the modulus (data not shown). A ratio higher than 0.1 bis(acid)-PEO per molecule of AU6UA led to a decrease in modulus and thus appears to have a negative effect on the network. A possible explanation for the behavior of the multi-component system could be the formation of a double network at high enough
bis(acid)-PEO concentrations, because this molecule can form a supramolecular polymer by interaction with $\text{Ca}^{2+}$. The formation of a double network can lead to either an increase or a decrease of the mechanical properties depending on parameters such as concentration and competition between components.\textsuperscript{28}

3.6 Polarized optical microscopy

The alignment and bundling of the individual U6U rods observed in CryoTEM (Figure 4) suggested that the overall structure of the system would be anisotropic. Therefore, optically detectable anisotropy, similar to what has been observed in (liquid) crystals, was characterized using polarizing microscopy.

![Polarized optical microscopy images](image)

*Figure 14:* Polarized optical microscopy images of A) U6U (2 wt%) with AU6UA (5 mol%), B) $\text{Ca}^{2+}$ at same concentration as 250 eq., pH = 13, C) U6U (2 wt%) with AU6UA (5 mol%), 250 eq. $\text{Ca}^{2+}$, pH 7.8, D) U6U (2 wt%) with AU6UA (5 mol%), 250 eq. $\text{Ca}^{2+}$, pH 13.
The U6U rods crosslinked by the AU6UA-carboxylic acid interaction at pH = 13 show birefringence (Figure 14D). This birefringence does not originate from the U6U rods (Figure 14A), the U6U rods with AU6UA and calcium at pH = 7.8 (Figure 14C) or precipitated Ca(OH)$_2$ (Figure 14B). Thus, the birefringence is only observed when the U6U rods in a highly crosslinked state are added to the solution with Ca(OH)$_2$. The presence of the rods leads to sufficient alignment and bundling to provide an ordered structure.

![Figure 14D](image)

**Figure 15**: Fan-like features in POM image Figure 14D.

Figure 15 shows fan-like features (also called Maltese crosses or extinction crosses) observed in the POM images of Figure 14D. Such features are generally ascribed to smectic phases or columnar (e.g. nematic) packing in liquid crystals, although the fans in Figure 15 are not perfectly symmetric. Taking the structure of the long U6U rods into consideration, there is no apparent morphological arrangement that allows for layers in the direction perpendicular to the bundles. Thus the smectic phase structure is less feasible in this system. But the columnar packing is supported by the TEM images in Figure 4 and thus the birefringence in the system with U6U, AU6UA and Ca(OH)$_2$ is likely due to the bundling into long fibers.

### 3.7 Discussion

Calcium-induced gelation of the aqueous U6U-AU6UA system yields a viscoelastic network. However, the increase in modulus upon gelation is much smaller than observed for crosslinking of U6U with a long tetra(urea) PEO crosslinker (see Chapter 2). Several factors appear to be involved in the low efficiency of crosslinking the AU6UA. The accessibility of the carboxylic acid is low, shown by the acidity that is lower than expected from theoretical considerations.$^{24}$ This shows the direct environment is less polar than water, which is
caused by PEO. Thus the PEO surrounding the carboxylic acid makes it less available for forming crosslinks. Moreover, the limited increase of the modulus upon addition of bis(acid)-PEO also indicates that the accessibility of the AU6UA carboxylate groups is low. The high number of equivalents of calcium needed also suggests not all of the carboxylic acid sites are available. Nevertheless, at higher pH, a significant number of carboxylic acids are available and the modulus increases significantly.

The interaction with PEO of both the carboxylic acid and metal ions also hinders the formation of crosslinks. The interaction of AU6UA with PEO was already discussed in paragraph 3.5.4. PEO and carboxylic acids can form hydrogen bonds, which because of the shielding of the carboxylic acid will be retained at high pH. Only when organic bases are added that can penetrate the surrounding PEO cloud better, a decrease in the opaqueness can be observed. This was tested with bis(amine)-PEO and triethylamine and the addition of both showed a clear solution at pH ≈ 9.

The cations can also interact with PEO, as has been shown in the comprehensive work of Yanagida et al. The interaction occurs between the free electron pairs of oxygen in the PEO backbone and is similar to the metal-oxygen interaction in crown ethers. However, the interaction with PEO is favored for heavier metals and less for calcium. These interactions with PEO are also a possible reason for the inability to form gels with copper and some other metals.

3.8 Conclusion

The interaction between rods of U6U bolaamphiphiles is weak and is not sufficient to form solid-like hydrogels. Crosslinking these rods with tetra(urea) PEO linkers does yield solid hydrogels, but they are not strain stiffening. One way to address this is to eliminate the flexible linker by using metal-carboxylic acid interactions between the rods instead.

This chapter shows that the dicarboxylic acid bis(urea) AU6UA can interact with Ca\textsuperscript{2+} and form crosslinks between U6U rods. The incorporation of AU6UA in the rods was shown by a lack of
incorporation of AU4UA into U6U rods. This shows that self-sorting can be used to indicate otherwise difficult to determine phenomena. Crosslinking these U6U and AU6UA rods was successful with calcium and zinc ions but was especially effective with Ca$^{2+}$. CryoTEM showed bundling of individual U6U rods into broad and long fibers. At an excess of 125 equivalents of Ca$^{2+}$ per carboxylic acid or more solid-like hydrogels were obtained. The formation of solid-like hydrogels was most efficient at high pH, when the carboxylic acids in AU6UA are deprotonated. Using polarized optical microscopy, birefringence of the crosslinked gel network could be shown, indicating a high degree of order within the fibers of the hydrogel.

The crosslinking of the U6U rods with metal ions was successful, however no strain stiffening was observed. Thus the elimination of the long PEO linker was not sufficient to obtain the predicted behavior. The chapter does show that supramolecular rods can be crosslinked by an orthogonal non-covalent interaction, yielding viscoelastic networks.
3.9 Experimental

Materials
All solvents used were of AR quality or better and purchased from Biosolve, Sigma-Aldrich or Acros. DMF and DCM were dried over molsieve (3 Å). All chemicals were purchased from Sigma-Aldrich, Acros or Fluka and used without any further purification.

General sample preparation
Samples were prepared by weighing AU6UA and U6U in the dry solid state, addition of 250 µL of milliQ water or the desired buffer solution and sonication until dissolved (between 12 and 48 hours, no degradation observed). Addition of ions occurred through addition of a few microliters of a highly concentrated solution of the desired cation in water or the appropriate buffer after dissolving AU6UA and U6U. The cation was always administered under the form of the metal chloride species.

Highly basic samples (pH > 13) were obtained by addition of 20.04 µL of a 3 N NaOH solution. The Tris-HCl buffer was obtained by dissolving 3.036 g of 2-amino-2-hydroxymethyl-propane-1,3-diol in 100 mL of milliQ water and titrating the solution with 1 M HCl until the desired pH was obtained. The resulting solution was further diluted to a volume of 250 mL with milliQ water in order to obtain a 0.1 M Tris-HCl buffer of the desired pH.

The reported weight percentages of the samples studied within this chapter are the weight percentages of the sample before addition of ions or strong base. The addition of ions and/or strong base induces a small dilution of the sample resulting in a real weight percentage that is 0.16 wt% (max) lower than the reported value.

Measurements
NMR spectra were acquired on a 400 MHz Varian Mercury Vx (400 MHz for $^1$H-NMR, 100 MHz for $^{13}$C-NMR). Proton and carbon chemical shifts are reported in ppm downfield of tetramethylsilane using the resonance of the deuterated solvent as internal standard. Splitting patterns are designated as singlet (s), doublet (d), triplet (t) and multiplet (m).

Rheology: Mechanical properties of these hydrogels were tested by oscillatory rheology. Dynamic viscoelastic measurements were performed using a stress-controlled rheometer (Anton Paar, Physicia
MCR501) equipped with a sand-blasted plate-plate geometry to prevent slippage. Measurement temperature was fixed at 20 °C.

**Cryogenic transmission electron microscopy**

Samples for cryogenic transmission electron microscopy (CryoTEM) were prepared in a ‘Vitrobot’ instrument (PC controlled vitrification robot, patent applied, Frederik et al 2002, patent licensed to FEI) at room temperature and a relative humidity >95%. In the preparation chamber of the ‘Vitrobot’ 3 μL sample was applied on a Quantifoil grid (R 2/2, Quantifoil Micro Tools GmbH; freshly glow discharged just prior to use), excess liquid was blotted away and the thin film thus formed was shot (acceleration about 3 g) into liquid ethane. The vitrified film was transferred to a cryoholder (Gatan 626) and observed at -170 °C in a Technai microscope operating at 120 kV. Micrographs were taken at low dose conditions.

**Matrix-assisted laser desorption ionization time-of-flight mass spectrometry** (MALDI-TOF MS) was performed with a PerSeptive Biosystems Voyager-DE PRO spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix.

**Infrared spectroscopy** (IR) spectra were recorded at RT on a PerkinElmer Spectrum One FT-IR spectrometer with a universal ATR sampling accessory.

**Synthetic procedures**

**Bis-1,6-(11-(polyethyleneglycol(monomethylether)-carbonyl)-undecyl)-ureido)-hexane (U6U)**

\(^1\)H NMR (399 MHz, CDCl\(_3\), T=295K) δ = 4.87 (d, 4H, NH), 4.22 (t, 4H, CH\(_2\)OCO), 3.73 – 3.51 (m, 60H, OC\(_2\)H), 3.38 (s, 6H, OCH\(_3\)), 3.15 (m, 8H, CH\(_2\)N), 2.32 (t, 4H, CH\(_2\)CO), 1.81 – 1.74 (m, 4H, NCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)N), 1.67 – 1.56 (m, 4H, NCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)), 1.47 (s, 4H, CH\(_2\)CH\(_2\)O), 1.27 (s, 24H, CH\(_2\)).

\(^13\)C NMR (100 MHz, CDCl\(_3\), T=295K) δ = 173.84, 158.78, 71.90, 70.59, 70.57, 70.49, 69.18, 63.35, 59.00, 40.43, 39.43, 34.18, 30.33, 29.82, 29.44, 29.30, 29.17, 29.04, 26.89, 25.47, 24.86

FT-IR (cm\(^{-1}\)): 3332, 2921, 2852, 1731, 1613, 1576, 1477, 1467, 1103

MALDI-TOF: [M+Na\(^{+}\)] =1325.87 ± 44

**13,22-Dioxo-12,14,21,23-tetraazatetratriacontane-1,34-dioic acid (AU6UA)**

A 100 mL round bottom flask containing 1.042 g (5.18 mmol) of 11-aminoundecanoic acid, 0.525 g (5.18 mmol) of triethylamine and 40
mL of dry dimethylformamide was heated to 80°C and stirred for 1 hour. 0.436 g (2.59 mmol) of 1,6-diisocyanatohexane dissolved in 2 mL of dry dimethylformamide was added dropwise to the solution under argon atmosphere. The reaction mixture was stirred and refluxed at 80°C for 3 hours and an additional 12 hours at room temperature. The mixture was diluted with 40 mL of methanol which yielded after filtration and washing with methanol 1.177 g (80%) of the pure product as a white solid.

\[ \text{1H NMR (399 MHz, CDCl}_3+ 2\muL \text{ trifluoroacetic acid-d per mg compound, T=295K)} \delta = 3.24 (m, 8H, CH}_2N), 2.44 (t, 4H, CH}_2CO), 1.72 – 1.56 (m, 12H, CH}_2CH}_2X \text{ with X=N or O), 1.41 – 1.23 (m, 36H, CH}_2). \]

\[ \text{13C NMR (100 MHz, CDCl}_3+ 2\muL \text{ Trifluoroacetic acid-d, T = 295 K)} \delta = 183.45, 158.65, 41.79, 41.40, 33.40, 28.72, 28.63, 28.43, 28.35, 25.92, 24.19FT-IR (cm}^\text{-1}) : 3326, 2922, 2849, 1690, 1612, 1568, 1476, 1458, 917 \]

\[ \text{MALDI-TOF: [M+Na}^+]=570.44 \]

**13,20-Dioxo-12,14,19,21-tetraazadotriacontane-1,32-dioic acid (AU4UA)**

A 100 mL round bottom flask containing 2.084 g (10.37 mmol) of 11-aminoundecanoic acid, 1.04 g (9.88 mmol) of triethylamine and 40 mL of dry dimethylformamide was heated to 80°C and stirred for 1 hour. 0.727 g (5.19 mmol) of 1,4-diisocyanatobutane dissolved in 2 mL of dry dimethylformamide was added dropwise to the solution under argon atmosphere. The reaction mixture was stirred and refluxed at 80°C for 3 hours and an additional 12 hours at room temperature. The mixture was diluted with 40 mL of methanol which yielded after filtration and washing with methanol, the pure product as a white solid in non-determined yield.

\[ \text{1H NMR (399 MHz, CDCl}_3+ 2\muL \text{ trifluoroacetic acid-d per mg compound, T=295K)} \delta = 3.24 (m, 8H, CH}_2N), 2.44 (t, 4H, CH}_2CO), 1.72 – 1.56 (m, 12H, CH}_2CH}_2X \text{ with X=N or O), 1.41 – 1.23 (m, 36H, CH}_2). \]

\[ \text{13C NMR (100 MHz, CDCl}_3+ 2\muL \text{ Trifluoroacetic acid-d, T = 295 K)} \delta = 182.23, 159.33, 41.86, 40.87, 33.92, 29.12, 28.91, 28.84, 28.75, 28.70, 26.35, 25.45, 24.41FT-IR (cm}^\text{-1}) : 3326, 2922, 2849, 1690, 1613, 1570, 1476, 1466 \text{ MALDI-TOF: [M+Na}^+]=542.44 \]
3.10 References


(7) Tasaki, I.; Byrne, P. M. Biopolymers 1994, 34, 209–215.


Chapter 4

Polymerizable networks of
diacetylene bis(urea) rod-like micelles
4.1 Introduction

In Chapter 2 it was shown that crosslinking of U6U bolaamphiphiles, using crosslinkers with two bis(urea) motifs at opposite ends of a long PEO linker, gives solid-like hydrogels with a storage modulus of 6 kPa. However, contrary to what was expected, the materials displayed no strain stiffening at high strains. One of the possible causes is that strain yielding of the system occurs due to failure of the supramolecular interactions between the bolaamphiphiles and/or the crosslinkers. An approach to counter failure of the weak interactions is presented in this chapter where the supramolecular interactions in the rod-like micelles are reinforced with covalent bonds.

Figure 1: Schematic representation of the mechanism discussed in this chapter. Above is the proposed mechanism of failure of the system described in chapter 2. At the bottom is the non-failing polymerized system discussed in this chapter.

