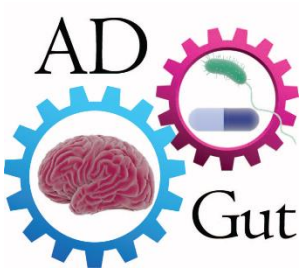

Max Planck Institute for Polymer Research

Department: Prof. Dr. T. Weil

„Designing hydrogel-based systems for the encapsulation of probiotic bacteria“

A thesis submitted as part of the requirements for

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Masterarbeit im Studiengang Chemie oder Biomedizinische Chemie an der Johannes Gutenberg-Universität Mainz

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Zusammenfassung:

Die folgende Arbeit befasst sich mit der Entwicklung eines Materials auf Hydrogelbasis zur Verkapselung anaerober Bakterien. Die Hydrogele werden als Teil eines interdisziplinären Forschungsprojektes (AD-Gut Projekt) entwickelt, um durch gezielte Modifikation der Darmflora Alzheimer vorzubeugen. Die Entwicklung einer dreidimensionalen Kulturmatrix, welche die Lagerung über einen längeren Zeitraum sowie die orale Vergabe und eine gezielte Applikation im Darm ermöglicht, eröffnet ein weites Einsatzgebiet in der modernen Medizin. Als Ausgangsmaterialen für die Hydrogelbildung wurde humanes Serum Albumin sowie Dextran verwendet. Das erste Hydrogel basiert auf oxidiertem Dextran, welches mit dem bivalenten Crosslinker Succinic dihydrazid einfach herzustellende Hydrogele bildet. Die Gelierung wird hierbei durch eine Bindung zwischen den Aldehyd Gruppen des modifizierten Dextrans und den Hydrazid Gruppen des Crosslinkers initiiert. Hierdurch können die Bakterien mit den Komponenten vermischt werden, bevor die Gelierung eingeleitet wird. Das Hydrogel wurde zu Beginn rheologisch charakterisiert und die Gelierungseigenschaften in den relevanten Medien untersucht. Um die Eignung für die gezielte Beeinflussung der Darmflora zu validieren wurde die Zersetzung der Hydrogele durch Änderung des pH Wertes und durch enzymatische Spaltung getestet. Um die Verträglichkeit der Gele mit den Bakterien zu ermitteln wurde *E.coli* für einen Zeitraum von 0, 1, 7, 14 Tagen verkapselt und danach ein Colony Formation Assay durchgeführt. Insgesamt stellte sich das oxD25 als ein hartes Hydrogel heraus, welches zwar durch enzymatische Spaltung jedoch nicht durch einen sauren pH zersetzbar war. Des Weiteren überlebte *E.coli* die Verkapselung nur in geringer Prozentzahl. Um ein flexibleres System zu erhalten wurde der bivalente Crosslinker durch ein modifiziertes humanes Serumalbumin (HSA) ersetzt. Durch Modifikationen mit PEG-Ketten, Diethylamin und Hydrazidkomponenten, wurde ein flexibles System erhalten. Mit dem entwickelten cHSA-PEG-Hyd/oxD25 Hydrogel wurden die gleichen Tests wie mit dem vorangegangenen durchgeführt. Das neue System zeigte enzymatische Zersetzung durch Trypsin und Dextranase. Die pH abhängige Zersetzung wurde jedoch ebenfalls nicht erreicht. Testreihen mit *E.coli* zeigten, dass die positive Oberfläche der Proteinkomponente zum Zelltod führte. Da die Verkapselung von Bakterien das Kernziel der Arbeit darstellt, wurde das System abgeändert. Wie bei der vorangegangenen Komponente wurden PEG-Ketten und

Hydrazide eingeführt. Jedoch wurde auf die Kationisierung mit Ethylendiamin verzichtet um das positive Zeta Potential zu verringern. Das erhaltene Hydrogel zeigte eine Verbesserung im Colony Formation Assay. Jedoch zeigte die neue Komponente eine schlechtere Löslichkeit. Durch diese Eigenschaft konnte das Hydrogel nicht rheologisch charakterisiert werden. Die Ergebnisse zeigten auf, dass die weniger positive Oberflächenladung zu einer verbesserten Viabillität der Bakterien führte. Die Löslichkeit der Proteinkomponente musste jedoch durch weitere PEG Ketten erhöht werden, um eine geeignete Verarbeitung zu gewähren. Beim vierten Hydrogel wurde die Anzahl der PEG Ketten erhöht, indem eine alternative Syntheseroute gewählt wurde. Hierdurch konnte ein System erhalten werden, welches ein negatives Oberflächenpotential aufweist und über genug PEG Ketten verfügt. Das erhaltene Gel zeigt positive Eigenschaften bei der Verkapselung der Bakterien. Des Weiteren wurde die Darm spezifische Zersetzung durch Trypsin und Dextranase experimentell bestätigt.

Insgesamt wurden im Rahmen dieser Arbeit vier Hydrogele entwickelt auf deren Basis weitere Systeme synthetisiert werden können. Die Studien haben gezeigt, dass das Oberflächenpotential einen großen Einfluss auf die Überlebensrate der Bakterien im Hydrogel hat. Weitere Faktoren wie Löslichkeit und Zersetzung müssen ebenfalls verbessert werden, um ein optimales Material für die Anwendung zu erhalten.

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1. Introduction

This Master´s thesis deals with the encapsulation and storage of bacteria in the 3D-matrix of hydrogels. Encapsulation of bacteria was done before with easy and conventional hydrogels like alginate [1] and milk proteins [2]. The thesis is part of a European project called Alzheimer´s disease-gut connection (AD-gut project). This project intends to find a treatment to prevent the plaque formation of the neurodegenerative illness Alzheimer's disease. For the clinical application a suitable hydrogel system has to be developed. As a model *E.coli* was used, but for the final application anaerobic strains like *Akkermansia* have to be encapsulated. These strains are more sensitive and react much faster to different environmental conditions. Facing the treatment, the bacteria have to be delivered orally into the colon. There the strains should establish in the gut microbiome. For a local application a controlled release is important. To fulfil this duty it is important to design a tuneable hydrogel with responsive elements. Therefore factors like pH value and enzymes have to be targeted. Hydrazones are such well known pH responsive crosslinker [3]. Hydrazone bonds tend to hydrolyse between a pH of 5 and 9. This corresponds to the pH value in the colon and can be used for hydrogel degradation [4]. Furthermore the degradation of the hydrogel could be achieved through the enzymatic cleavage of the polymer backbone. Polysaccharides derived from dextran were used for hydrogels before. Dextran is used as a core component since it is degradable by dextranase, an enzyme which is localized in the human colon. This enzyme is produced by *Bacteroides* which are located at the application site, the colon [5].