In the top half of Figure 1, one of the possible mechanisms of network failure due to shear is displayed. After dissolving the bolaamphiphiles and the crosslinker, the components self-assemble into a viscoelastic network. Under shear, the gels show strain yielding, caused by failure of the supramolecular interaction in the network. Failure can occur at a random position in the rod or specifically at the crosslinking sites. The aim of this chapter is to use self-assembly to obtain the viscoelastic network and subsequently
‘freeze’ this structure by covalent cross-polymerization. The resulting network, shown in the bottom part of Figure 1, remains a network under shear because the non-covalent interactions are reinforced with stronger covalent bonds. For cross-polymerization between the bolaamphiphiles, polymerization of the diacetylene motif was selected.

The cross-polymerization of diacetylenes requires close proximity of the acetylene motifs at a proper orientation, to enable the 1-4-addition reaction shown in Figure 2.\textsuperscript{1} The reaction has a high activation energy and is therefore often initiated with X-ray or UV exposure. In this chapter, the cross-polymerization was initiated with 254 nm UV light. Diacetylenes within a bis(urea) motif have been cross-polymerized in the solid state before, showing that the distance of hydrogen bonding of the ureas and the distance required for efficient cross-polymerization of the acetylene are compatible.\textsuperscript{2,3} The perfect order present in the crystalline solid state is not a requirement for the cross-polymerization to proceed, as polydiacetylenes have also been prepared in aqueous environments.\textsuperscript{4–6} Nevertheless, also in solution there is a need for preorganization of the diacetylenes.

Figure 2: 1,4-Addition cross-polymerization of bis(urea) diacetylenes.

Rod-like aggregation of bis(urea) bolaamphiphiles as presented in Chapter 2 provides preorganization in solution.\textsuperscript{7} Structurally, the introduction of a diacetylene group in the bis(urea) bolaamphiphiles is a minor modification (Figure 3). As a result of inserting
diacetylenes in the center of the hydrophilic part, the distance
between the urea groups is increased from 4 or 6 carbon atoms in
U4U and U6U, to 10 or 12 carbon atoms in the diacetylene bis(urea)
segments designated as UD10U and UD12U, in which the letter D is
used to indicate the polymerizable diacetylene group.

\[
\text{polUD10U}_{8k} \quad p = 2, \ M_w(\text{PEO}) = 8000 \\
\text{polUD10U}_{20k} \quad p = 2, \ M_w(\text{PEO}) = 20000
\]

**Figure 4:** Molecular structure of the segmented polymer crosslinkers with the introduction of the diacetylene group.

In order to closely match the structure of the analogous nonpolymerizable crosslinkers in Chapter 2, diacetylene crosslinkers should have two terminal bis(urea) segments. These molecules were synthesized, but with the same bis(urea) diacetylene motif as the UD10U and UD12U they were not soluble enough to achieve a sufficient crosslink density. Therefore, the more soluble segmented polymers polUD10U_{8k} and polUD10U_{20k} were synthesized (Figure 4). The segmented polymers have the same hydrophobic part as the UD10U bolaamphiphiles, but the PEO polymer is longer (\(M_w = 8000\) and 20000 Da) and bifunctional. The names of the crosslinkers include the prefix ‘pol’ to indicate that it is a segmented polymer, with the suffix ‘8k’ or ‘20k’ indicating the molecular weight of the PEO segments.

This chapter will present the characterization of the cross-polymerization of the rods of bolaamphiphiles with CryoTEM and an investigation of the mechanical properties of the crosslinked viscoelastic networks before and after cross-polymerization using oscillatory rheology.

### 4.2 Synthesis

The synthesis of the diacetylene bolaamphiphiles follows the literature procedure of the bis(urea) bolaamphiphiles of Chapter 2 in which an amine-functionalized PEO and a diisocyanate are coupled to obtain the bis(urea) bolaamphiphiles.\(^7,\)\(^8\) This requires the precursor for the bis(urea) diacetylene motif as a diacetylene diisocyanate. The synthesis was performed by Dr. Asish Pal.
The synthesis of the diacetylene diisocyanate was performed by first forming the diacetylene from 5-hexynoic acid (1) with an oxidative coupling. Subsequently the carboxylic acids were converted into acid chlorides, which yielded a diisocyanate via a Curtius rearrangement. The 1,10-diisocyanatodeca-4,6-diyne (and 1,12-diisocyanatododeca-5,7-diyne) (4) were then used in the previously described bolaamphiphile synthetic pathway. 

Thus the diacetylene diisocyanate was used for the synthesis of UD10U and UD12U, using the previously synthesized PEO with the hydrophobic spacer and amine end-group used in Chapter 2.
The diacetylene diisocyanate was also used for the synthesis of the segmented polymers by polymerizing with a bis(amine) PEO, yielding a diacetylene bis(urea) PEO polymer (Scheme 2). Crosslinkers with two bis(urea) diacetylene motifs with a long PEO linker were synthesized as well, according to the synthetic pathway in chapter 2, but the solubility of these crosslinkers (0.1 mg/mL) was too low for the applications in this chapter.

4.3 Characterization of the topology of supramolecular rods before and after cross-polymerization

CryoTEM was used to image the formation of rod-like aggregates. First, the assembly of the diacetylene bis(urea) rods was investigated with CryoTEM. Subsequently, the topology of the rods after cross-polymerization was studied to determine whether cross-polymerization induces morphological changes in the rod-like micelles.

![Figure 5: Semi-flexible rods of UD10U (left) and UD12U (right) at 1 mM. Scale bars are 100 nm.](image)

Diacetylene bolaamphiphiles UD10U and UD12U were dissolved at 1 mM in water and the solutions were studied with CryoTEM. Formation of rod-like aggregates was observed in the electrographs, with a diameter of 6-9 nm and length of 100 nm to 1 µm.
Polymerizable networks of diacetylene bis(urea) rod-like micelles

After irradiation with 254 nm UV light, the solutions turned a deep blue color. Cryo-TEM showed that the rod-like morphology was retained (Figure 6). There were no apparent changes in the morphology of the rods, since the diameter and length were similar to the uncrosspolymerized rods.

The UV spectra of 1 mM solutions of UD10U and UD12U after cross-polymerization showed peaks at 604 and 660 nm, indicative for the diacetylene crosspolymerization. Further characterization by Asish Pal, using static light scattering, gave an approximate degree of polymerization of 250 which corresponds to a length of 110 nm (assuming 0.46 nm bis(urea) distance). \(^9\)
4.4 Rheological characterization of the rod-like micelles before and after cross-polymerization.

Uncrosslinked rods

The mechanical properties of solutions containing the diacetylene based bolaamphiphiles were investigated with oscillatory rheology. First the effect of cross-polymerization of the rods on the storage and loss modulus was determined. Subsequently, the addition of segmented polymers to crosslink the rod-like micelles was investigated.

![Figure 8: Strain-dependent storage and loss modulus of UD10U (1 wt%) before (left) and after (right) UV irradiation.](image)

A solution of UD10U was characterized using oscillatory rheology at a concentration of 1 wt% before and after 30 min UV irradiation (Figure 8). The absolute value of the moduli is low and approaches the detection limit of the instrument. However, even with the limited accuracy of the measurements at low modulus, it is clear that the differences between the mechanical properties of the solution before and after cross-polymerization are small.

![Figure 9: Strain-dependent storage and loss modulus of UD12U (1.5 wt%) before (left) and after (right) UV irradiation.](image)
The storage and loss modulus of UD12U were determined at a concentration of 1.5 wt% after 30 min UV irradiation. Increasing the concentration compared to the measurements of UD10U, did not increase the value of the moduli. After cross-polymerization the moduli were slightly higher (from 0.1 Pa to 0.2 Pa on average) and the measurement showed less noise than before.

**Rods with segmented polymer crosslinker**

In Chapter 2 it was shown that the addition of cross-linkers to U6U bolaamphiphiles resulted in the formation of a viscoelastic network of semi-flexible rods. Analogous experiments with the diacetylene-containing bolaamphiphiles could not be performed, because the corresponding bifunctional crosslinkers were insoluble. Therefore, segmented polymers polyUD10U_{8k} and polyUD10U_{20k} were used as crosslinkers.

![Figure 10: Strain-dependent storage and loss modulus of UD10U (1 wt%) with polUD10U_{8k} (0.2 wt%) before and after irradiation.](image)

In a strain sweep experiment, the storage and loss modulus of a solution with 1 wt% of UD10U and 0.2 wt% polUD10U_{8k} were determined. Before cross-polymerization, the system has a storage modulus of 6 Pa, significantly higher than either the uncrosslinked system, or the plateau storage modulus of a 0.2 wt% solution of segmented copolymer, which is 0.5 Pa (not shown). Upon irradiation with UV a deep blue solution was obtained, indicating polymerization. Oscillatory rheology experiments showed a decrease
of the storage modulus to $G' < 0.1$ Pa. Thus there is no sign of retaining the mechanical properties after irradiation and the moduli approach the values for uncrosslinked rods.

The same strain sweep experiment was performed with UD10U (1 wt%), but now with polUD10U$_{20k}$ (0.2 wt%) as crosslinker. The plateau modulus of the unpolymerized system is 1.5 Pa. This is lower than the system with the polUD10U$_{8k}$ crosslinker, which has a higher fraction of bis(urea) per weight in the crosslinker. However, upon UV irradiation a decrease of the moduli was observed, similar to the experiment with polUD10U$_{8k}$ as crosslinker. Although the decrease is not as large as for the system with polUD10U$_{8k}$, the effect is the opposite of the intended increase in modulus due to crosslinking.

### 4.5 Discussion

The aim of this chapter was to develop a viscoelastic material that after UV cross-polymerization shows strain stiffening. This was based on the hypothesis that the strain yielding occurred due to breaking of the rod-like micelles, which are held together by the non-covalent interactions of the bis(urea) motifs. Reinforcing the weak bonds with covalent bonds by cross-polymerization would strengthen the weakest link in the system.
Cross-polymerizing the semi-flexible UD10U and UD12U rods resulted in only small changes in the storage and loss moduli. However, considering the rods are semi-flexible and not crosslinked, no significant change in the mechanical properties is expected. Possibly, the difference in modulus is caused by a stiffening of the rods, since their length is not altered (Figure 5 and Figure 6). The addition of the segmented polymers as crosslinkers showed an increase in the moduli of approximately 1.5 orders of magnitude. Thus, the moduli of the mixed system are higher than of the individual components showing that a viscoelastic network is formed. This is comparable to the 2 orders of magnitude increase after crosslinking of micelles of non-polymerizable bolaamphiphiles described in Chapter 2. Upon irradiation of the networks a deep blue color develops, indicating that a reaction takes place, but the moduli of the viscoelastic networks of crosslinked UD10U decrease by a factor of 2, and strain stiffening is not observed. The failure to obtain a crosslinked network might be explained by the polymerization of the diacetylenes which stops at the rod-crosslinker interface. Although the modulus of the network before cross-polymerization shows that the segmented polymer co-aggregates with the rod-like micelles, termination of the cross-polymerization at the point where the crosslinkers are incorporated into the rods would explain the decrease in modulus. A minor difference in diacetylene geometry would be sufficient to terminate the reaction. Precipitation or coagulation of (parts of) the network is also a possible reason for the observed decrease in moduli, but no opaqueness or solid particles were observed after irradiation.

4.6 Conclusion
Chapter 2 showed that crosslinking semi-flexible rods with crosslinkers of two bis(urea) motifs at opposite ends of a long PEO chain will not yield a strain stiffening network. One of the possible reasons for this lack of strain stiffening is failure of the non-covalent interaction between the bola amphiphiles in the rods. This chapter shows that reinforcing this interaction with crosspolymerized diacetylenes has no beneficial effect on the mechanical properties. An analysis with CryoTEM showed that the cross-polymerization of the self-assembled bola amphiphiles has no influence on the morphology of the rods. However, after crosspolymerization with
crosslinkers a decrease of moduli and no strain stiffening were observed.
The approach of reinforcing the non-covalent interactions is not discarded, since one possible difficulty could be the interaction between the rods and the crosslinker. A more soluble crosslinker with two bis(urea) diacetylene motifs at opposite ends could be synthesized, which is effectively incorporated into the rods following the design of Chapter 2. More solubility could be obtained by changing the methoxy end group of the poly(ethylene glycol) into a hydroxy group.
4.7 Experimental

**Materials**: Solvents used in synthesis were reagent grade. CH$_2$Cl$_2$, CHCl$_3$, Et$_3$N and pyridine were distilled from CaH$_2$. All PEO derivatives were dried in vacuum over P$_2$O$_5$ during at least 12 h. The reagents 11-aminoundecanoic acid, poly(ethylene glycol)-monomethyl ether ($M_n = 350$), 5-hexynoic acid and 6-heptynoic acid were purchased from Aldrich, Fluka, or Acros and were used without additional purification. 11-aminoundecanoyl-(poly(ethylene glycol)-monomethylether)-ester was prepared according to literature procedures.$^8$

**General Methods**: NMR spectra were acquired on a 400 MHz Varian Mercury Vx (400 MHz for $^1$H-NMR, 100 MHz for $^{13}$C-NMR). Proton and carbon chemical shifts are reported in ppm downfield of tetramethylsilane using the resonance of the deuterated solvent as internal standard. Splitting patterns are designated as singlet (s), doublet (d), triplet (t) and multiplet (m). Infrared spectra were measured on a Perkin Elmer 1600FT-IR. Matrix assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF) was performed on a Perseptive DE PRO Voyager MALDI-TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid as the calibration matrix.

**Cryogenic transmission electron microscopy**
Samples for cryogenic transmission electron microscopy (CryoTEM) were prepared in a ‘Vitrobot’ instrument (PC controlled vitrification robot, patent applied, Frederik et al 2002, patent licensed to FEI) at room temperature and a relative humidity >95%. In the preparation chamber of the ‘Vitrobot’ 3 μL sample was applied on a Quantifoil grid (R 2/2, Quantifoil Micro Tools GmbH; freshly glow discharged just prior to use), excess liquid was blotted away and the thin film thus formed was shot (acceleration about 3 g) into liquid ethane. The vitrified film was transferred to a cryoholder (Gatan 626) and observed at -170 °C in a Technai microscope operating at 120 kV. Micrographs were taken at low dose conditions.