1.1. Alzheimer´s Disease (AD)

The Alzheimer´s disease is the most spread neurodegenerative disease, medicine has to face at the moment. More than 80% of all patients with neurodegenerative pathology are diseased with Alzheimer´s. In 2014 five million people at the age of 65 and older suffered from this illness. In 2050 it is expected, that every 33 seconds a new case of Alzheimer´s disease develops [6]. This disease is mainly based on the aggregation of amyloid β structures. Amyloid β40 and amyloid β42 are two proteins which appear after cleavage of the amyloid-precursor-protein (APP) through beta and gamma secretase [7]. Aβ proteins form soluble oligomers which emerge to be neurotoxic. Similar to prions, misfolded Aβ proteins induce an accumulation of the peptides. This behaviour

leads to plaque formation in the cortex of AD patients [8]. The second pathology of AD are neurofibrillary tangles (NFTs), which consist of misfolded Tau proteins. Other than the amyloid plaques the NFTs do not form plaques in the extracellular matrix but aggregate inside the neurons. It is believed that the misfolding of Tau proteins is induced by Aβ oligomers [9]. These two pathological appearances lead to neuronal degradation and symptoms of dementia in Alzheimer´s patients. Until 2014 only five treatments for Alzheimer´s disease were approved by the US Food and Drug Administration (FDA) [6]. All these treatments are not able to cure the illness or slow down the neuronal damage. The only possibility is the increasing of neurotransmitters in the brain. All pharmacological treatments in 2017 address this way of dealing with the illness [10]. The AD-gut project facing the research to handle this medical problem. Therefore it wants to prevent formation of amyloid β structures in the brain which are suspected to be a key factor of Alzheimer´s disease. It is shown that a connection between the intestine and the brain is given through the enteric nervous system (ENS). With the ENS it is possible for the brain to control and change the behaviour of the gastrointestinal tract. Studies have documented that the connection between these two organs can work in both directions and is called the gut-brain axis (GBA). It has been previously shown, that the microbiome has an influence on this bidirectional communication system [11]. Changes in the microbiome can activate proinflammatory factors like cytokines and some bacteria excrete amyloids and lipopolysaccharides, which are suspected to have an effect on other organs like the brain. The mechanisms are investigated in different experiments to see if the excreted bacterial amyloids can lead to plaque formation in the brain through the GBA [10]. Studies by T. Harach and coworkers have shown differences in the microbiome between conventionally raised wild-type mice and transgenic mice (APPPS1), which tend to develop Alzheimer´s (see **figure 1**)[13].

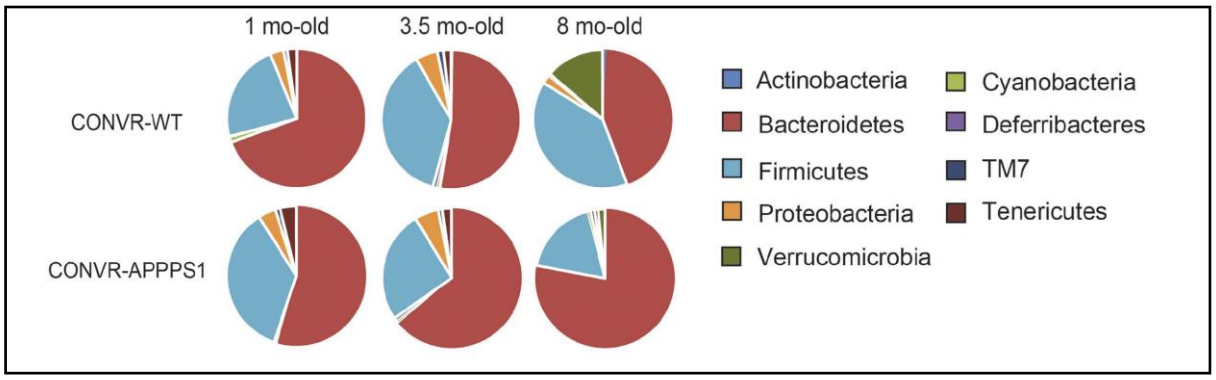

Figure 1: Microbiome of transgenic and WT mice - microbiome strains of 1, 3.5 and 8 month old mice compared between conventional raised wild type mice and transgenic APPPS1 mice [13].

Based on this discovery Harach and coworkers investigated if the composition of the microbiome changes the tendency to develop Alzheimer´s. Comparing germ free (GF) transgenic mice (APPPS1) with conventionally raised (CONVR) transgenic mice showed, that the amount of amyloid β proteins in the brain is significantly less in GF mice than in CONVR mice. Harach and coworkers showed that GF APPPS1 mice have a reduced amount of amyloid β inside the brain, although the level of APP is comparable to conventionally raised WT mice. The next step was the investigation of the influence of the microbiome on the progression of Alzheimer's symptoms. Therefore the microbiome of aged wild-type and APPPS1 mice was transferred to GF APPPS1 mice, respectively, and compared with CONVR transgenic mice [13].

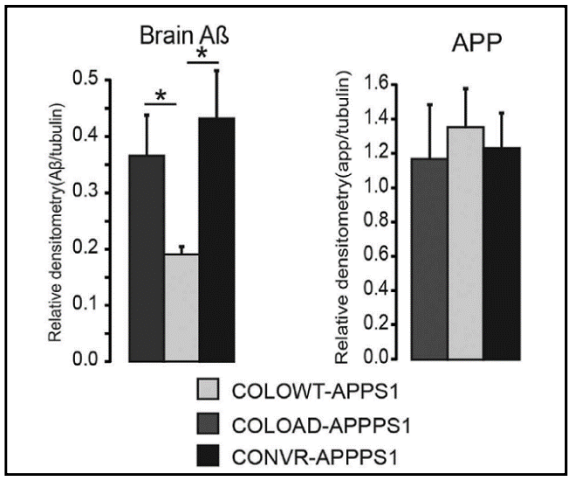

*Figure 2: A*myloid composition in microbiome transplanted mice – amyloidβ protein in the brain and total APP in APPPs1 mice transplanted with feces of WT mice (COLOWT) and APPPS1 mice (COLOAD) compared with CONVR APPPS1 mice [13].

The amount of brain amyloid β dropped significantly in the mice that were transplanted with the microbiome of wild type mice compared to the CONVR mice and the mice that were transplanted with the microbiome of APPPS1 mice [13]. These results indicate that a regulation of the microbiome could influence the progression of the Alzheimer´s

disease in a preventive manner. As part of the AD gut project workgroups are screening the microbiome to analyse which bacteria might be relevant for the progression of Alzheimer's disease. Following to these screenings the relevant bacteria have to be cultured successfully. Therefore the cultivation and storage conditions have to be improved for these bacteria. To optimize the storage and application and minimize the stress for the bacteria through environmental conditions an encapsulation in three dimensional cultures is targeted. Hydrogels are a promising material for such encapsulation and cell culture.