**Rheology**: Mechanical properties of these hydrogels were tested by oscillatory rheology. Dynamic viscoelastic measurements were performed using a stress-controlled rheometer (Anton Paar, Physicia MCR501) equipped with a sand-blasted plate-plate geometry to prevent slippage. Measurement temperature was fixed at 20°C.
**5,7-Dodecadiynedioic acid.** A suspension of copper(I) chloride (2.63 g, 26.6 mmol) and ammonium chloride (4.78 g, 89.3 mmol) in 14 mL water was added to a solution of 5-hexynoic acid (1 g, 8.92 mmol) in 14 mL of water. The green reaction mixture was heated to 60 °C, and air was bubbled through the solution while stirring vigorously for 2 h. The reaction mixture was quenched with 20 mL of concentrated hydrochloric acid, and the resulting precipitate was collected by suction filtration immediately, washed with a 1/1 mixture of hydrochloric acid/water, and recrystallized from a water/methanol mixture (2/8), resulting in white crystals of compound **1** (650 g, 65%). 1H NMR (400 MHz, CD3OD): δ: 2.43 (t, 4H, C=OC\(_2\)H\(_2\)), 2.35 (t, 4H, CH\(_2\)-C), 1.81 (m, 4H, CH\(_2\)-CH\(_2\)-CH\(_2\)). 13C NMR (100 MHz, CD3OD): δ: 175.3 (C=O), 75.7 (CH\(_2\)-C), 65.3 (C-C), 32.2 (C=O-CH\(_2\)), 23.5 (CH\(_2\)-CH\(_2\)-CH\(_2\)), 17.7 (CH\(_2\)-C).

**5,7-Dodecadiynedioic acid dichloride.** 5,7-Dodecadiynedioic acid (730 mg, 3.3 mmol) was dissolved in an ethanol/tetrahydrofuran (5 mL/5 mL) mixture. To this solution was added an ethanolic NaOH solution (262 mg, 6.57 mmol) at room temperature. Immediate precipitation was observed. After stirring for 5 min the solvent was evaporated in rotavapor and in vacuum, and the residue was dried under vacuum for 5 h. The residue was dissolved in 10 mL of dry ether and 1 drop of dry DMF and was cooled to 0 °C. At this temperature oxalyl chloride (2.5 mL, 32 mmol) was added, and the solution was stirred for 2 h. The resulting suspension was filtered, and the filtrate was evaporated to dryness, resulting in 0.86 g of compound **2**, which was used in the next step without purification. 1H NMR (400 MHz, CDCl\(_3\)): δ: 3.05 (t, 4H, O=CC\(_2\)H\(_2\)), 2.40 (t, 4H, CH\(_2\)-C), 1.92 (m, 4H, CH\(_2\)-CH\(_2\)-CH\(_2\)). FT-IR (cm\(^{-1}\)): 2945, 1793.

**1,10-Diisocyanatodeca-4,6-diyne.** To 5,7-dodecadiynedioic acid dichloride (870 g, 3.3 mmol) in dry acetonitrile (15 mL) was added sodium azide (472 mg, 7.3 mmol) with stirring under an argon atmosphere. The reaction mixture was heated to 65 °C. N\(_2\) started to evolve. After the nitrogen evolution had become negligible (1 h), a white solid was filtered off, and the filtrate was purified by distillation. The diisocyanate (**3**) was collected as a colorless oil (0.35 g, 50%). 1H NMR (400 MHz, CDCl\(_3\)): δ: 3.45 (t, 4H, O=C=N-CH\(_2\)), 2.39 (t, 4H, CH\(_2\)-C), 1.80 (m, 4H, CH\(_2\)-CH\(_2\)-CH\(_2\)). 13C NMR(100 MHz, CDCl\(_3\)): δ 122.4 (O=C=N), 79.4 (CH\(_2\)-C), 65.9 (C-C), 42.2 (N-CH\(_2\)), 31.8 (CH\(_2\)-CH\(_2\)-CH\(_2\)), 13.7 (C-CH\(_2\)). FT-IR (cm\(^{-1}\)): 2959, 2253(-N=C=O).
Polymerizable networks of diacetylene bis(urea) rod-like micelles

**UD10U.** A solution of 11-aminoundecanoyl-(poly(ethylene glycol)-monomethylether)-ester (550 mg, 0.91 mmol) and triethylamine (91 mg, 0.91 mmol) in 2 mL dichloromethane was added to 1,10-diisocyanatodeca-4,6-diyne (94 mg, 0.43 mmol) 2 mL dichloromethane and stirred overnight. The solution was concentrated and the product was purified using column chromatography (silica gel, CHCl₃/methanol 19:1 v/v). Finally, precipitation from diethyl ether yielded 0.85 g (68%) of the product as a white solid.

**1H-NMR (400 MHz, CDCl₃):** δ = 4.82, 4.69 (bs, 4H, NH), 4.22 (t, 4H, J(H,H) = 4.0 Hz, CH₂OCO), 3.71-3.54 (m, 52H, OCH₂), 3.38 (s, 6H, OCH₃), 3.30-3.26 (m, 4H, CH₂N), 3.18-3.14 (m, 4H, CH₂N), 2.31 (t, 4H, J(H,H) = 4 Hz, CH₂CO), 1.74-1.68 (m, 4H, CH₂C), 1.63-1.58 (m, 4H, CH₂CH₂NH), 1.47-1.32 (m, 4H, CH₂CH₂CO), 1.28 (bs, 24H, CH₂).

**13C-NMR (100 MHz, CDCl₃, T=295K):** δ =173.85, 158.55, 71.90, 70.53, 69.17, 65.89, 63.35, 58.99, 40.45, 40.38, 39.21, 39.04, 34.17, 30.32, 30.27, 29.45, 29.42, 29.28, 29.18, 29.15, 29.02, 28.77, 28.70, 26.91, 26.87, 24.86, 16.65. **FT-IR (cm⁻¹):** 3335, 2921, 2852, 1731, 1615, 1579, 1468.

**GPC (THF; PS standards):** Mₙ = 1707 g/mol, PDI = 1.10.

**MALDI-TOF [M+Na⁺] = 1373.74 ± n*44 (calcd: 1373.88 ± n*44).**

**6,8-Tetradecadiynedioic acid.** A suspension of copper(I) chloride (2.63 g, 26.5 mmol) and ammonium chloride (4.78 g, 89.3 mol) in 14 mL water was added to a solution of 5-heptynoic acid (1.12 g, 8.9 mmol) in 14 mL of water. The green reaction mixture was heated to 60 °C, and air was bubbled through the solution while stirring vigorously for 2 h. The reaction mixture was quenched with 20 mL of concentrated hydrochloric acid, and the resulting precipitate was collected by suction filtration immediately, washed with a 1/1 mixture of hydrochloric acid/water, and recrystallized from a water/methanol mixture (2/8), resulting in white crystals (600 g, 65%). **1H NMR (400 MHz, CD₃OD):** δ: 2.33-2.26 (m, 8H, C=OCH₂, CH₂-C), 1.69 (dt, 4H, CH₂-CH₂C=O), 1.54 (dt, 4H, CH₂-CH₂-C). **FT-IR (cm⁻¹):** 3036, 2950, 1699.

**6,8-Tetradecadiynedioic acid dichloride.** 6,8-Tetradecadiynedioic acid (772 mg, 3.1 mmol) was dissolved in an ethanol/tetrahydrofuran (5 mL/5 mL) mixture. To this solution was added an ethanolic NaOH solution (247 mg, 6.17 mmol) at room temperature. Immediate precipitation was observed. After stirring
for 5 min the solvent was evaporated and the residue was dried under vacuum for 5 h. The residue was dissolved in 10 mL of dry ether and 1 drop of dry DMF and was cooled to 0 °C. At this temperature oxalyl chloride (2.5 mL, 32 mmol) was added, and the solution was stirred for 2 h. The resulting suspension was filtered, and the filtrate was evaporated to dryness, resulting in 0.86 g of 6,8-tetradecadiynedioic acid dichloride, which was used in the next step without purification. FT-IR (cm⁻¹): 2939, 1790.

**1,12-Diisocyanatododeca-5,7-diyne.** To 6,8-tetradecadiynedioic acid dichloride (870 g, 3.3 mmol) in dry acetonitrile (15 mL) was added sodium azide (440 mg, 6.8 mmol) with stirring under an argon atmosphere. The reaction mixture was heated to 65 °C. N₂ started to evolve. After the nitrogen evolution had become negligible (1 h), a white solid was filtered off, and the filtrate was purified by distillation. The diisocyanate was collected as a colorless oil (0.45 g, 55%). ¹H NMR (200 MHz, CDCl₃): δ: 3.35 (t, 4H, O=C=N-C₄H₂), 2.32 (t, 4H, CH₂-C), 1.75-1.70 (m, 4H, CH₂CH₂CH₂N), 1.65-1.59 (m, 4H, CH₂CH₂C=O). FT-IR (cm⁻¹): 2950, 2257 (-N=C=O stretch).

**UD12U.** A solution of 11-aminoundecanoyl-(poly(ethylene glycol)-monomethylether)-ester (1 g, 1.65 mmol) and triethylamine (0.27 mL, 1.97 mmol) in 2 mL dichloromethane was added to 1,12-diisocyanatododeca-5,7-diyne (194 mg, 0.787 mmol) 2 mL dichloromethane and stirred overnight. The solution was concentrated and the product was purified using column chromatography (silica gel, CHCl₃/methanol 19:1 v/v). Finally, precipitation from diethyl ether yielded 0.7 g (62%) of the product as a white solid.

¹H-NMR (400 MHz, CDCl₃): δ = 4.60 (bs, 4H, NH₂), 4.22 (t, 4H, J(H,H) = 4.0 Hz, CH₂OCO), 3.71-3.54 (m, 52H, OCH₂), 3.37 (s, 6H, OCH₃), 3.18-3.12 (m, 8H, CH₂N), 2.31 (t, 4H, J(H,H) = 4 Hz, CH₂CO), 1.63-1.57 (m, 8H, CH₂C & CH₂CH₂NH), 1.45-1.38 (m, 4H, CH₂CH₂CO), 1.28 (bs, 24H, CH₂).

¹³C-NMR (100 MHz, DMSO-d₆, T=295K): δ = 173.98, 158.37, 71.90, 70.55, 69.18, 65.75, 63.48, 59.00, 40.54, 39.82, 34.18, 30.19, 29.46, 29.40, 29.26, 29.14, 29.02, 18.92.

FT-IR (cm⁻¹): 3326, 2922, 2852, 1732, 1613, 1578, 1458.

GPC (THF; PS standards): Mₙ = 1939 g/mol, PDI = 1.03.

**Bis(11-ammonium chloride undecanoyl)-poly(ethylene glycol)(8k):** To 30 mL of a 4 M HCl solution in dioxane was added to the solution
of 10 g (1.1 mmol) of bis(N-(tert-butyloxycarbonyl)-11-
aminoundecanoyl)-poly(ethylene glycol) in 30 mL of dioxane and
stirred at 0°C for 1 h and subsequently at room temperature for 12
h. The solvent was evaporated to yield 9.0 g (100%) of the product
as its hydrochloric salt. \(^1\)H-NMR (400 MHz, CDCl\(_3\), T=295K): \(\delta = 7.78\)
(bs, 6H, NH\(_3\)), 4.22 (bs, 4H, CH\(_2\)OCO), 3.90-3.20 (m, OCH\(_2\)), 2.89 (bs,
4H, CH\(_2\)N), 2.32 (t, 4H, CH\(_2\)CO), 1.75-1.48 (m, 8H, CH\(_2\)CH\(_2\)N and
CH\(_2\)CH\(_2\)CO), 1.28 (bs, 28H, CH\(_2\)). MALDI-TOF [M+Na\(^+\)] = 9350.27 ±
n*44.

Bis(11-ammonium chloride undecanoyl)-poly(ethylene glycol(20k)):
Bis(11-ammonium chloride undecanoyl)-poly(ethylene glycol(20k))
was synthesized according to the above mentioned procedure of 8k
material. \(^1\)H-NMR (400 MHz, CDCl\(_3\), T=295K): \(\delta = 7.76\) (bs, 6H, NH\(_3\)),
4.24 (bs, 4H, CH\(_2\)OCO), 3.90-3.20 (m, OCH\(_2\)), 2.92 (bs, 4H, CH\(_2\)N),
2.34 (t, 4H, CH\(_2\)CO), 1.75-1.55 (bs, H\(_2\)O+CH\(_2\)CH\(_2\)N +CH\(_2\)CH\(_2\)CO), 1.28
(bs, 26H, CH\(_2\)).

polUD10U8k. To a solution of 25 mg (0.05 mmol) of 1,10-
Diisocyanatodeca-4,6-diyne in 1 mL of chloroform, solution of
bis(11-aminoundecanoyl)-poly(ethylene glycol) (1.0 g, 0.05 mmol)
and triethylamine (1 mL) in 20 mL of chloroform was added and
stirred for 3 days. The solution was concentrated and precipitated
by addition of diethyl ether, filtered off, and dried.
GPC (DMF; PEO standards): \(M_w =127000\ g/mol, PDI = 1.9\).

polUD10U20k. To a solution of 12.2 mg (0.024 mmol) of 1,10-
Diisocyanatodeca-4,6-diyne in 1 mL of chloroform, solution of
bis(11-aminoundecanoyl)-poly(ethylene glycol) (1.0 g, 0.024 mmol)
and triethylamine (1 mL) in 20 mL of chloroform was added and
stirred for 3 days. The solution was concentrated and precipitated
by addition of diethyl ether, filtered off, and dried.
GPC (DMF; PEO standards): \(M_w =104000\ g/mol, PDI = 1.9\).
4.8 References


Chapter 5

Structure, morphology and mechanical properties of bis(urea)-based hydrogels
5.1 Introduction
The mechanical properties of hydrogels are part of the vast research area of hydrogels, with important applications in fields ranging from oil recovery to medicine. Recently, hydrogels with biomimetic, non-linear mechanical behavior have received considerable attention.\(^1,2\) In the nonlinear regime, stress is no longer proportional to strain. For example, the modulus can decrease or increase when the strain is increased above a critical value. These behaviors are called strain softening and strain stiffening, respectively. In oscillatory shear measurements, which are often used to characterize viscoelastic hydrogels, mechanical characteristics such as the storage and loss modulus are not accurately defined outside the linear regime, but they can still be used to establish the onset of nonlinearity in a qualitative manner.
This Chapter focuses on hydrogels that display strain stiffening, a non-linear elastic response, where the stress increases stronger than linear with the applied strain deformation. In contrast to Chapters 2 to 4 where a design based, bottom-up approach is taken, this chapter uses a top-down approach by characterizing a strain stiffening hydrogel and by elucidating the mechanism and molecular origin of the mechanical behavior.
In biological tissues, strain stiffening plays an important role in rupture prevention of blood vessels and soft lung tissue and is a consequence of the semi-flexible nature of filamentous proteins.\(^3\) The phenomenon of strain stiffening and its physical origins have been widely studied in literature, both theoretically and experimentally.\(^4-6\) Due to its prevalence in biological systems, the mechanism of strain stiffening is relevant in the development of biomimetic scaffolds in tissue engineering.\(^7\) Recent results have shown that the differentiation of stem cells can be influenced by the mechanical properties of the scaffold and thus a material with tunable biomimetic mechanical properties is of key importance in biomedical applications.\(^1,8\)
Strain stiffening in hydrogels has been observed before, but it has mostly been discussed in terms of material properties on the macroscopic scale.\(^6,9\) In gels of biopolymers such as actin and collagen, strain stiffening originates from the semi-flexible nature of the filamentous proteins.\(^4,10-13\) In engineering materials, strain stiffening is well known in rubbers, but its mechanism has less
significance for gel networks, which generally have a high volume fraction of solvent playing an important role in the mechanical properties. The origin of strain stiffening in synthetic gel networks varies and includes stretching of biomimetic rigid filaments, reorganization of reversible bonds and strain-induced entanglement constraints.