1.2. Hydrogels

Hydrogels are materials, which are capable to absorb water without dissolving. This ability can be explained by the structure of these materials. Hydrophilic polymers, like polyhydroxyethylmethacrylate (pHEMA), build a three dimensional network through tangles or ionic and covalent bonds to become insoluble. It can be distinguished between physical hydrogels, which are often reversible through environmental changes, and chemical hydrogels. This differentiation is based on the network formation mechanism. In physical hydrogels this happens through entanglements and secondary interactions, like ionic force, hydrogen bonds or hydrophobic interactions. An example for such a hydrogel would be an alginate hydrogel, which gelates through ionic interactions between the carboxylic groups of the polymer backbone and the bivalent calcium ions Ca^{2+}. These hydrogels often show a self-healing effect, when their three dimensional structure rebuilds after mechanical collapse [14]. Chemical hydrogels build their network through covalent bonds. The branched structure can be introduced via polymerisation as shown by Wichterle and Lim, who copolymerised HEMA with the bivalent monomer ethylene glycol dimethyl acrylate (EGDMA) [15]. Additionally polymer chains can be connected by using chemical crosslinkers. An example would be the hydrogel formation of oxidized dextran with dihydrazides similar to the experiments in this work. The hydrophilic character which appears through carboxyl, hydroxyl, amine and other polar groups of the polymers allows the hydrogel to swell in a liquid without dissolving and build a viscous material [16]. By varying the polar groups and the amount of crosslinking points the swelling properties and the rheological characteristics like storage modulus G' and loss modulus G" can be tailored [18].

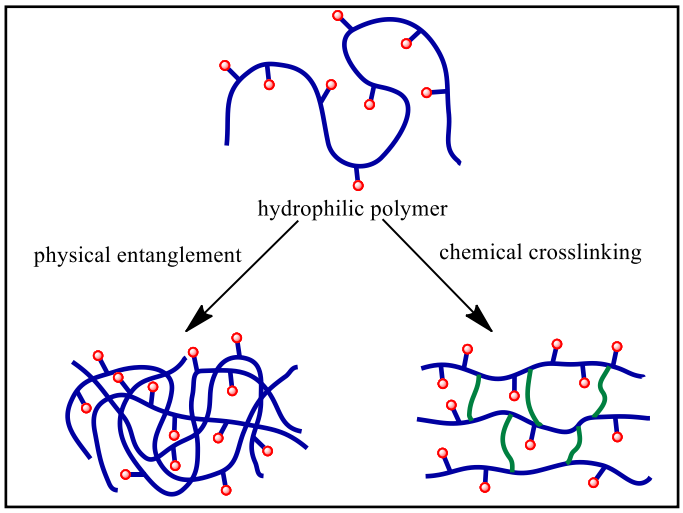

Figure 3: Formation of physical and chemical hydrogels

These abilities make hydrogels suitable for a wide range of applications. Through their elasticity and optical behaviour, which is comparable to glass the previously mentioned pHEMA was used as a material for the first contact lenses in 1960 [15]. Other possible functionalities of hydrogels are given as wound dressings and three dimensional scaffolds. Here different functions can be achieved by changing the hydrogels characteristics. Their softness can be used to build up a protective layer on large superficial wounds and the consistency makes it easy to remove the batches without causing new damages. The hydrogel can protect the wound from drying and prevent infections through bacteria. On the other hand hydrogels offer a possibility for the treatment of deeper wounds to support cell grow and regeneration of tissue [17]. Another usage for hydrogels is the encapsulation of drugs inside the hydrogel to deliver them to a specific area. Therefore the drug can be released through diffusion out of the hydrogel, which allows a release of the drug over a longer time period. Different to this a release after a fast degradation of the hydrogels or swelling can appear. In this case the drug is often surrounded by a hydrogel, which then degrades or swells and releases the drug faster [17]. A further interest in medicine is the encapsulation of human cells for tissue engineering. Hydrogels can be used as an artificial extracellular matrix for cells to grow and assemble [21]. With this technique it is already possible to encapsulate for example osteoblasts in different hydrogels to repair bone defects [22,23]. Besides bone defect hydrogels could be used as a matrix for neuronal repair in the spine. Here the drug carrying properties and the structural properties of a hydrogel are combined [24]. The ability of encapsulation can not only be used for mammalian cells. The usage of bacteria opens a wide spectrum of medical and experimental possibilities. Hydrogels

offer an effective way to deliver probiotic bacteria into the intestine. For example *Lactobacillus* was already successfully encapsulated in alginate and milk protein hydrogels [1,2]. Other groups used hydrogels as a scaffold to encapsulate *E.coli* to form a 3D culture [18]. Furthermore anaerobic bacteria like *Bifidobacterium* strains were successfully encapsulated in hydrogels within the last years [20]. These investigations are similar to the target of our work. During this thesis hydrogel systems are designed to encapsulate anaerobic bacteria for storage and oral application targeting a colon specific release.

1.3. Hydrogels from Natural Polymers

Considering all these possible applications for hydrogels new fields for research are spreading. Natural polymers can be used to form bio-based hydrogels, resulting in an increasing research topic of the last decades. Hydrogels can be synthesised out of many natural compounds, as example a chitosan derived gel [25]. This polysaccharide structure is obtained by deacetylation of chitin and can be crosslinked by glutaraldehyde to form hydrogels. Another well-known bio-based hydrogel component is alginate [26]. This natural polymer from brown algae and some bacteria gels through ionic interactions between carboxylic groups and calcium ions. Alginate is a god example for biodegradability of some bio-based hydrogels [27]. Different factors which are affecting the hydrogel inside the organism, for instance the enzymatic cleavage of the polymer backbone or the repeal of the crosslink, disrupt the three dimensional structure. In the case of alginate the diffusion of the calcium ions leads to the annulment of the three dimensional structure. A broad spectrum of bio-based hydrogels does not only consists of polysaccharides. Proteins and short peptides can also be used for hydrogels. Natural peptides like gelatine and collagen are able to form fibrillary hydrogels, but even globular proteins like bovine serum albumin are used to form gels [27]. Bio-based hydrogels do not have to be polymers from natural sources. Peptides and proteins can be synthesised and/or modified with synthetic compounds [28]. Especially peptides are synthesised to form, for example, fibrillary structures resulting in a hydrogel network [30]. Proteins are rather obtained from biological material and subsequently modified with functional groups. An example would be denatured human serum albumin (HSA) which can be functionalised with polyethylene glycol (PEG) chains to increase solubility [31]. The process of

biodegradation has to be faced in every hydrogel, which is designed for medical applications. Using a specific backbone and suitable crosslinker results in hydrogels with specific release mechanism [32]. Enzymes like dextranase, collagenase or trypsin cause a degradation of hydrogels through cleaving of the backbone [31,33]. Another way of degrading a hydrogel would be the cleavage of crosslinker and/or the entanglement. The degradation of alginate hydrogels which occurs over a long period of several weeks [34]. The choice of the right backbone and crosslinker can affect the duration of the degradation process. This makes hydrogels suitable for both burst releases and a delivery over a long period. The degradation conditions do not only influence the release time of an encapsulated molecule or cell, it can also determine the location [34]. PH responsive bonds like the previously described hydrazone bond could be used to target specific sections in the gastrointestinal tract. The change from a basic to a more acidic pH can hydrolyse the hydrazone and degrade the hydrogel which leads to a bacteria release.