In this chapter, the properties of supramolecular bis(urea) hydrogels on multiple length scales are investigated in order to relate the molecular structure and morphology (studied with CryoTEM) to the mechanical properties determined with oscillatory rheology. The hydrogels used for this study consist of amphiphilic segmented copolymers based on hydrophilic poly(ethylene glycol) (PEO) and hydrophobic bis(urea) blocks, of which the molecular structures are shown in Figure 1.

![Figure 1: Structures of amphiphilic segmented poly(ethylene glycol) bis(urea) based segmented polymers.](image)

The polymers are named according to the bis(urea) and PEO length, where the bis(urea) segment is designated with UnU (with n the number of CH₂ groups between the ureas) and the suffix 8k or 20k is used to indicate the Mw of the PEO segments. At low concentrations in water, these polymers aggregate into discrete supramolecular nanoparticles. Water soluble nanoparticles are an active field of research, where such particles have recently been investigated as enzyme mimics. However, this chapter reports the behavior of these polymers at and above the overlap concentration, i.e. when the
behavior of the particles is better described as a semi-dilute than as a dilute system. Our experiments indicate that above a critical concentration the amphiphilic polymers form solid-like hydrogels. However, these hydrogels do not generally display strain-stiffening behavior, which is only observed at sufficiently high concentrations and temperature. Possible origins of this behavior are discussed in the framework of literature models for strain stiffening in supramolecular gels. The physical chemistry of supramolecular gels that forms the foundation of strain stiffening has recently been reviewed by Seiffert and Sprakel. They emphasize that, as a result of the dynamic nature of non-covalent interactions and their sensitivity to environmental parameters, an understanding of the relation between the morphology and the mechanical properties of supramolecular networks is not straightforward, as multiple timescales and various processes originating from the non-covalent nature of the interactions are involved. This chapter aims to describe the mechanical behavior of hydrogels of segmented bis(urea) polymers in terms of the physical chemistry that governs the assembly, morphology and strain stiffening of these materials.
5.2 Synthesis

The segmented polymers used in this chapter were prepared by a step-growth polymerization by the reaction of free amines and isocyanates to form urea groups (Scheme 1). The synthesis was performed by dr. G.M. Pawar. The poly(ethylene glycol) was functionalized with a Boc-protected aminoundecanoic acid. The long aliphatic segment provides hydrophobic shielding for the bis(urea) interaction, as was shown before for many supramolecular motifs. After deprotection of the amine, the bis-amino PEO was polymerized by adding the appropriate diisocyanate. By varying the molecular weight of the poly(ethylene glycol) and the number of CH2-groups between the isocyanates the four segmented polymers U4U8k, U4U20k, U6U8k and U6U20k were obtained.

The degree of polymerization (DP) was increased by performing the reaction under dry and inert conditions in a glove box. Low DPs were obtained under less strict anhydrous conditions. This is most probably due to the hygroscopic nature of the PEO. Water will cause hydrolysis of the isocyanate, which in turn decreases the ratio of isocyanate to amine resulting in a lower DP. The molecular weights obtained for these polymers were $M_w(U4U8k)=84$ kDa, $M_w(U4U20k)=59$ kDa, $M_w(U6U8k)=86.5$ kDa and $M_w(U6U20k)=45$ kDa according to GPC. After precipitation, the polymers had a high purity and could be used for the formation of nanoparticles and hydrogels as discussed in this chapter.

5.3 Morphology below the gelation concentration

The synthesized polymers were dissolved in water at relatively low concentrations between 1 and 40 mg/mL and their behavior was investigated using dynamic light scattering (DLS) and CryoTEM.
Poly(ethylene glycol) forms random coils in water with a radius of hydration that depends on the molecular weight. This experimental relation is well described as a power law of \( R_h = 0.145M_W^{0.571} \). Intramolecular physical and chemical bonds can transform the polymer chains from random coils into single-chain nanoparticles, with a concomitant reduction of their radius. This has previously been shown using a step-wise decrease in size of the particles that fold by a sequential activation of supramolecular motifs. The segmented PEO bis(urea) polymers have been shown to form nanoparticles at concentrations below the critical gelation concentration. The nanoparticles are approximately 15 nm in diameter, according to dynamic light scattering (DLS). However, it is not clear what the equilibrium size of the nanoparticles is, since the preparation method has a strong influence on the final size of the nanoparticles. The diameter of 15 nm was generally the lowest obtained, but poor sonication or freeze-drying and redissolving the particles sometimes gave particles up to 80 nm in diameter. It is unclear whether this variation in size has to do with a folding process that is not completed, as was previously observed for similar systems or that the aggregation number of the polymers in the nanoparticles can vary. Based on the molecular weight of the polymers, PEO with a similar molecular weight would give a diameter of 9.5 nm (for the higher \( M_w = 85 \) kDa, using the relation given above). Since this is lower than the 15 nm found for the nanoparticles, the number of polymers per particle must be higher than one.
The formation of nanoparticles was also investigated with CryoTEM. At a concentration of 2 wt% of U6U8k and 2 wt% of U4U8k the viscosity of the solutions was low enough to allow normal CryoTEM sample preparation. For both U4U8k and U6U8k samples, aggregates with an approximate core size of 13-15 nm are observed, as shown in the CryoTEM electrographs in Figure 2. As these samples were dissolved by sonication, the observed aggregate sizes are not completely in agreement with the DLS results, where for a similar preparation method results indicated the presence of much larger aggregates. Such big aggregates of up to 80 nm diameter, along with an increased size polydispersity were not observed with CryoTEM. The size of the particles was determined by manually measuring the diameter of the dark circular objects. As well-dissolved PEO is not visible under these conditions, these manually extracted aggregate diameters could be smaller than the true aggregate sizes.

Figure 2: CryoTEM images of concentrated nanoparticles of U6U8k (2 wt%) (left) and U4U8k (2 wt%) (right).
Looking with a higher magnification at the nanoparticles of U6U8k than in Figure 2, a close packing can be observed (Figure 3). It has been shown before that classical close sphere packing in the hexagonal close packing (HCP) arrangement is also possible for micelles. In a horizontal cross-section (the TEM image is a horizontal projection of one layer of the sample) the HCP is characterized by a hexagonal unit cell and lattice axes at a 60° angle. Therefore the concentration of the nanoparticles is at least equal to the overlap concentration $c^*$, since the indication of a HCP structure means that the nanoparticles overlap at this concentration. Moreover, the close packing shows that there is a relatively high resistance to interparticle aggregation, because there is no sign of the formation of bigger clusters. While this hexagonal order could be a result of the confinement in a thin film during sample preparation, the indication of a HCP structure shows that the interactions between particles are relatively weak.

5.4 Formation of fibers

5.4.1 CryoTEM
When the concentration is increased above the regime where nanoparticles are formed, an increase in viscosity is observed and
above a critical concentration a solid-like hydrogel is formed. The morphology of these hydrogels was investigated with CryoTEM.

At 4 wt% the sample is just below the concentration at which a solid-like gel is formed; the material thus still behaves like a viscous liquid. Results indicate the presence of nanoparticles in the material, with a small number of fibrous structures also visible, as shown in Figure 4. Such fiber formation is found predominantly around an area where there is a local deposit of poorly dissolved polymer, as shown in the combined image in Figure 4, right. The bottom part shows mostly the carbon film with the polymer deposit. The top part is the sample well with two fibers that originate from the deposit in the bottom part. Most of the fibers originate from the deposit and the deposit itself has a very high fiber density. The fact that in these images the fibers appear to originate from a deposit of higher concentration indicates that their formation is concentration-dependent, occurring more readily at higher concentrations.

At higher concentrations of the polymers it is not possible to use the cryogenic preparation technique normally used for CryoTEM. The blotting procedure that is used to reduce the height of the sample by absorbing the fluid sample does not work properly when the sample does not flow quickly. The resulting thick sample reduces both the amount of vitrified water and the transmission of electrons because of scattering and absorption of non-vitrified water. These
effects prevent the imaging of features below 100 nm in size. However, it was possible to image a U6U20k solution at 10 wt%, just above its gelation concentration (Figure 5).

![Figure 5: CryoTEM of U6U20k at 10 wt%](image)

Some artifacts of the poor sample preparation are visible in the CryoTEM images, mostly appearing as black circular objects. This is most probably non-vitrified water that is less transparent to the electron beam. Nevertheless, a higher density of fibers is present in this sample, compared to the U4U8k sample at 4 wt% in Figure 4. The difference in fiber density agrees with the apparent concentration-dependent fiber formation seen in Figure 4. While the images in Figure 5 do not show any nanoparticles, the lower sample quality prohibits exclusion of their presence in these samples. The fibrous aggregates in Figure 5 may coexist with nanoparticles.

5.4.2 Atomic force microscopy
Since the sample preparation method of CryoTEM is not suitable for solid gels, atomic force microscopy (AFM) was used to investigate gels with a higher concentration of polymers. Furthermore, to give an impression of the necessity to characterize hydrogels in their wet state in general, a comparison was made between a wet and dried hydrogel. The dried sample was prepared by drop casting a 5 wt% U4U8k gel on freshly cleaved mica and letting the sample dry while exposed to air. This process strongly increases the concentration of the polymer during the preparation of the sample. The other sample was prepared by drop casting the same hydrogel on freshly
cleaved mica and was stored in a closed container saturated with water vapor in order to prevent dehydration of the hydrogel.

Figure 6: Height (left) and phase mode (right) images of a dried 5 wt% U4U8k hydrogel.

In the AFM images of the dried hydrogel (Figure 6) a fibrous structure can be seen. The fibers’ coverage of the mica surface was not complete. In the images above, some of the mica surface is exposed, which is characteristic for inhomogeneous drying of the material. In the phase mode image, in some of the fibers a dark core with a white shell can be seen. This indicates fibers with a difference in the phase angle of the response of the AFM tip and indicates a difference in the softness of the material. This is possibly due to the phase-separation of the hard bis(urea) block and soft PEO block.

Figure 7: Height (left) and phase mode (right) images of a wet 5w% U4U8k hydrogel.
The mica surface coverage by the wet hydrogel was complete and showed no sign of drying. The height image in Figure 7 shows that the gel surface is much flatter than that of the dried hydrogel. This can be explained by the effect of water on the gel, since it will create a smoother surface than in a dried sample. In the height image no features with a high aspect ratio are visible. The overall height profile is visible in the phase image since a difference in height will also have an effect of the phase response of the AFM tip. In the phase mode, elongated features are visible that follow the height profile. This indicates that these features are due to a difference in the structure at the surface of the gel.31 Therefore, even though the aggregates can be identified with AFM their exact shape cannot be determined unambiguously. The structural information on the fibers from AFM is less detailed than that obtained from the CryoTEM images. For the AFM samples, drying-induced artifacts are easily obtained and may lead to incorrect interpretation of the hydrogel morphology. Furthermore, at the surface the network will not be as fully developed as inside a sample, due to the boundary conditions at the surface.

5.5 Small angle X-ray scattering
Small-angle X-ray scattering (SAXS) can give information about structure inside a sample, which is prominent in crystalline and liquid crystalline systems. Gel networks, in contrast, are generally disordered and too irregular to display characteristic distances within the network. However, some examples where a prominent characteristic length scale is observed exist in literature, but which specific distance is represented can differ from gel to gel.32 Generally, the length scales in gel networks are not uniform enough in size to result in sharp peaks in SAXS. However, the hydrogels of the segmented PEO bis(urea) polymers showed enough regularity to display peaks in the SAXS intensity plot. The effect of concentration and molecular composition of the polymers on the position of the peaks were investigated. The measurements were performed by placing the gels in the X-ray beam in an aluminum plate with holes and held in place between Kapton tape.
The intensity profiles for three hydrogels based on U4U8k, U6U8k and U6U20k at 10 wt% all show a clear shoulder or peak, indicating that there is a characteristic distance in the gel network (Figure 8). The magnitude of the scattering wave vector $q$ of the peak can be used to determine the correlation length $d$ with $d = 2\pi/q_{\text{max}}$ where $q_{\text{max}}$ is the peak position. The peaks for the bis(urea) polymers with a PEO of $M_w = 8\text{kDa}$ are at a significantly higher $q$-value than the peak of U6U20k. Thus the distance in the 8k PEO gels is shorter than the U6U20k gel with the longer PEO chains. Furthermore, the distance $d$ in the U4U8k and U6U8k gels is similar. This indicates that the PEO chains influence the correlation length observed for these hydrogels.
Hydrogels with varying concentrations of U4U8k were analyzed with SAXS, to determine the concentration dependence of the scattering profile. The concentration dependent scattering profiles show a shift to higher values of $q$. Thus the distance $d$ in the gel is decreasing with increasing concentration of U4U8k. Not considering the underlying structural features that give rise to the maximum, this can be explained by the system becoming more confined at higher concentrations, which results in closer packing.

Comparison of CryoTEM image analysis and SAXS
The morphology of the sample at 4 wt% is known from CryoTEM (Figure 4). Image analysis was performed on the CryoTEM images to determine if there is a relationship with the correlation length determined by SAXS. The distance between the nanoparticles in Figure 4 and similar images was determined along several random lines in the images. This was achieved by taking the intensity profile of the line and determining the number of pixels (or nm) between the peaks (i.e. the peaks are the centers of the nanoparticles were there is a maximum of blackness). The distances between two neighboring nanoparticles were then plotted against the number of the particles in the analysis, giving a plot such as in Figure 10.
The distance between the nanoparticles was then averaged over the number \((n = 300)\) of particles. This gives an average distance between the nanoparticles of 14.5 nm, but the deviation from the mean is quite large with ± 5nm. This distance corresponds to a \(q\) value of \(q = 0.43\) nm\(^{-1}\); a peak at this \(q\) value would be at higher \(q\) than the peak observed for the 15 wt% solution of U6U8k. This means the nanoparticles in the CryoTEM images are closer together than the smallest distance observed with SAXS. Therefore, the \(q_{\text{max}}\) values of the SAXS data do not correspond to the distance between nanoparticles.

The distance obtained from the image analysis does not necessarily correspond to the nearest neighbor distance. The random lines will (despite the dense packing) at some point miss the nearest nanoparticle. As a result the distance obtained from the image analysis could be different from the nearest neighbor distance, but the nearest neighbor distance will always be smaller than the distance obtained from image analysis. The distance obtained from SAXS is larger than the distance obtained from image analysis, thus the difference with the nearest neighbor distance will be even larger.