1.4. Motivation

Alzheimer´s disease is one of the most common illnesses leading to dementia. Until now no treatment is developed to stop this disease or help to prevent it. The worst thing about Alzheimer´s disease is the development of dementia. Besides being a medical problem, dementia poses a heavy social and economic burden on the patient and the family. The forecasts for this disease are disturbing today. In 2017 every 66 seconds someone is developing this disease. Until 2050 this number is going to increase to one patient every 33 seconds [6]. This Master´s thesis offers the possibility to participate the development of Alzheimer´s disease prevention. Hydrogels are used in a wide field of medical applications. The challenge will be to find a hydrogel which assembles all needed characteristics. The desired hydrogel has to be nontoxic for both patients and the bacteria, furthermore it has to be degradable in the colon. This can be achieved by either a responsiveness to pH changes inside the intestine or by cleavage of the hydrogel components by specific enzymes. All characteristics have to be achieved by choosing the right backbone, an adequate crosslinker and the right modification. This tailoring must be done while the viability of the bacteria has to be guaranteed which marks the hardest part of the project. The work will be based on the design of a modified human serum albumins and oxidized dextran. These systems are

expected to be pH responsive, enzymatically cleavable and biocompatible. This thesis can lead to new materials, which have the possibility to bring new opportunities in modern medicine.

2. Material and Instruments

All chemicals were obtained by commercial suppliers and used without further purification unless indicated otherwise. Dextran (from *Leuconostoc mesenteroides*), human serum albumin, dimethyl sulfoxide (DMSO), trypsin (from bovine pancreas), dextranase (from *Chaetonium erraticum*), $PEG_{(2000)}$-maleimide and dinitrophenyl hydrazide in phosphoric acid were purchased from Sigma-Aldrich. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) was purchased from GL Biochem LTD. (Shanghai, China). Sodium acetate was received from Riedel de Haen. Acetic acid was received from Merck. Sodium hydroxide solution and hydrochloric acid were ordered from VWR International. Disodium hydrogen phosphate, sodium periodate, tris(2-carboxyethyl)phosphine (TCEP) and hydroxylamine hydrochloride were purchased from Carl Roth. Sodium dihydrogen phosphate, urea, ethylenediaminetetraacetic acid (EDTA) and ethylenediamine were ordered from Acros. Succinic dihydrazide was used from TCI. N-β-maleimidopropionic acid hydrazide (BMPH) was purchased from Thermo-Fisher Scientific. The MeO-$PEG_{(2000)}$-NHS ester was ordered from Rapp Polymere

For purification by ultracentrifugation Vivaspin®20 tubes with MWCO 30,000 and MWCO 5,000 from Sartorius were used. MALDI-ToF measurements were performed with a Reflex II, Reflex III and Rapiflex from Bruker. Rheological measurements were executed with a Discovery HR-3 hybrid rheometer from TA Industries. The microscopic images were measured with a Leica DMi8 microscope which was equipped with a Leica DFC 3000 GT as well as a Leica MC170 HD camera and a lumencor sola light engine as light source. The zeta potential was measured with a Zetasizer Nano Series from Malvern. For NMR analysis a Bruker Biospin 850 MHz AVANCE III was used.

3. Experimental

3.1. Preparation

3.1.1. Synthesis of Oxidized Dextran

Besides human serum albumin, oxidized dextran represents the core of this work. It was used in every hydrogel within the scope of the project.

Oxidized dextran with a theoretical oxidation degree of 25 % (oxD25) was mainly used. Therefore 0.25 equivalent sodium periodate per dextran ring were used. With an average chain length of 61 saccharide rings the reaction should lead to 15 oxidized dextran rings.

$$\frac{10{,}000u}{162\frac{u}{rings}} \approx 61\ rings \rightarrow 61rings * 0.25 \approx 15\ oxidized\ rings$$

For the synthesis 500 mg of dextran (molecular wt 9,000-11,000) was dissolved in 40 ml Milli-Q water. Then 162 mg (0.25 eq) of sodium periodate was dissolved in 10 ml H_2O. The periodate solution was added dropwise to the polysaccharide solution and stirred in the dark for 24 h at RT. The reaction was stopped by adding 42 µl of ethylene glycol. The product was purified using ultrafiltration (MWCO 5,000) several times against water. The resultant solution was then lyophilized to yield modified dextran as a colourless solid.

3.1.2. Quantification of Aldehyde Groups

To determinate the degree of oxidation the hydroxylamine hydrochloride method was used as follow. In the first step a 0.25 M hydroxylamine hydrochloride solution had to be prepared. Therefore 1.74 g hydroxylamine hydrochloride was dissolved in 100ml Milli-Q water. After completely dissolving the pH was adjusted to pH 4 with sodium hydroxide and hydrochloric acid. Then 50 mg of the sample to be analysed were dissolved in 25 ml of the reagent solution prepared previously. The mixture was stirred for 3 h at 50 °C to react completely. After cooling to room temperature the solution was titrated with 0.1 M sodium hydroxide solution and the pH change was monitored using a pH-meter.

For analysing the degree of oxidation via NMR the dextran was modified with dinitrophenylhydrazine. Therefore a reagent solution in phosphoric acid and ethanol was used. 5 mg dextran was dissolved in 700 µl d6-DMSO. To modify the hydrazide reactive groups 200 µl of the reagent was added. The solution was added in an NMR-tube and 1H, 13C, and HSQC spectra were measured. To calculate the oxidation degree the amount of -CH_2 protons of the dextran backbone and shifted aromatic protons were compared.

3.1.3. Preparation of oxD25 hydrogels

To form hydrogels a 20 mM oxD25 solution had to be prepared. Therefore 2 mg oxD25 were dissolved in 10 µl Milli-Q water. To initiate the crosslinking, a 0.15 M succinic dihydrazide solution was used. To prepare the crosslinker, 0.219 mg succinic dihydrazide (SucHyd) were dissolved in 10 µl Milli-Q water. For hydrogel formation 3 µl oxD25 solution was mixed with 3 µl SucHyd solution in a PCR tube. This synthesis leads to a ratio of 2:1 aldehydes to hydrazide groups.

3.1.4. Gelation of oxD25 in Different Bacteria Media

The gelation in microbiological medium was first evaluated to determine the suitability for the encapsulation of bacteria within the project. To test the gelation, the two components were dissolved in LB- and fastidious medium. 2 mg oxD25 were dissolved in 10 µl LB- or fastidious medium and 0.219 mg SucHyd were dissolved in the same amount of the corresponding medium. To initiate the gelation 3 µl of the oxD25 solution and 3 µl of the SucHyd mixture were added to a 96well plate. The gelation was monitored microscopically and images were taken.