Usually the slope in the Porod region\(^{33}\) of the scattering profile gives information about the structure factor of the aggregates; however for the SAXS data in this section, the peaks in this region prevent an accurate determination of the slope. Nevertheless, it is postulated that the correlation length obtained from the \(q_{\text{max}}\) values of the
SAXS measurements corresponds to the distance between the hydrophobic domains. The decrease of the distance with increasing concentration is then related to the fibers coming closer to each other.

The dependence of the distance $d$ on the $M_w$ of the PEO can be explained by increased volume taken up by the PEO chains between the fibers, which leads to an increased separation between the fibers. Combining the SAXS and CryoTEM images gives more information on the morphology of the hydrogels, indicating that fibers play an important role in the gel network and that the distance between nanoparticles is not measured in SAXS.

## 5.6 Rheology

Since the CryoTEM experiments indicate qualitative changes in morphology as a function of the polymer concentration, the mechanical properties of the hydrogels are expected to exhibit a non-trivial behavior as well. To study this behavior in detail, the macroscopic mechanical properties of hydrogels of the segmented polymers were studied using oscillatory rheology.

A shear thinning behavior of these hydrogels has been reported previously, which is relevant to their applicability as injectable scaffolds for tissue engineering. At high concentrations (10 wt%) these gels show a storage modulus of $G' \approx 10^4$ Pa, a pronounced shear thinning behavior, and quick recovery after extrusion from a syringe.

![Figure 11: Storage and loss modulus of U4U8k at different concentrations.](image)

100
To study the effects of polymer concentration on the linear viscoelastic response of the material, oscillatory measurements of the U4U8k hydrogels were performed at 1 % strain at an oscillation frequency of 1/s. The resulting storage and loss moduli exhibit a qualitative change at a critical concentration $c_{gel} \approx 4.5$ w%; in the same concentration regime the appearance of fibers was observed in the CryoTEM images. Below $c_{gel}$ both moduli increase with increasing concentration and at 4 w% $G'' > G'$. Upon increasing the concentration from 4 w% to 5 w%, a dramatic increase of both $G'$ and $G''$ is observed, corresponding to 2.5 to 3 orders of magnitude. Moreover, in this regime above $c_{gel}$, the storage modulus now dominates, indicating a solid-like behavior as expected for a space-spanning gel network.

![Figure 12: Frequency (left) and strain (right) dependent moduli of U4U8k (5 wt%).](image)

The mechanical behavior of U4U8k based hydrogels just above the critical gelation concentration $c_{gel}$ at 5 w%, was further characterized with frequency and strain sweep experiments. In the frequency sweeps a solid-like behavior, characteristic for a gel network is observed. In the entire range of frequencies studied, the storage modulus dominates the viscoelastic behavior and depends only weakly on frequency, as shown in Figure 12 for a measurement performed at a strain amplitude of 1 %. The corresponding loss modulus is about one order of magnitude lower in magnitude, and also exhibits only a weak dependence on frequency. However, $G''$ does show an increase towards lower frequencies, which could indicate the presence of a slow structural relaxation process at low frequencies, beyond the range of frequencies accessed in our experiments. Nevertheless, within the range of time frequencies
studied here the material shows a solid-like behavior, as is typical for a gel network.\textsuperscript{35–37}

In strain dependent measurements, the changes in mechanical behavior as a function of the applied strain deformation are studied. At sufficiently small deformations, within the linear viscoelastic regime, the stress is always proportional to the applied strain deformation, which corresponds to strain-independent values of the storage and loss moduli $G'$ and $G''$. For the U4U8k samples at 5 w\% concentration, the linear regime extends to strain amplitudes of around 70\%, as shown in Figure 12 in strain sweep performed at a oscillation frequency of $1/s$. As the definitions of the moduli $G'$ and $G''$ are based on a linear viscoelastic response, they are no longer defined outside the linear regime. The values outside the linear regime that are displayed in Figure 12 are based on sinusoidal fits to the, generally anharmonic, nonlinear stress response. However, the level of strain where these deviations from linear behavior are observed, are often used to define the extents of the linear viscoelastic regime and to define the so-called yield strain, where usually a transition from solid-like to liquid-like behavior is observed. This interpretation is confirmed by the observation of changes in the linear viscoelastic response of samples that have been deformed beyond this yield point. When a new measurement was started immediately after a measurement up to 1000 \% strain, the storage modulus was approximately 30 \% lower than the equilibrium storage modulus (not shown). Waiting for 20 minutes before starting a new measurement showed a storage modulus equal to Figure 12. Moreover, previously reported results for these hydrogels also show non-immediate recovery of the modulus after subjecting the samples to a continuous shear deformation.\textsuperscript{34} Thus it is reasonable to interpret the deviation from linear behavior as the yield strain for these hydrogels.
At concentrations above 5 w%, the U4U8k hydrogels also behave like a strain softening supramolecular hydrogel. The storage modulus of the U4U8k hydrogel at 10 wt% is $10^4$ Pa and the yield strain is around 30 %, as shown in Figure 13 for a measurement performed at a frequency of 1/s. The observed yield strain is slightly lower than that found for the same systems at lower concentrations. Similar behavior was also observed when manipulating the gel by hand, as it appeared more brittle than the lower concentration gels.

5.6.1 Strain stiffening

A much more dramatic feature than the increase of the moduli up to $10^4$ Pa at 10 wt%, is the concentration and temperature dependent mechanical behavior of the gels at high strains. Figure 14 shows strain stiffening of a U4U8k 4 wt% hydrogel (below $c_{gel}$), where the moduli $G'$ and $G''$ increase as the strain deformation increases beyond the linear viscoelastic regime. This behavior is in stark contrast with the strain yielding that was observed above 5 wt%. Because in the non-linear regime the moduli are not properly defined, they can only be used to qualitatively establish non-linearity of the mechanical response.
It should be noted that at 4 wt% in the linear regime, the storage modulus is lower than the loss modulus, indicating the viscoelastic behavior is more liquid-like. The origin of the reversed values of the moduli at this concentration is not understood and is especially remarkable since both at lower and higher concentrations the loss modulus is lower than the storage modulus (Figure 11).

**Figure 14:** Strain-dependent moduli of U4U8k (4 wt%).

**Figure 15:** Strain-dependent moduli of U4U8k (5 wt%). a) T=20°C. b) T=45°C. c) T=20°C, after cooling.
Strain stiffening was also observed in a 5 wt% U4U8k hydrogel when the temperature was increased to 45°C. In the series of experiments shown in Figure 15, the effect of temperature on the mechanical behavior can be clearly seen. At 20 °C, the material exhibits yielding behavior, where the moduli show a transition to a liquid-like behavior (Figure 15a). To study the behavior of the material at higher temperature, the sample was heated to 45°C and equilibrated for 15 min., after which strain sweep measurement was performed. As shown in Figure 15b, the resulting moduli in the linear viscoelastic regime are lower than those found at 20 °C. Interestingly however, instead of the yielding behavior observed at room temperature, samples at this elevated temperature exhibit strain stiffening. Both the apparent storage modulus $G'$ and the apparent loss modulus $G''$ increase continuously as the strain amplitude is increased beyond the linear viscoelastic regime.

The experiments indicate that this strain-stiffening behavior is brought about by a change in structure of the sample and not by a direct influence of the temperature on the mechanical properties of the sample. This is illustrated by measurements (after equilibrating for 5 min.) at 20 °C, on a sample that had previously been subjected to a strain sweep measurement up to 500 % strain at 45 °C (Figure 15c). Strain stiffening is observed, but the effect is less pronounced and yields at a lower strain. However, it occurs at higher moduli than Figure 15b, with a plateau storage modulus of 170 Pa.

The response of the material thus depends not only on the temperature, but also on the history of the sample. The sample history has no permanent effect, since equilibrating at room temperature for 40 minutes after heating to 45 °C and a strain sweep experiment, shows the same behavior as in Figure 15a.

The strain stiffening behavior of the U4U8k hydrogel is thus both concentration and temperature dependent. Possible origins of the observed strain stiffening behavior are discussed in the next section.

5.7 Discussion

5.7.1 Origin of strain stiffening
The concentration range where a sudden increase in dynamic moduli (Figure 11) and strain stiffening are observed (Figure 14) coincides with the transition from spherical nanoparticles to
elongated fibers (from 4 wt% up), as indicated by CryoTEM measurements. It is tempting to therefore conclude that strain stiffening is *caused* by the appearance of fibers, but strain stiffening due to the presence of nanoparticles should also be taken into account. Therefore the following paragraph will discuss the theoretical background of strain stiffening in supramolecular materials using the broad categorization into non-linear stretching and structural reorganization. Therefore, both of these mechanisms will be discussed for both nanoparticle and fiber dominated systems.

### 5.7.2 Strain stiffening of crosslinked nanoparticles

The concentration at which strain stiffening occurs is around 4 wt%, above the concentration where close packing as shown in Figure 3 is observed. Thus, the system is above \( c^* \), and has entered the semi-dilute regime, where the polymer chains interact. These interactions may consist of (non-covalent) crosslinks or entanglements.

![Figure 16: Strain applied to a solution of nanoparticles acting as crosslinked flower-like micelles.](image)

A well-characterized class of strain stiffening systems that shows parallels with the copolymer nanoparticles, consists of flower-like micelles cross-linked by flexible linkers (Figure 16). These linkers are often composed of polymers with telechelic ‘sticky’ groups. In such systems, stiffening can be caused either by non-linear stretching of the flexible linkers, or by strain induced structural reorganization that leads to an increase in the number of elastically active chains. In both mechanisms, interaction starts at a critical concentration, where the interparticle distance is small enough to be bridged by the flexible linkers. In the segmented copolymer system, the linkers
are the PEO segments that bridge aggregates (nanoparticles) of bis(urea) segments.

5.7.3 Strain stiffening of a fibrous network

Non-linear stretching

Strain stiffening in non-ideal fibrous networks has been subject of extensive theoretical studies.\textsuperscript{4,6,10,19,41,42} Like in crosslinked micelles, strain stiffening in fibrous networks is caused either by non-linear stretching, or as the result of structural reorganization of the network.\textsuperscript{6,19}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{non-linear_stretching}
\caption{Two modes of non-linear stretching in fibrous networks. Stretching of in the axial direction of the fibers (left) and stretching of the PEO linker (right)}
\end{figure}

Non-linear stretching can occur both in the axial direction of the fibers or in the PEO chains that crosslink the fibers (Figure 17). In the segmented copolymer gels, axial stretching of the fibers could explain the observed temperature dependence of strain stiffening if aggregation within the fiber would become stronger at higher temperatures.\textsuperscript{10,13} A higher aggregation in the fiber can be caused by the increased aggregation of PEO (i.e. decreased solubility in water), which counters the weakening of the bis(urea) interactions. Non-linear stretching in the PEO linkers is entropic in nature and the uncoiling of the polymer chains will require more force at higher temperatures. However, both of these mechanisms cannot satisfactorily explain the concentration dependence of the strain stiffening. Therefore, the non-linear stretching of the fibrous
network is not a satisfactory explanation for the cause of strain stiffening in the hydrogel networks.

**Structural reorganization**
According to literature, structural reorganization of deformed fibrous networks results in strain stiffening if the number of elastically active elements increases,\(^{43-45}\) either by a strain-induced increase in number of active crosslinks\(^{43,44}\) or by recruitment of fibers that actively resist the deformation, possibly through alignment into the direction of deformation.\(^{45}\)

![Figure 18: Structural reorganization of fiber due to an applied strain leading to an increase in the number of crosslinks and alignment of the fibers.](image)

Figure 18 shows both effects (alignment and increase in crosslink density) simultaneously, however the mechanism of strain stiffening in the segmented copolymer gels could also be the result of one of these effects alone. As they both belong to the broad category of structural relaxation, both modes of reorganization have the same characteristics with respect to temperature and concentration dependence. More detailed measurements are required to distinguish between these modes, such as experiments that determine the concentration dependence of the crosslink density.
A strain stiffening mechanism of the segmented copolymer solutions based on structural reorganization accounts for the observed temperature dependence. Following the broad categories of strain induced reorganization, the following mechanism can be proposed. At 45 °C, the exchange of hydrophobic bis(urea) segments between fibers is faster and results in a relatively rapid increase in the number of elastically active linkers when the fibers align at high strains. At 20 °C, the exchange is slower, and therefore alignment of fibers at high strains does not lead to a fast increase in active crosslinks. Furthermore, this mechanism also explains why the strain stiffening remains for a short period of time after cooling down to 20 °C (Figure 15c). When a sample is quickly cooled from 45 to 20 °C, reorganization to the equilibrium network structure is slowed down, and initially the strain stiffening corresponding to the higher temperature is observed. The eventual relaxation to the strain yielding behavior of Figure 15a indicates the presence of two separate processes with different timescales.

Faster exchange at high temperature also lowers the modulus in the linear regime, because stress is released when the bis(urea) segments on stress-bearing linkers exchange (Figure 15). The disappearance of the stiffening above 5 wt% can be explained by limited orientational freedom of fibers in the network at higher concentrations. At the higher concentration there are more crosslinks ($G'$ increase to 600 Pa at 5 wt%) and as a result the fibers are attached at more points. This prevents the fibers from aligning in the direction of strain and reorientation leading to an increase in the number of fibers resisting the deformation of the network.

Thus the structural reorganization of a fibrous network satisfactorily explains both the temperature and concentration dependence of the strain stiffening phenomenon. The alignment of fibers in the direction of the applied strain and a reorganization of the crosslinks in the network cause an increase in modulus above a critical strain.

Assuming that both the decrease in concentration and the increase in temperature decrease the number of crosslinks, can be validated because the plateau modulus drops (Figure 11 and Figure 15). General gel network theory describes the minimal number of crosslinks in a network and a critical gel concentration above which the network spans the entire sample volume.
concentration, gels also have a critical gel temperature under (or sometime above) which the gel is an infinite percolated network. It has been shown that phenomena close to these critical parameters $p_c$, described as low values for $(p-p_c)^\alpha$, often show dramatically different behavior than at high values of $(p-p_c)^\alpha$. Further research will have to show if the strain stiffening phenomenon is found only in a regime close to $p_c$, where the number of crosslinks is just sufficient to form an infinite network. If this is the case, it would mean that the number of crosslinks is the important parameter that determines in which regime the strain stiffening behavior is observed. Furthermore, this would mean both the concentration and temperature dependency have the same origin.

### 5.7.4 Biomimetic nature of the strain stiffening

The segmented polymers studied in this chapter show favorable properties for biomedical applications as injectable scaffolds. Under high shear, the gels flow, but they quickly set once they have been ejected from the syringe. Further exploitation of these hydrogels in tissue engineering could be sought as scaffolds with biomimetic mechanical properties. However, the onset of strain stiffening in U4U8K at a strain of 100% is much later than in the relevant natural tissues where stiffening starts at 0.1 to 1% strain. Thus the strain stiffening is not biomimetic in that sense and will therefore not have additional benefits regarding stem cell differentiation. Furthermore, the mechanism of strain stiffening is significantly different from the proposed mechanism for strain stiffening of semi-flexible proteins, but this is not necessarily problematic when applied in tissue engineering. The response of stem cells to mechanical cues is a relatively new field of science and the exact method of interaction has not been determined, thus a study with a hydrogel with non-biomimetic strain stiffening would introduce more unknown parameters.

### 5.8 Conclusion

The synthesis of segmented copolymers based on hydrophobic bis(urea) and hydrophilic poly(ethylene glycol) yielded organic hydrogelators with varying morphological and mechanical properties. The origin of the strain stiffening was explained by taking the morphology and physical interactions of the polymers into
consideration. This gives more insight in how morphology and supramolecular motifs play a role in the mechanical behavior of supramolecular hydrogels.

Despite the straightforward molecular structure, the behavior of the polymers in water was is complex. At dilute to semi-dilute concentration the polymers aggregate into nanoparticles of approximately 15 nm in diameter. CryoTEM images showed that in this regime the nanoparticles stay spherical and act as hard spheres with close packing that is indicative for a hexagonal close packing structure.

Above this concentration, fiber formation is observed that appears to result from aggregation of nanoparticles. Increasing the polymer concentration further shows a network composed of a dense fibrous network that shows high storage and loss moduli of $10^4$ Pa. The mechanical behavior showed concentration and temperature dependent strain stiffening. At a concentration of 4 wt%, which results in weak gels, strain stiffening was observed, changing into a strain yielding behavior at 5 wt%. Increasing the temperature above room temperature for a 5 wt% gel also yielded strain stiffening behavior. After comparing several mechanisms for strain stiffening, it was shown that these gels most likely exhibit strain stiffening due to a structural reorganization at high strains.
5.9 Experimental

**Materials:** Solvents used in synthesis were reagent grade. CH$_2$Cl$_2$, CHCl$_3$, Et$_3$N and pyridine were distilled from CaH$_2$. All PEO derivatives were dried in vacuum over P$_2$O$_5$ for at least 12h. The reagents 11-aminoundecanoic acid, 1,4-diisocyanatobutane, 1,6-diisocyanatohexane and N-BOC-1,3-propanediamine were purchased from Aldrich, Fluka, or Acros and were used without additional purification. Di-tert-butyl tricarbonate was prepared according to literature procedures.$^{47}$

**General Methods:** All reactions were performed under a nitrogen atmosphere in a glove box (MBraun LabMaster 130, MBraun, Garching, Germany) to achieve high molecular weight segmented block-copolymers. NMR spectra were acquired on a 400 MHz Varian Mercury Vx (400 MHz for $^1$H-NMR, 100 MHz for $^{13}$C-NMR). Proton and carbon chemical shifts are reported in ppm downfield of tetramethylsilane using the resonance of the deuterated solvent as internal standard. Splitting patterns are designated as singlet (s), doublet (d), triplet (t), multiplet (m) and broad (b). Infrared spectra were measured on a Perkin Elmer 1600FT-IR.

**GPC and MALDI-TOF analysis:** GPC in dimethyl formamide (DMF) was performed on a PL-GPC 50 Plus of Polymer Laboratories with integrated refractive index detector, using a Polymer Standards Service Gram Linear M 8 × 300 mm, 10 μm particles column, and DMF with 10 mM LiBr as eluent at a flow rate of 1 mL/min (27 °C). Poly(ethylene glycol) standards were used for calibration at 50 °C. Matrix assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF) was performed on a Perseptive DE PRO Voyager MALDI-TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix.

**Rheology:** Mechanical properties of these hydrogels were tested using rheology. Dynamic viscoelastic measurements were determined using a stress-controlled rheometer (Anton Paar, Physicia MCR501) equipped with a sand-blasted plate-plate geometry to prevent slippage. Measurement temperature was fixed at 20°C.

**Cryogenic transmission electron microscopy**

Samples for cryogenic transmission electron microscopy (CryoTEM) were prepared in a ‘Vitrobot’ instrument (PC controlled vitrification robot, patent applied, Frederik et al. 2002, patent licensed to FEI) at
room temperature and a relative humidity >95%. In the preparation chamber of the ‘Vitrobot’ 3 μL sample was applied on a Quantifoil grid (R 2/2, Quantifoil Micro Tools GmbH; freshly glow discharged just prior to use), excess liquid was blotted away and the thin film thus formed was shot (acceleration about 3 g) into liquid ethane. The vitrified film was transferred to a cryoholder (Gatan 626) and observed at -170 °C in a Technai microscope operating at 120 kV. Micrographs were taken at low dose conditions.

**Small-angle X-ray scattering (SAXS):** SAXS experiments were performed at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France, at the high brilliance beamline ID02. An X-ray energy of 12.46 keV and one sample-to-detector distance of 1.5 m was used to cover a q-range of 0.07 < q < 3.5 nm⁻¹ with q being the magnitude of the scattering wave vector. The samples were contained in an aluminum plate with small holes sandwiched between Kapton tape at a temperature of 20.1°C. The scattering data were corrected for background scattering, detector response and primary beam intensity fluctuations. The instrument scattering vector was calibrated using a silver behenate standard.

**Atomic force micrographs:** Atomic force micrographs were recorded under ambient conditions with silicon cantilever tips (PPP-NCH, 300-330 kHz, 42 N/m from Nanosensors) using an Asylum Research MFP-3D-Bio in non-contact mode. For the atomic force microscopy, 2 μL of the solution at ambient temperature was drop-cast on freshly cleaved mica and allowed to dry or 2 μL was dropcast and kept under a saturated water atmosphere until the measurement.

**Synthesis of PEO-bis(urea) segmented block copolymers.**

**11-tert-butoxycarbonylamino-undecanoic acid:** To a stirred solution of 11-aminoundecanoic acid (5 g, 24.8 mmol) in a mixture of THF/H₂O (130 mL/130 mL) was added NaOH (2.18 g, 54.5 mmol). After 10 min di-tert-butyl dicarbonate was added and the reaction mixture let to stir for 20 h. The solution was reduced in volume, taken up with CHCl₃ (150 mL) and washed with 1N HCl (3 x 100 mL). The organic layer was then dried over MgSO₄, filtered and the filtrate reduced in volume to obtain a colorless powder that was recrystallized from hexane (7.0 g, 95%); m.p.: 68 °C; ¹H NMR (400 MHz, CDCl₃) 4.55 (br, 1H, NH), 3.12 (br, 1H, NH-CH₂), 2.37 (t, 2H, CH₂-CO), 1.66 (m, 2H, CH₂-CO), 1.54-1.41 (m, 11H, C(CH₃)₃ and CH₂), 1.40-1.24 (m, 12H, CH₂); ¹³C NMR (100 MHz, CDCl₃) 179.1 (CO-
OH), 155.7 (NH - CO-O), 79.1 (C(CH3)3), 40.6, 34.0, 30.0, 29.4, 29.2, 29.1, 29.0, 28.9, 28.4 (C(CH3)3), 26.7, 24.7.

**Bis(N-(tert-butyloxy carbonyl)-11-aminoundecanoyl)-poly(ethyleneglycol (8k)):** In a 250 mL two-neck round-bottom flask 2.26 g (7.49 mmol) of N-(tert-butyloxy carbonyl)-11-aminoundecanoic acid, 1.44 g (7.49 mmol) of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride and 0.92 g (7.48 mmol) of dimethylaminopyridine were stirred in 100 mL of dry dichloromethane under argon for 20 min. To the resulting solution was added 15 g (1.87 mmol) of poly(ethylene glycol) (Mn ca. 8000) and the reaction mixture was stirred for 3 days. The solution was washed with 10% citric acid and precipitated by addition of diethyl ether, filtered off and dried. \(^1\)H NMR (400 MHz, CDCl3): \(\delta = 4.22\) (t, 4H, CH2OCO), 3.8-3.3 (m, OC\(\text{H}_2\)), 3.08 (br, 4H, CH2N), 2.31 (t, 4H, CH2CO), 1.60 (m, 4H, CH2CH2CO), 1.43 and 1.26 (s, 46H, C(CH3)3 and CH2). MALDI-TOF [M+Na+] = 9642.83 ± n*44. GPC (CHCl3; PS standards): \(M_n = 10100\) g/mol, PDI = 1.28.

**Bis(N-(tert-butyloxy carbonyl)-11-aminoundecanoyl)-poly(ethyleneglycol(20k)):** Bis(N-(tert-butyloxy carbonyl)-11-aminoundecanoyl)-poly(ethyleneglycol(20k)) was synthesized according to the above mentioned procedure of 8k material. \(^1\)H NMR (400 MHz, CDCl3): \(\delta = 4.21\) (bs, 4H, CH2OCO), 3.8-3.3 (m, OC\(\text{H}_2\)), 3.10 (br, 4H, CH2N), 2.31 (t, 4H, CH2CO), 1.61 (m, 4H, CH2CH2CO), 1.43 and 1.26 (s, 46H, C(CH3)3 and CH2).

**Bis(11-ammonium chloride undecanoyl) -poly(ethylene glycol(8k)):** 30 mL of a 4 M HCl solution in dioxane was added to the solution of 10 g (1.1 mmol) of bis(N-(tert-butyloxy carbonyl)-11-aminoundecanoyl)-poly(ethyleneglycol) in 30 mL of dioxane and stirred at 0°C for 1 h and subsequently at room temperature for 12 h. The solvent was evaporated to yield 9.0 g (100%) of the product as its hydrochloric salt. \(^1\)H-NMR (400 MHz, CDCl3, T=295K): \(\delta = 7.78\) (bs, 6H, NH3), 4.22 (bs, 4H, CH2OCO), 3.90-3.20 (m, OCH2), 2.89 (bs, 4H, CH2N), 2.32 (t, 4H, CH2CO), 1.75-1.48 (m, 8H, CH2CH2N and CH2CH2CO), 1.28 (bs, 28H, CH2). MALDI-TOF [M+Na+] = 9350.27 ± n*44.

**Bis(11-ammonium chloride undecanoyl)-poly(ethylene glycol(20k)):** Bis(11-ammonium chloride undecanoyl)-poly(ethylene glycol(20k)) was synthesized according to the above mentioned procedure of 8k material. \(^1\)H-NMR (400 MHz, CDCl3, T=295K): \(\delta = 7.76\) (bs, 6H, NH3),
Structure, morphology and mechanical properties of bis(urea)-based hydrogels

4.24 (bs, 4H, CH₂OCO), 3.90-3.20 (m, OCH₂), 2.92 (bs, 4H, CH₂N), 2.34 (t, 4H, CH₂CO), 1.75-1.55 (bs, H₂O+CH₂CH₂N+CH₂CH₂CO), 1.28 (bs, 26H, CH₂).

U4U8k: To a solution of 100.0 mg (0.58 mmol) of 1,4-diisocyanatobutane in 1 mL of dichloromethane, a solution of bis(11-aminoundecanoyl)-poly(ethylene glycol) (5.0 g, 0.55 mmol) and triethylamine (0.1 mL, 0.96 mmol) in 2 mL dichloromethane was added and stirred for 3 days. The solution was concentrated and precipitated by addition of diethyl ether, filtered off and dried. Yield: 4.1 g (80%). ¹H-NMR (400 MHz, CDCl₃, T=295K): δ = 5.01 and 4.80 (bs, 2H, NH), 4.23 (bt, 4H, CH₂OCO), 3.90-3.10 (m, OCH₂), 3.15 (bm, 8H, CH₂N), 2.32 (t, 2H, CH₂CO), 1.65-1.40 (m, 12H, CH₂CH₂N and CH₂CH₂CO), 1.27 (bs, 24H, CH₂). ¹³C NMR (100 MHz, CDCl₃): δ = 173.7, 158.7, 71.6, 70.5, 69.4, 69.1, 63.3, 40.4, 39.7, 34.1, 30.3, 29.4, 29.2, 29.1, 29.0, 27.5, 26.8, 24.8. FT-IR (ATR mode, cm⁻¹): ν=2880, 2742, 1734, 1620, 1573, 1466, 1359, 1342, 1279, 1241, 1145, 1100, 1060, 962, 841. GPC (DMF; PEO standards): Mₙ = 84000 g/mol, PDI = 1.90.

U6U8k: To a solution of 60.0 mg (0.35 mmol) of 1,6-diisocyanatohexane in 1 mL of dichloromethane, a solution of bis(11-aminoundecanoyl)-poly(ethylene glycol) (3.0 g, 0.33 mmol) and triethylamine (0.1 mL, 0.96 mmol) in 2 mL dichloromethane was added and stirred for 3 days. The solution was concentrated and precipitated by addition of diethyl ether, filtered off and dried. Yield: 2.8 g (85%). ¹H-NMR (400 MHz, CDCl₃, T=295K): δ = 4.76 (bd, 2H, NH), 4.22 (bt, 4H, CH₂OCO), 3.90-3.33 (m, OCH₂), 3.14 (bm, 8H, CH₂N), 2.32 (t, 2H, CH₂CO), 1.65-1.48 (m, 14H, CH₂CH₂N and CH₂CH₂CO), 1.27 (bs, 35H, CH₂). ¹³C NMR (100 MHz, CDCl₃): δ = 173.7, 158.7, 71.6, 70.5, 69.4, 69.1, 67.2, 63.3, 40.3, 39.3, 34.1, 30.3, 29.4, 29.2, 29.1, 29.0, 26.8, 24.8. FT-IR (ATR mode, cm⁻¹): ν=3386, 2877, 1734, 1644, 1567, 1466, 1359, 1342, 1280, 1241, 1145, 1097, 1060, 961, 841. GPC (DMF; PEO standards): Mₙ = 86500 g/mol, PDI = 1.55.

U4U20k: To a solution of 30.0 mg (0.17 mmol) of 1,4-diisocyanatobutane in 1 mL of dichloromethane, a solution of bis(11-aminoundecanoyl)-poly(ethylene glycol) (3.0 g, 0.15 mmol) and triethylamine (0.1 mL, 0.96 mmol) in 2 mL dichloromethane was added and stirred for 3 days. The solution was concentrated and precipitated by addition of diethyl ether, filtered off and dried. Yield: 2.8 g (85%). ¹H-NMR (400 MHz, CDCl₃, T=295K): δ = 4.19 (bt, 4H,
\(CH_2OCO\), 4.04-3.24 (m, OCH\(_2\)), 3.15 (bm, 8H, CH\(_2\)N), 2.29 (b, H\(_2\)O+CH\(_2\)CO), 1.65-1.35 (m, 11H, CH\(_2\)CH\(_2\)N and CH\(_2\)CH\(_2\)CO), 1.27 (bs, 19H, CH\(_2\)). FT-IR (ATR mode, \(cm^{-1}\)): \(\nu =2882, 1723, 1619, 1574, 1466, 1359, 1342, 1279, 1241, 1147, 1102, 1060, 962, 842\). GPC (DMF; PEO standards): \(M_n = 59000\) g/mol, PDI = 1.46.