3.1.5. Synthesis of Cationized Human Serum Albumin cHSA

For cationization of human serum albumin an ethylenediamine solution (2.5 M; pH 2.5) had to be prepared. Therefore 41.67 ml ethylenediamine were dissolved in 208.33 ml Milli-Q water and the pH was regulated with hydrochloric acid. Then 1.2 g (18.06 µmol) human serum albumin were dissolved in 150 ml of the prepared ethylenediamine solution. The reaction was initiated by adding 6.136 g

EDC·HCl (32 mmol) and stirred for 2 h at RT. To stop the reaction 8 ml acetate buffer (4 M; pH 4.75) were added. The product was purified by ultracentrifugation (Vivaspin20; MWCO 30,000) and washed two times with acetate buffer (0.1 M; pH 4.75) and five times with Milli-Q water. After lyophilisation the product was received as a colourless solid. MALDI-ToF m/z: 71,734; Zeta Potential: 33.4 mV

3.1.6. Preparation of cHSA-PEG(2000)$_{14}$

To introduce polyethylene glycol to cHSA with a MeO-PEG$_{(2000)}$-NHS ester a phosphate buffer (50 mM; pH 8) was needed. Therefore 2.999 g sodium dihydrogenphosphate and 3.549 g disodium hydrogenphosphate were dissolved in 1 l Milli-Q water and the pH was regulated with sodium hydroxide solution. Then 50 mg (0.7 µmol) cHSA were dissolved in 50 ml of the degassed phosphate buffer. 37.87 mg MeO-PEG$_{(2000)}$-NHS (27 eq) were dissolved in 500 µl DMSO. During stirring the NHS-ester was added dropwise to the cHSA solution. The reaction was stirred for 2 h and then the product was purified by ultracentrifugation (Vivaspin20 MWCO 30,000) and washed eight times against water. The product was received as a colourless solid by lyophilisation. MALDI-ToF m/z: 100,728; Zeta Potential: 17.8 mV

3.1.7. Denaturation of the cHSA-PEG

First a denaturation buffer had to be prepared. Therefore 60.06 g Urea and 116.89 mg ethylenediaminetetraacetic acid were dissolved in 200 ml sodium phosphate buffer (50 mM) and the pH was adjusted to pH 7.4 with sodium hydroxide and hydrochloric acid. First 10 ml of the previously prepared buffer had to be degassed, before 23.90 mg (230 nmol) cHSA-PEG were added. After the protein was dissolved the reaction mixture was stirred for 15 min at RT. Then 6.3 mg (22 µmol) tris(2-carboxyethyl)phosphine were added and the mixture was stirred for further 30 minutes. In the last step 6.53 mg BMPH were added and the reaction was stirred overnight under argon atmosphere. The product was purified by ultracentrifugation (Vivaspin20 MWCO 30,000) and washed 8 times with Milli-Q water. After the lyophilisation the cHSA-PEG-Hyd was achieved as a colourless powder. Zeta Potential: 20.6 mV

3.1.8. Preparation of cHSA-PEG-Hyd/oxD25 Hydrogels

Two solutions for hydrogel formation were prepared: A 1 mM cHSA-PEG-Hyd solution and a 20 mM oxD25 solution. For the protein solution 1.1 mg cHSA-PEG-Hyd were dissolved in 10 µl Milli-Q water and for the dextran solution 0.2 mg OxD25 were dissolved in 2 µl Milli-Q water. For gelation 5 µl protein solution was mixed with 1 µl dextran solution in a PCR-tube. The mixture was allowed to gel for 20 minutes before further usage.

3.1.9. Gelation of cHSA-PEG-Hyd/oxD25 in Different Bacteria Media

To test the ability of the compounds to gel in different media, hydrogels were prepared in LB-medium and Fastidious medium. Therefore 1.1 mg cHSA-PEG-Hyd was dissolved in 10 µl LB or fastidious medium. 0.2 mg oxidized dextran was dissolved in 2 µl LB or fastidious medium. The compounds were pipetted into a 96-well plate and analysed under a microscope.

3.1.10. Preparation of HSA-PEG(2000)$_3$

For a higher solubility of human serum albumin PEG$_{(2000)}$ was introduced. Therefore the previously prepared phosphate buffer was used. 100 mg (1.51 µmol) HSA were dissolved in 100 ml phosphate buffer (50 mM; pH 8). Then 81 mg (27 eq) MeO-PEG$_{(2000)}$-NHS Ester are dissolved in 1 ml DMSO. The DMSO solution was added dropwise to the HSA solution. The mixture was stirred for 4 h before the purification via ultracentrifugation (Vivaspin20 MWCO 30,000) was implemented. The product was washed eight times with water and received as a colourless solid by lyophilisation. MALDI-ToF m/z: 71,614; Zeta Potential -2.30 mV

3.1.11. Denaturation of the HSA-PEG

The denaturation was performed in the previously prepared denaturation buffer. 10 ml urea buffer (5 M urea, 50 mM phosphate, pH 7.4) were degassed by ultrasonic bath and vacuum. Then 15 mg HSA-PEG were added. After dissolving the mixture was stirred for 15 min at RT. To reduce the protein 6.3 mg (22 µmol) tris(2-carboxyethyl)phosphine were added and stirred for further 30 min. To complete

the denaturation 6.53 mg BMPH were added to the reaction. The mixture was stirred overnight under argon atmosphere. The product was purified by ultracentrifugation (Vivaspin20 MWCO 30,000) and washed several times with Milli-Q water. After lyophilisation the HSA-PEG-Hyd was accomplished as a colourless powder. Zeta Potential: -23.6 mV

3.1.12. Preparation of cHSA-PEG-Hyd/oxD25 Hydrogels

To achieve the new hydrogel two solutions were prepared: A 1 mM HSA-PEG-Hyd solution and a 20 mM oxD25 solution were processed. For the first solution 0.848 mg cHSA-PEG-Hyd were dissolved in 10 µl Milli-Q water and for the second solution 0.2 mg OxD25 were dissolved in 2 µl Milli-Q water. For gelation 5 µl protein solution was mixed with 1µl dextran solution in a PCR-tube. The protein solution had to be handled quickly because it gelated by itself. The protein did not solve completely and aggregate through hydrophobic interactions. When it is handled quick and mixed directly a hybrid hydrogel could be achieved.

3.1.13. Gelation of cHSA-PEG-Hyd/oxD25 in Different Bacteria Media

The need of the new hydrogel to gel in different media is beneficial for the progress of the project. Therefore 0.848 mg HSA-PEG-Hyd were mixed with 10 µl LB or Fastidious medium. Then 5 µl were directly mixed with 1 µl of the 10 mmol oxD25 solution in a 96-well pate. The state of gelation was analysed under the microscope.

3.1.14. Preparation of cHydHSA

To introduce the succinic dihydrazide to the Serum Albumin the carboxyl groups of the aspartic acids and the glutamic acids had to be activated with carbodiimides. Therefore 365 mg succinic dihydrazide were dissolved in 10 ml Milli-Q water and the pH was regulated to pH 4.7 with hydrochloric acid. Then 100 mg human serum albumin were dissolved in the reaction mixture. To initiate the reaction 511mg EDC·HCl were added and the mixture was stirred for 4 h at RT. To stop the reaction 0.66 ml acetate buffer (4 M, pH 4.75) was added. For purification the product was concentrated via ultracentrifugation (MWCO 30,000), washed two times with acetate buffer

(0.1 M; pH 4.75) and several times with water. After lyophilisation the product was received as a colourless solid. MALDI-ToF m/z: 72,178; Zeta Potential: -0.0864 mV

3.1.15. Denaturation of cHydHSA

The denaturation is performed in the previously prepared denaturation buffer. 10 ml urea buffer (5 M urea, 50 mM phosphate, pH 7.4) was degassed. Then 15.71 mg cHydHSA were dissolved in the buffer. After the protein was completely dissolved the mixture was stirred for 15 min at RT. Then 6.3 mg (22 µmol) tris(2-carboxyethyl)phosphine were added and stirred for further 30 minutes. To introduce the PEG chains 46.46 mg PEG(2000)-maleimide were added to the reaction and the mixture was stirred overnight under Argon atmosphere. The product was concentrated by ultracentrifugation (Vivaspin20 MWCO 30,000) and washed seven times with Milli-Q water. After lyophilisation the cHydHSA-PEG was received as a colourless powder. Zeta Potential: -2.39 mV

3.1.16. Preparation of cHydHSA-PEG/oxD25 Hydrogels

First two solutions had to be prepared. For the protein solution 2.16 mg cHydHSA-PEG were dissolved in 10 µl water to receive a 1.5 mM solution. For the second solution 0.4 mg oxD25 were dissolved in 2 µl Milli-Q water to obtain a 20 mM solution. To prepare a hydrogel 5 µl of the cHydHSA-PEG solution was pipetted in a PCR tube before 1 µl of the polysaccharide solution was added. The mixture was inverted several times to distribute the components.

3.1.17. Gelation of cHydHSA-PEG/oxD25 in Different Bacteria Media

To check the suitability of the compounds for the project, the gelation in different media has to be tested. Therefore a 1.5 mM solution of cHydHSA-PEG in LB and Fastidious medium was prepared by mixing 2.16 mg modified protein with 10 µl of the respective medium. The oxD25 compound was dissolved to obtain a 20 mM solution with the different media. To monitor the gelation the solutions were mixed like described in **3.1.16** inside a 96-well plate and images were taken with a microscope.

3.2. Analysis

3.2.1. Rheological Measurements

For the rheological experiments a Discovery HR-3 hybrid rheometer from TA Industries was used. The hydrogels were freshly prepared on a sample plate with a diameter of 8 mm. The hydrogel was positioned by mixing the solutions like described in the following table. The measurement of HSA-PEG-Hyd was not possible, due to the spontaneous gelation of the modified protein solution.

Table 1: Hydrogel compositions and preparation for rheological measurements

Hydrogel	**Component A**	**Volume A**	**Component B**	**Volume B**
oxD25	20 mM oxD25	15 µl	0,15 M SucHyd	15 µl
cHSA-PEG-Hyd / oxD25	20 mM oxD25	25 µl	1 mM cHSA-PEG-Hyd	5 µl
HSA-PEG-Hyd /l oxD25	-	-	-.	-
cHydHSA-PEG / oxD25	20 mM oxD25	25 µl	1,5 mM cHydHSA-PEG	5 µl

After the mixture was distributed over the sample plate, the gap between the measurement plate and the sample plate was closed to a gap of around 400 µm to fill the whole gap with hydrogel. The frequency was fixed to 1 Hz for the following experiments. In the first experiment the change of the storage modulus (G') and the loss modulus (G") was measured over a time frame of 1 h. In this experiment the process of gelation was monitored. An increase of both moduli was expected, until the gelation process was finished. The second experiment was the measurement of the hydrogel stability. Therefore an amplitude sweep was performed. In this experiment the oscillation strain was increased slowly. At a certain point the loss modulus (G') becomes higher than the storage modulus (G"). At this crossover point the hydrogel structure is destroyed and the viscous behaviour outweighs. After the amplitude sweep a time sweep was performed to monitor potential self-healing effect.

3.2.2. Degradation Behaviour of Hydrogels

For the degradation, hydrogels were prepared like described previously (**chapter 3.1**) and placed in a 96well-plate. 75 µl of 50 mM phosphate buffer with pH2 and pH 4,

respectively, were added and the samples were incubated at 37 °C. The process was monitored with the microscope after 0, 5, 30, 60, 90 and 120 minutes.

For the degradation through enzymatic cleavage a dextranase solution had to be prepared. Therefore the commercially obtained dextranase solution with a concentration of 500 mg/ml was diluted with 50 mM phosphate buffer (pH 5.4) to a concentration of 1.5 mg/ml. The hydrogels were prepared as described in the previously chapters and placed in 96 well plates. 75 µl of the enzyme solution was added and the samples were incubated at 37 °C for 0, 5, 30 and 60 minutes. The degradation process was monitored by microscope.

For the trypsin dependent degradation 0.5 mg trypsin were dissolved in 50 mM phosphate buffer with pH 7 to give a 1 mg/ml solution. The hydrogels were prepared as described earlier and placed in 96well plates. They were incubated at 37 °C for 0, 5, 30 and 60 minutes. The degradation process was observed by using microscope.

3.2.3. Colony Formation Assay

For the experiment 1.5 ml of the cultured bacteria were centrifuged for 5 minutes at 1,000 g to form a wet bacteria pellet. The surfactant was taken off and the *E.coli* pellet was diluted with fresh LB-Medium to a concentration of 3 mg/ml. For the encapsulation the hydrogels were prepared as described in the previous chapters using the bacteria mixture as solvent. The formed hydrogel was covered with 50 µl water and stored at 4 °C for 0, 1, 7 and 14 days. After incubation the hydrogels were dissolved mechanically and diluted to a total volume of 150 µl with LB-medium. Then a serial dilution was prepared like seen in the figure below. The dilutions with the factor x1,000 and x10,000 were plated on conventional agar plates and incubated overnight at 37 °C. On the next day the grown bacteria colonies were counted manually and the CFU/ml was calculated.

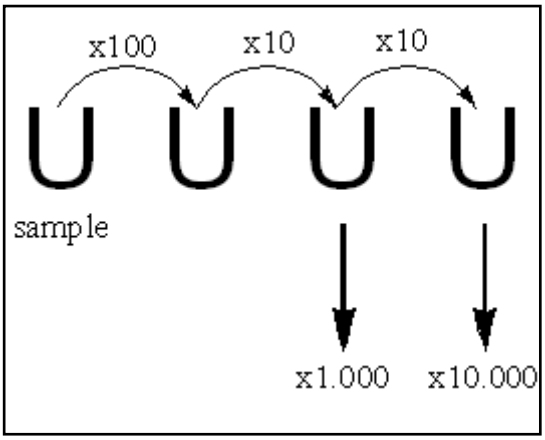

Figure 4: Serial dilution for CFU-assay – preparation of serial dilution to validate the CFU/ml

Besides *E.coli* as an aerobic model, the project-related anaerobic strains were tested. These experiments were carried out by cooperation partners at Ulm University.