**U6U20k**: To a solution of 40.0 mg (0.23 mmol) of 1,6-diisocyanatohexane in 1 mL of dichloromethane, a solution of bis(11-aminoundecanoyl)-poly(ethylene glycol) (4.0 g, 0.20 mmol) and triethylamine (0.1 mL, 0.96 mmol) in 2 mL dichloromethane was added and stirred for 3 days. The solution was concentrated and precipitated by addition of diethyl ether, filtered off and dried. Yield: 3.0 g (75%).\(^3\)H-NMR (400 MHz, CDCl\(_3\), T=295K): \(\delta = 4.22\) (bt, CH\(_2\)OCO), 3.98-3.20 (m, OCH\(_2\)), 3.12 (bm, CH\(_2\)N), 2.32 (t, CH\(_2\)CO), 1.70-1.05 (m, CH\(_2\)CH\(_2\)N and CH\(_2\)CH\(_2\)CO, CH\(_2\)). FT-IR (ATR mode, \(cm^{-1}\)): \(\nu =2879, 1650, 1549, 1466, 1359, 1342, 1279, 1241, 1146, 1099, 1060, 961, 841\). GPC (DMF; PEO standards): \(M_n = 45000\) g/mol, PDI = 2.05.
5.10 References


(2) Brandl, F.; Sommer, F.; Goepferich, A. Biomaterials 2007, 28, 134–146.


Chapter 6

Fatty acid based hydrogels with non-directional supramolecular interactions
6.1 Introduction
Currently, there is a strong interest in nonionic surfactants made from affordable building blocks for industrial and biomedical applications as e.g. rheology modifiers and hydrogelators. Many of these amphiphiles have oligo-ethylene glycol or poly-ethylene glycols as hydrophilic group. Two important classes of these surfactants are poly(ethylene glycol) (PEO) alkyl ethers and triblock copolymers of PEO with poly (propylene glycol) (PPO). Poly(ethylene glycol) alkyl ethers form micelles in aqueous solution with a wide variety of morphologies including spherical, disc-like, cylindrical, lamellar and can also form vesicles. Their properties have been studied extensively and are often used as modeling systems for morphological studies.

The triblock copolymer of PEO with poly (propylene glycol), commercially available as Pluronics, Poloxamers and Synperonics, has been studied extensively and is known for the wide variation of geometries of its aggregates. Furthermore, the gels of these triblock copolymers have a broad application in drug delivery and solubilization of hydrophobic compounds. The difference in temperature dependence of the PEO block versus the PPO is often used to trigger capture and release, as well as more general morphological changes in the polymer aggregates.

The goal of this chapter is to describe the synthesis of bolaamphiphilic hydrogelators obtained by esterification of PEO monomethyl ethers with dimerized fatty acids (DFAs), in order to establish their capability to form hydrogels. The dimerized fatty acid of linoleic acid and PEO both are inexpensive building blocks and therefore offer an accessible source for supramolecular hydrogels. The hydrogenated form of DFA was used to prevent any side-reactions such as oxidation of the double bonds. Dimerization of unsaturated fatty acids is an unspecific process. Therefore, the product is a mixture of several isomers with very similar properties (Figure 1). Dimer fatty acids have a broad range of industrial applications such as hot-melt adhesives and coatings. Their commercial availability and affordability make them ideal for industrial use in applications where compounds with long aliphatic chains are required. Furthermore, their availability as a mixture of isomers has been used to prevent crystallization in self-healing polymeric materials. Analogous to the hydrogelation by the
Pluronic polymers, nano-phase separation between the hydrophobic fatty acid and the hydrophilic PEO should result in the formation of a non-covalent network in water.

![Figure 1: Most abundant isomers of hydrogenated dimer fatty acid (DFA).](image)

The molecular weight of the PEO was varied from 350 to 5000, yielding a series with varying hydrophilic/hydrophobic ratio. The effect of this variation on the critical gelation concentration and the general mechanical behavior is studied with rheological measurements. Furthermore, thixotropic behavior of the hydrogels is investigated with oscillatory rheology.

### 6.2 Synthesis

The amphiphilic polymers used in this chapter were prepared from methoxy-PEO and dimer fatty acid in the melt according to a literature procedure for esterification of dimer fatty acids (Scheme 1).\textsuperscript{19,20} The reactions were performed under dynamic vacuum to ensure the removal of water from the viscous reaction mixture. At 180 °C, PEO in the used molecular weight range is a viscous liquid. The average molecular weight of PEO was 350, 550, 750, 2000 and 5000. All reactions yielded the desired products in high yields and purity.

![Scheme 1: Synthesis of dimer fatty acid based hydrogelator.](image)
The esterification was catalyzed by SnCl₂. This is the preferred catalyst in the literature procedure because the salt also acts as an antioxidant preventing the oxidation of the double bonds.¹⁹ Although the DFA used in the reaction in Scheme 1 does not have double bonds, the other catalysts used in literature (sulfuric acid and p-toluene sulfonic acid) caused blackening of the reaction mixture similar to the findings for the unhydrogenated fatty acids. Although tin chloride was the most successful catalyst, residual tin in the final product can be toxic even when it is used in catalytic amounts, and it is therefore less suitable for biomedical applications.²¹ The yields were good and varied from 84 to 93 % after extraction with ethyl acetate/brine. The procedure provided dimer fatty acid-PEO compounds of high purity according to ¹H-NMR, and allowed for an investigation into their hydrogelating capabilities. The compounds are abbreviated with DFA-PEO and when needed the molecular weight of the PEO is added in subscript (e.g. DFA-PEO₃₅₀).

6.3 Characterization of morphology with CryoTEM
Aggregation of DFA-PEO₇₅₀ at low concentration was investigated with CryoTEM. At a concentration of 2 mg/mL the viscosity of the samples was low enough to ensure proper sample preparation.²² The amphiphile with a PEO of Mₘ = 750 is shown in Figure 2, as a representative sample for all DFA-PEO hydrogelators studied.

The CryoTEM images of DFA-PEO₇₅₀ at 2 mg/mL show small irregularly shaped aggregates. The cross section of the mostly non-
spherical objects varies from 20-50 nm. Images of samples of the other amphiphiles displayed similar features.

### 6.4 Rheological characterization of hydrogels

The rheological characterization of DFA-PEO based hydrogels focused on the effect of the molecular weight of the PEO and the typical supramolecular mechanical properties of self-healing and thixotropy. At concentrations below the gelation concentration, the DFA-PEO solutions are clear viscous solutions. Above the gelation concentration, clear hydrogels were obtained by a short heating-cooling cycle to homogenize the sample. First vial inversion tests and oscillatory rheology measurements were used to determine the influence of the length of PEO on the gelation concentration of the hydrogels.

![Figure 3: Comparison of minimal gelation concentrations determined with vial inversion tests.](image)

Using vial inversion tests, the lowest concentration at which the hydrogel does not flow in an upturned vial was determined. At 20 °C, DFA-PEO with a PEO of 2000 g/mol has the lowest gelation concentration (19 wt%) of the series, while DFA-PEO\(_{350}\) did not yield a gel that remained in the upturned vial at any concentration, which could be related to the liquid state of the compound at room temperature. The results of the vial inversion tests can only be used in a qualitative manner, since the value of the critical gelation concentration found is somewhat arbitrary. The reason is that the critical gelation concentration depends on the diameter and geometry of the vial in which the experiment is performed. A comparison between the different PEOs can still be made since the
dimensions of the vial and gel were kept constant in these measurements.

**Figure 4:** Frequency sweep of DFA-PEO\textsubscript{350} (left) and DFA-PEO\textsubscript{2000} (right) at 38 wt%.

Mechanical properties of the gels were determined in a more quantitative manner using oscillatory shear experiments in plate-plate geometry. First, frequency sweeps were performed to determine the frequency dependence of the gels. In Figure 4, the results for hydrogels of 38 wt% of DFA-PEO\textsubscript{350} and DFA-PEO\textsubscript{2000} are shown. Both hydrogels showed a dependence of the modulus on the frequency, within the range that was measured. The storage modulus is above the loss modulus over several decades indicating a weak dependency on the frequency. For the strain sweep experiments a frequency of 1 s\textsuperscript{-1} was chosen.

**Figure 5:** Storage and loss modulus of 23 wt% hydrogels of DFA-PEO with varying molecular weight of PEO.
The effect of the molecular weight of PEO was also investigated with oscillatory rheology in hydrogels with 23 wt% of the respective DFA-PEO. DFA-PEO\textsubscript{2000} had the highest storage and loss modulus at 23 wt% (Figure 5), confirming the trend observed in the vial inversion tests (Figure 2). Thus the qualitative relation of the minimal gelation concentration, follows the same trend as the quantitative determination of the storage and loss modulus at constant concentration.

![Graph A](image1)

![Graph B](image2)

**Figure 6:** Storage and loss modulus of a DFA-PEO\textsubscript{2000} 23 wt\% hydrogel after preshear (A) and at variable strain (B).

The thixotropic behavior\textsuperscript{23} of DFA-PEO\textsubscript{2000} was investigated by performing a shear recovery experiment in which the storage and loss moduli were measured in time after preshearing at 100 s\textsuperscript{-1} for 5 sec. Preshearing led to a strong decrease in moduli (Figure 6A),

126
which recovered to the values measured before preshearing (Figure 6B) in approximately 500 s. The recovery experiment shows the dynamic nature of the DFA-PEO$_{2000}$ hydrogel, because the network is built up by the non-covalent interactions between the hydrophobic fatty acid moieties. Furthermore, the dynamic behavior enables a more specific form of thixotropy, which for solids and solid-like materials is often called ‘self-healing’.\textsuperscript{24–26} Self-healing concerns the recovery of crosslinks in a material to regain its original properties after (network) failure. A difference with general thixotropic recovery after shear is the physical separation of the crosslinks, preventing reformation of the same crosslinks.\textsuperscript{24} Due to the high volume fraction of solvent, self-healing is an inherent property of supramolecular hydrogels. The dynamic nature of the non-covalent interaction combined with relative ease of diffusion through the solvent, create optimal conditions for the formation of new crosslinks.

![Figure 7: Jointed bars of DFA-PEO$_{2000}$, showing self-healing.](image)

Self-healing of a 30 wt% DFA-PEO$_{2000}$ hydrogel was studied by manually joining two separate samples, one of which contained a blue dye for visualization (Figure 7). After 5 minutes, the joined sample had similar mechanical properties as a similar sized bar of gel without cutting and showed no memory (local weakness) of the location of adhesion.

6.5 Discussion and conclusion
The synthesis of amphiphiles based on dimerized fatty acids and PEO was performed successfully, using a range of PEO with an average
M<sub>w</sub> of 350, 550, 750, 2000 and 5000. Except DFA-PEO<sub>350</sub> these compounds form hydrogels, among which DFA-PEO<sub>2000</sub> showed the lowest gelation concentration and the highest modulus at a given concentration.

CryoTEM showed irregularly shaped aggregates, which might be due to the aggregation of the isomeric mixture of fatty acids that have been shown to lack macroscopic phase separation in supramolecular systems.<sup>17</sup>

The DFA-PEO hydrogels show that there is no need for directional non-covalent interactions in the molecules to obtain hydrogels with complex dynamic mechanical properties such as thixotropy. The diffusion and reversibility of the crosslinks is sufficient to result in a gel with mechanical properties generally associated with supramolecular hydrogels.

These interesting mechanical properties also justify further investigation in for example the temperature dependent behavior of these hydrogels. The lower critical solution temperature of the DFA-PEO gels for example, should vary based on the length of the PEO. This gives a method of varying the sol-gel behavior of at higher temperature.
6.6 Experimental

Materials
All solvents used were of AR quality or better and purchased from Sigma-Aldrich. DMF and DCM were dried over molsieves (3 Å). All chemicals were used without any further purification.

Measurements
Proton Nuclear Magnetic Resonance ($^1$H NMR) spectra were recorded at room temperature (RT) on a 400 MHz Varian Mercury Vx NMR spectrometer. Proton chemical shifts are reported in ppm downfield from tetramethylsilane (TMS, 0 ppm). Splitting patterns were assigned as singlet (s), doublet (d), triplet (t), quartet (q), quintet (quint.) or multiplet (m).

Carbon Nuclear Magnetic Resonance ($^{13}$C NMR) spectra were recorded at RT on a 400 MHz Varian Mercury spectrometer with a corresponding frequency of 100 MHz. Carbon chemical shifts are reported downfield from TMS using the resonance of deuterated chloroform (CDCl$_3$) as an internal standard (77.2 ppm).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was performed with a PerSeptive Biosystems Voyager-DE PRO spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix.

Rheology: Mechanical properties of these hydrogels were tested by oscillatory rheology. Dynamic viscoelastic measurements were performed using a stress-controlled rheometer (Anton Paar, Physicia MCR501) equipped with a sand-blasted plate-plate geometry to prevent slippage. Measurement temperature was fixed at 20°C.

Cryogenic transmission electron microscopy
Samples for cryogenic transmission electron microscopy (CryoTEM) were prepared in a ‘Vitrobot’ instrument (PC controlled vitrification robot, patent applied, Frederik et al 2002, patent licensed to FEI) at room temperature and a relative humidity >95%. In the preparation chamber of the ‘Vitrobot’ 3 µL sample was applied on a Quantifoil grid (R 2/2, Quantifoil Micro Tools GmbH; freshly glow discharged just prior to use), excess liquid was blotted away and the thin film thus formed was shot (acceleration about 3 g) into liquid ethane. The vitrified film was transferred to a cryoholder (Gatan 626) and
observed at -170 °C in a Technai microscope operating at 120 kV. Micrographs were taken at low dose conditions.

**Synthetic procedures**

All compounds were prepared according to literature procedure. Additionally the reactions were performed under vacuum.

**General synthetic procedure**

A round bottom flask was charged with PEO of respective molecular weight (1 eq.) and the temperature was increased to 180 °C. The vessel was evacuated for 15 minutes and subsequently the hydrogenated dimer fatty acid (1 eq.) and catalyst (0.03 eq.) was added. The reaction was then continued overnight under dynamic vacuum. After cooling 40 mL ethyl acetate/brine was added and the product was obtained after separation.

**DFA-PEO350**

Yield 93%, 8.8 g. \(^1\)H NMR (400 MHz, CDCl\(_3\)) δ 4.22 (t, 4H, CH\(_2\)OCO), 3.73 – 3.51 (m, 60.5H, OCH\(_2\)), 3.38 (s, 6H, OCH\(_3\)), 2.32 (t, 4H, CH\(_2\)CO), 1.62-0.88 (m, isomeric aliphatic peaks) \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) δ 70.43, 63.29, 58.95, 34.05, 31.74, 29.71, 24.82, 22.42, 14.04.