4. Oxidized Dextran Hydrogel

4.1. Introduction

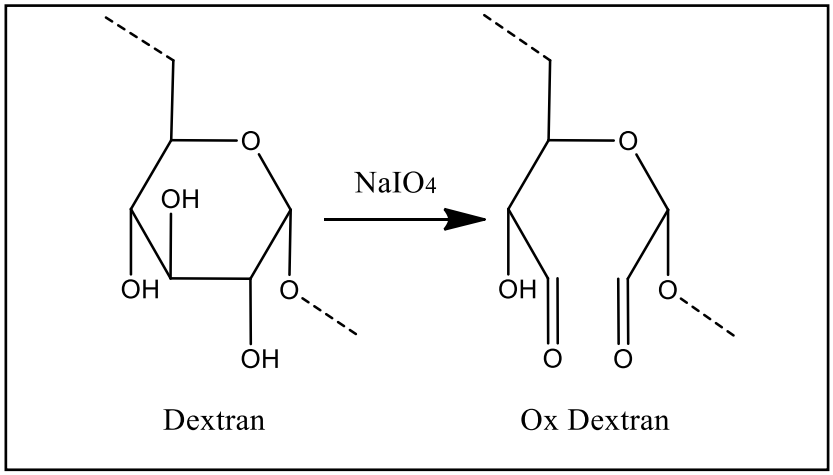

Figure 5: Oxidation of dextran – oxidation of dextran with sodium periodate

Polysaccharides are one of the oldest components known for hydrogel formation. Natural saccharides like alginate and chitosan are commonly used for hydrogels [1,35]. They are considered as biocompatible and nontoxic. Furthermore they are cheap and biodegradable and in most cases, the hydrogels are easy to prepare. They have functional groups like amines, hydroxy- and carboxygroups and can be gelated through several mechanisms. The polysaccharide which is used is dextran, which can be degraded specifically in the colon through dextranase[36]. Dextran is often used in its oxidized form to prepare hydrogels with aldehyde responsive crosslinkers. For this

system succinic dihydrazide was chosen as a crosslinker, due to its solubility. This hydrazine derivative of the bivalent succinic acid reacts with the aldehyde groups of the oxidized dextran. The hydrazone bond formation appears in a basic pH and is cleaved changing to more acidic conditions.

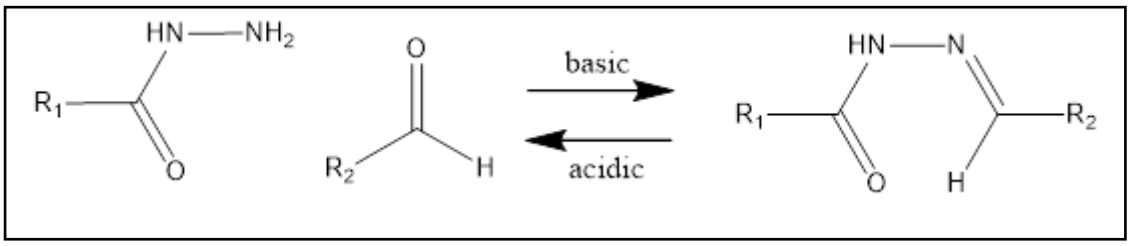

Figure 6: Hydrazone bond formation – reaction of aldehydes with hydrazides to form hydrazones

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4.2. Results and Discussion

4.2.1. Preparation of Hydrogels in Water and Media

The hydrogel preparation showed positive results. After mixing the oxD25 solution and the succinic dihydrazide crosslinker solution the first gelation appeared upon inverting the mixture. After a few minutes it became harder. A clear, sticky hydrogel was obtained, as shown **figure 7**.

Figure 7: oxD25 hydrogel

As depicted in the figure below the LB medium, used to culture *E.coli*, and the Fastidious medium, which is used for culturing *Akkermansia*, did not interfere with the gelation of the hydrogels. The side reactions of amine groups, which could react with the aldehydes to form imines, occur in strong basic conditions only. Since the media have a pH value of 7 no interference was observed in agreement with the expectation.

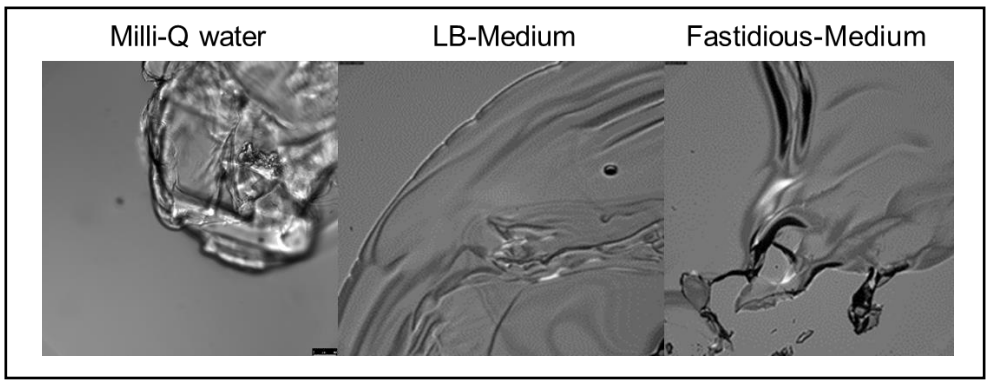

Figure 8: oxD25 hydrogel in different solvents - oxD25 hydrogels prepared with LB and Fastidious medium as solvent. Milli-Q water was used as control (scale 50 µm).

A gelation to a clear hydrogel can be seen in the figure above, substantiating that the microbiological media will not prevent hydrogel formation. The gelation appeared as expected in water and the growth media and further investigations could be initiated.

4.2.2. Determination of Aldehyde Groups

To determine the oxidation degree of the modified dextran the pH change of the hydroxylamine solution with oxD25 samples is monitored. The result of the titration can be seen in the graph below.

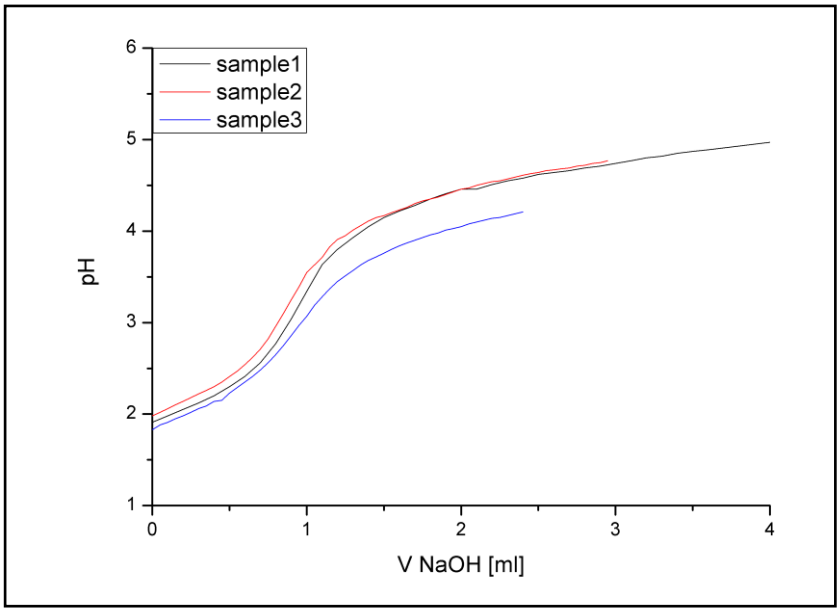

Figure 9: pH titration of oxD25 samples – titration of HCl with NaOH for evaluating the amount of hydroxylamine reacted with the aldehydes.

The graph shows the pH value over the volume of 0.1 M sodium hydroxide solution used. The average equivalent point appeared at 1.02 ml. The oxidation degree can be calculated using the following formula.

$$\% = \frac{V_{NaOH} * C_{NaOH} * 162{,}14 \frac{g}{mol}}{m_{oxD25} * 2}$$

Using titration an oxidation degree of 16.5 ± 0.3 % was determined.