**DFA-PEO550**

Yield 84%, 5.56 g. \(^1\)H NMR (400 MHz, CDCl\(_3\)) δ 4.22 (t, 4H, CH\(_2\)OCO), 3.73 – 3.51 (m, 71.5H, OCH\(_2\)), 3.38 (s, 6H, OCH\(_3\)), 2.32 (t, 4H, CH\(_2\)CO), 1.62-0.88 (m, isomeric aliphatic peaks) \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) δ 70.44, 63.29, 58.95, 34.05, 31.74, 29.71, 24.82, 22.42, 14.04.

**DFA-PEO750**

Yield 92%, 7.09 g. \(^1\)H NMR (400 MHz, CDCl\(_3\)) δ 4.22 (t, 4H, CH\(_2\)OCO), 3.73 – 3.51 (m, 124.9H, OCH\(_2\)), 3.38 (s, 6H, OCH\(_3\)), 2.32 (t, 4H, CH\(_2\)CO), 1.62-0.88 (m, isomeric aliphatic peaks) \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) δ 70.44, 63.29, 58.95, 34.05, 31.74, 29.71, 24.82, 22.42, 14.04.

**DFA-PEO2000**

Yield 92%, 9.46 g. \(^1\)H NMR (400 MHz, CDCl\(_3\)) δ 4.22 (t, 4H, CH\(_2\)OCO), 3.73 – 3.51 (m, 305.6H, OCH\(_2\)), 3.38 (s, 6H, OCH\(_3\)), 2.32 (t, 4H, CH\(_2\)CO), 1.62-0.88 (m, isomeric aliphatic peaks) \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) δ 70.47, 63.18, 58.75, 34.09, 31.81, 29.54, 24.74, 22.58, 13.54.

**DFA-PEO5000**

Yield 64%, 2.07 g, gelation during work-up. \(^1\)H NMR (400 MHz, CDCl\(_3\)) δ 4.22 (t, 4H, CH\(_2\)OCO), 3.73 – 3.51 (m, 662.2H, OCH\(_2\)), 3.38 (s, 6H, OCH\(_3\)), 2.32 (t, 4H, CH\(_2\)CO), 1.62-0.88 (m, isomeric aliphatic peaks)
$^{13}$C NMR (100 MHz, CDCl$_3$) δ 70.44, 63.29, 58.95, 34.05, 31.74, 29.71, 24.82, 22.42, 14.04.
6.7 References


Summary

Supramolecular gels are gel networks that consist of (supramolecular) polymers connected via physical aggregation of the polymer chains, either with ‘sticky’ groups in the main chain of the polymers or as supramolecular crosslinks between the polymers. The dynamic nature of supramolecular gels gives rise to desirable functional properties, which can be used in for example tissue engineering.

In this thesis, the structure, morphology and mechanical properties of supramolecular hydrogels are studied, focusing on strain stiffening, which is characteristic for fibrous biopolymers in their native environment. The supramolecular motif of the hydrogelators in this thesis is typically the bis(urea) motif situated in a larger hydrophobic segment, whereas poly(ethylene) glycol provides the solubility in water.

In Chapter 1, an overview is given of the common properties and characterization of supramolecular hydrogels. Moreover, the mechanical properties of gel networks are described, especially strain stiffening in both biological and synthetic systems. Furthermore, the behavior of the bis(urea) motif is described, emphasizing its interactions in aqueous solutions. Finally, the context of the mechanical properties of hydrogels for tissue engineering is discussed.

Chapter 2 shows how the crosslink density of a hydrogel can be increased. The hydrogel consists of crosslinked semi-flexible rod-like micelles that are formed by PEO and bis(urea) based bolaamphiphiles. The crosslinkers are long, flexible poly(ethylene glycol) chains, functionalized at both ends with two of the same hydrophobic bis(urea) segments as in the bolaamphiphiles. Using the self-sorting of the bis(urea) motif, the efficiency of crosslinking is improved by making use of heterocrosslinkers with two different hydrophobic ends. These heterocrosslinkers have less tendency to form mechanically inactive loops within the same rod and thus form more bridges between rods a the same crosslinker concentration. At equal concentrations the hydrogels with the heterocrosslinker have a storage modulus that is 15 times higher than with the
homocrosslinker, showing that this is an efficient method to improve the crosslink density.

Chapter 3 introduces an alternative method for crosslinking the semi-flexible rods of bolaamphiphiles. Carboxylic acid crosslinking sites are introduced with a dicarboxylic acid bis(urea) bolaamphiphile that is incorporated into the rods. The system of rod-like micelles is subsequently crosslinked by the interaction between carboxylic acids and calcium ions. Upon the addition of calcium or zinc, the rods start forming bundles in which the individual rods can still be seen with CryoTEM.

In Chapter 4 a diacetylene group is introduced between the ureas of the bis(urea) motif. The aim is to covalently polymerize the rods in the axial direction, to remove the weak non-covalent interactions. The rod-like morphology is retained after polymerization according to CryoTEM with a sufficiently high degree of polymerization. However, the crosslinked system shows a loss of the viscoelastic network upon crosspolymerization.

Chapter 5 presents the characterization of a strain stiffening hydrogel based on segmented polymers of PEO and the bis(urea) motif. Below the critical gelation concentration these polymers form nanoparticles, which upon increasing concentration form fibers. With the formation of fibers, a change from strain yielding to strain stiffening is observed in the rheological characterization of the hydrogel. Using descriptive theoretical models from literature, the cause for strain stiffening is attributed to a structural reorganization of the fibrous network.

The final Chapter 6 shows the synthesis of hydrogelators out of the inexpensive building blocks PEO and dimerized fatty acids. These hydrogels show that the mechanical properties can be tuned by the molecular weight of the PEO and furthermore express desirable mechanical properties such as self-healing.
Publication list


Curriculum vitae

Marcel Koenigs was born on the 23rd of December 1982 in Tegelen, Netherlands. After he finished his secondary education at the Marianum/Valuascollege in Venlo in 2001, he studied Chemical Engineering at the Eindhoven University of Technology. During his study he performed an internship at TNO in Eindhoven and successfully completed the certificate program ‘Technology for Sustainable Development’. In 2009 he graduated from the group of prof. dr. E.W. Meijer in the laboratory of Macromolecular and Organic Chemistry under the supervision of dr. ir. A.R.A. Palmans and dr. ir. T. Mes. Afterwards, he started his PhD research project in the group of prof. dr. R.P. Sijbesma. The most important results of this research are described in this thesis.
Dankwoord

Bij het schrijven van het dankwoord kijk je terug op je promotie en realiseer je je dat vier mooie en leuke jaren alweer voorbij zijn. In deze periode hebben vele mensen mij met raad en daad bijgestaan en hun wil ik in dit gedeelte van het proefschrift hiervoor bedanken.
Hierbij is de eerste plaats voor mijn promotor prof. dr. Rint Sijbesma. Beste Rint, toen ik vier jaar geleden naar je toe kwam voor een promotieonderzoek met een duidelijke toepassing hadden we beiden niet kunnen vermoeden hoe complex en ‘anders dan anders’ supramoleculaire hydrogels zijn kunnen. Ik wil je bedanken voor het feit dat ik hierin mijn eigen weg kon zoeken en heb onze wekelijks geplande gesprekken hierover zeer op prijs gesteld. Veel succes gewenst met nieuwe uitdagingen en hopelijk mooie resultaten.
Als tweede wil ik mijn tweede promotor prof. dr. Bert Meijer bedanken. Bert, ik waardeer het zeer dat mijn afstudeerhoogleraar ook mijn tweede promotor is geworden. Ondanks dat dit door de nieuwe regelgeving komt, ben ik de laatste jaren steeds zeer betrokken geweest bij het onderzoek in jouw groep. Dit heeft ons ook meerdere mooie resultaten en publicaties opgeleverd, waardoor ik me altijd nog een beetje lid van de Meijer groep voel. Hartelijk bedankt en veel succes met het verder stimuleren van open wetenschappelijke discussies binnen het ICMS.
Verder wil ik twee leden van mijn commissie, dr. Kees Storm en dr. Hans Wyss, die nauw betrokken waren bij mijn onderzoek graag bedanken. Kees, hartelijk bedankt voor alle discussies door de jaren heen. Ik heb het vooral op prijs gesteld dat je altijd de moeite neemt om proberen de vertaalslag te maken van de natuurkundige kant, die veel met formules en modellen werkt, naar een plaatje of grafiek waardoor de discussie van beide zijdes met begrip gevoerd kan worden. Hans, thank you very much for your interest and input throughout the years in my project. Your knowledge of mechanical properties and oscillatory rheology has increased my insight into the field a lot and has also generated my enthusiasm for the characterization of materials.
Ik wil ook graag prof. dr. van Esch bedanken voor het plaatsnemen in mijn promotiecommissie. Ik heb tijdens mijn promotie kunnen profiteren van meerdere reviews en inzichten in supramoleculaire hydrogels die mijn begrip zeker hebben vergroot. Furthermore, I would like to thank dr. Oda for taking place in my committee. I appreciate your open and enthusiastic view of science. Verder wil ik ook prof. dr. ir. Luc Brunsveld bedanken voor het plaats nemen in mijn commissie. Ik heb gedurende mijn promotie veel te maken gehad met jouw groep en dit heeft me zowel binnen als buiten heb lab veel opgeleverd. Hartelijk bedankt voor het creëren van een groep die mij hartelijk ontvangen heeft en verantwoordelijk is voor een goede sfeer op onze verdieping.
Na mijn promotiecommissie zijn er nog meer mensen die belangrijk zijn geweest tijdens mijn promotie. First of all, I would like to thank dr. Asish Pal. Asish you helped me kick start my project and I have benefitted from your work throughout my four years. Your synthetic skills are remarkable, as well as your ability to explain and teach them to
other people. Next to this, you are a very kind and warm person and I wish you all the best in the future. Dr. Gajanan Pawar thank you for the synthesis of the polymers that are featured in chapter 5. Although the synthesis and their structure was straightforward, we could have never imagined that they display such a complex and interesting behavior as hydrogels. Good luck with your further career. Geert Pirotte, mijn afstudeerder, jouw werk heeft een mooie plaats gevonden in hoofdstuk 3. Het was mooi om de transformatie te zien van iemand die in het begin goed was in synthese en zijn chemie begrijpt naar iemand die zelfstandig zijn onderzoek beheerst. Hierdoor heb ik er ook vertrouwen in dat je met succes aan je promotie zult werken. Zahra and Hamed, my two colleagues in the hydrogel project. Thank you for all the discussions on the hydrogels, in which I learned a lot on the viewpoint of people from different scientific backgrounds, how to meet in the middle and work on problems from a different perspective.

Het gebruik van SAXS is een belangrijk element is mijn proefschrift. Hiervoor wil ik dr. Ilja Voets bedanken zonder wie ik de uitwerking en analyses niet zo makkelijk had kunnen maken. De hulp hierbij van dr. ir. Martijn Gillissen was ook van grote waarde. Martijn bedankt dat je me met de SAXS en andere algemene discussie hebt geholpen. Ik ben blij dat ik je tegen terug heb kunnen betalen met mooie CryoTEM plaatjes, waarbij we zelfs organische oplosmiddelen niet hebben geschuwd. Verder ook bedankt voor alle gezelligheid bij diverse metingen, conferenties en ook daarbuiten. Samen met Janus Leenders heb ik ook enige mooie resultaten geproduceerd, waarbij het raakvlak met mijn eigen onderzoek het grootst is binnen SMO. Janus bedankt voor alle discussies over water oplosbare systemen en veel succes met het afsluiten van je promotie en het daarbij ontwijken van ‘gedetailleerde’ karakterisatie van je hydrogels. Paul Bomans, de meester van transmissie elektronenmicroscopie, hartelijk bedankt voor alle tips, trucs en oplossing van problemen. Elk van mijn hoofdstukken heeft TEM plaatjes en jou hulp hierbij was van onschafbare waarde. Here I would also like to take the opportunity to thank dr. Pol Besenius for his introduction into the world of CryoTEM.


Anja en Tristan, eigenlijk zouden jullie helemaal bovenaan in dit dankwoord moeten staan. Zonder jullie begeleiding en stimuleren om iets beter te doen was ik waarschijnlijk nooit een promotie gaan doen. Het feit dat jullie intussen al veel mensen hebben begeleid die uiteindelijk veel beter worden, toont aan dat dit geen toeval is.
Part of my warm welcome on the third floor was provided by Katja. Thank you very much for everything, from being the bad cop when I was the good cop to organizing so many things such as parties and presents. The fact that we have already said goodbye to each other four times in the last two months is I hope just a good sign that we continue to meet each other now that we do not share an office anymore. Good luck with the new job. Dung, you are missed here in Eindhoven, especially on Wednesday evening going from the office to the football with me.

A special thanks to all members, past and present, of the Sijbesma group for discussions and sharpening the critical mind. Vooral Bob en Ramon als generatie genoten, bedankt voor al jullie steun en leuke momenten. Berry, zowel als kantoorgenoot en als opvolger van lab verantwoordelijke, sta je altijd klaar en dat waardeer ik zeer. This thanks is also extended to all the members of the STO 3.55 lab.

A big thank you to all the SMO football players. I think we showed that even with a big difference in football quality, everyone can have a fun evening and a good game.

Dit is ook het moment om het SMO retreat movie team te bedanken. Heren, hartelijk bedankt voor alle gezellige avonden en weekenden. De filmpjes zijn altijd goed ontvangen en ik ben ervan overtuigd dat als we alles hadden gedaan wat tijdens de brainstorm sessies besproken is, dit niet zo zou zijn.

SMO is een mooie diverse groep die de laatste jaren alleen maar groter is geworden. Dit is niet mogelijk zonder mensen die alles in goede banen leiden. Ik wou graag alle secretaresses bedanken, vooral Joke, die op de een of andere manier alles doet en toch altijd tijd heeft om een vraag of verzoek te beantwoorden. Hans, bedankt voor alle bestellingen en ook vooral het oplossen van problemen met apparaatuur en reparaties waar ik als labverantwoordelijke vaak genoeg mee aan kwam zetten. Ik was er niet vaak te vinden maar ook de mensen van het analytisch lab bedankt.


Zonger de hulp van mien elders waas ’t gaar neet meugelik gewees um oek maar aan mien studie te beginne, loat sjtoan det ik ’t ooit tot un succesvol eind had kenne bringe. ’t is gaar neet te doon um op te sjriewe wie belangriek ge veur mich ziet. Dankjewel.

Tradition ist es, dass die wichtigste Person am Ende steht. Dana, wir haben uns am Anfang von der Arbeit, die in diesem Buch steht, gefunden und ich hoffe, dass wir noch ganz lange danach zusammen sind. Die ersten Herausforderungen sind schon wieder da, aber zusammen mit dir ist alles möglich.

Marcel