To verify the result of the titration NMR studies were performed. Here the aromatic protons of dinitrophenyl hydrazine are compared to the proton of the CH_2 group which connects the saccharide rings. The spectrum can be seen in the appendix. Using NMR measurements an oxidation degree of 17 ± 1 % could be calculated, further substantiating the result from the titration.

4.2.3. Rheological Measurements

To determine the time required for complete gelation and the physical characteristics of the resultant hydrogel, rheological measurements were performed in triplicates. The figures below summarize the data obtained from time-sweep and amplitude measurements.

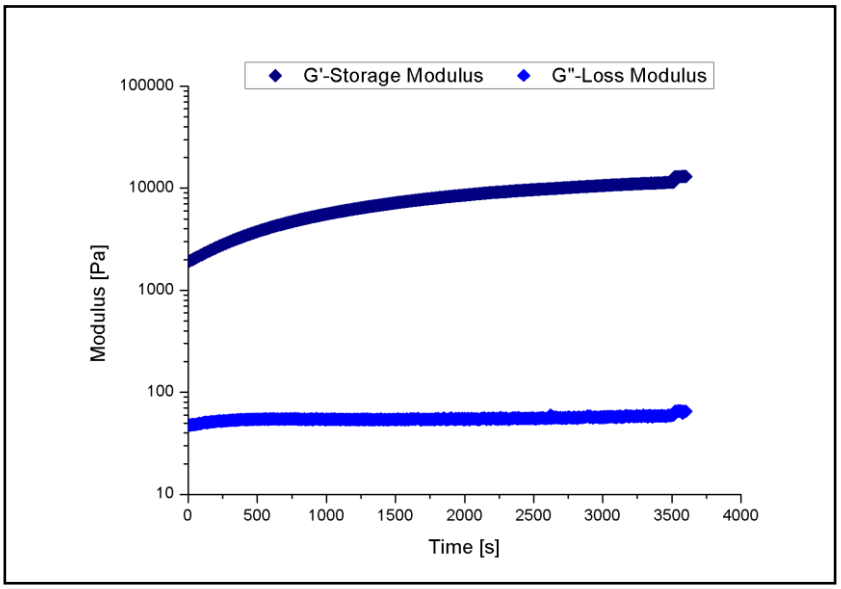

Figure 10: Time sweep oxD25 – time sweep performed with a strain of 1 % at 1 Hz to monitor the gelation time and moduli.

In **figure 10** we can see the storage (G') and loss modulus (G") over the time frame of 1 h. After 15 minutes a plateau can be observed. After this point the moduli did not

change significantly. This implies that the gelation of the hydrogel is completed after 15 minutes. The gel shows a storage modulus (G') of 12524 Pa and a loss modulus (G") of 63 Pa.

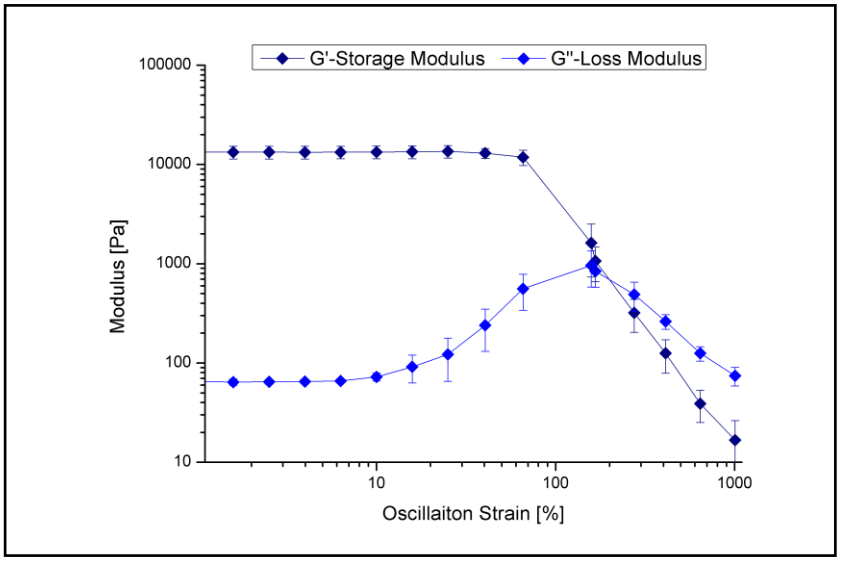

Figure 11: Amplitude sweep oxD25 – amplitude sweep to investigate the collapse point of the hydrogel

In **figure 11** we can see the storage and loss modulus over an increasing oscillation strain. A crossover of the moduli can be observed at a strain of 200 % and a modulus of 693 Pa. After the amplitude sweep an additional time sweep was performed to determine, if there is a self-healing effect (see Appendix). The results shown, that the collapse of the hydrogel was not irreversible.

In general it can be postulated, that a hard hydrogel is obtained. This can be seen through the high difference between storage modulus G' and loss modulus G", which represents the solid like and liquid like behaviour. In addition no self-healing effect could be observed. These observations fit to the expected results. The storage modulus G' can be correlated to the amount of crosslinking. In this hydrogel every 6th dextran ring is crosslinked which can lead to such a high storage modulus.

4.2.4. Degradation Assays

The degradation of the hydrogel as a response of a pH change or an enzymatic cleavage of the polymer backbone is necessary for the colon specific release of bacteria. The results for degradation assays through pH change can be observed in the figures below.

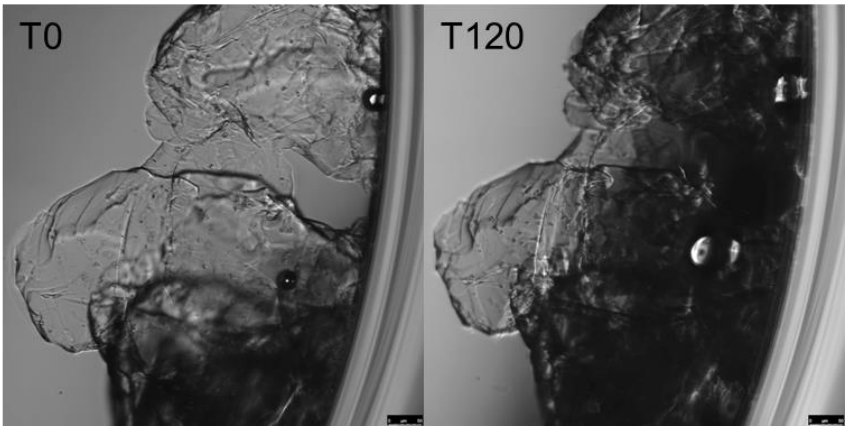

Figure 12: pH 4 dependent degradation oxD25 – hydrogel incubated in 50mM phosphate buffer pH4 to investigate solution behaviour (scale 50 µm)

It can be seen that the exposure to a solution of pH 4 does not have an influence on the hydrogel. The hydrazone bonds remained and the gel was intact after 120 minutes of treatment. In these experiments, the hydrogels were incubated at 37 °C to stimulate the human body temperature.

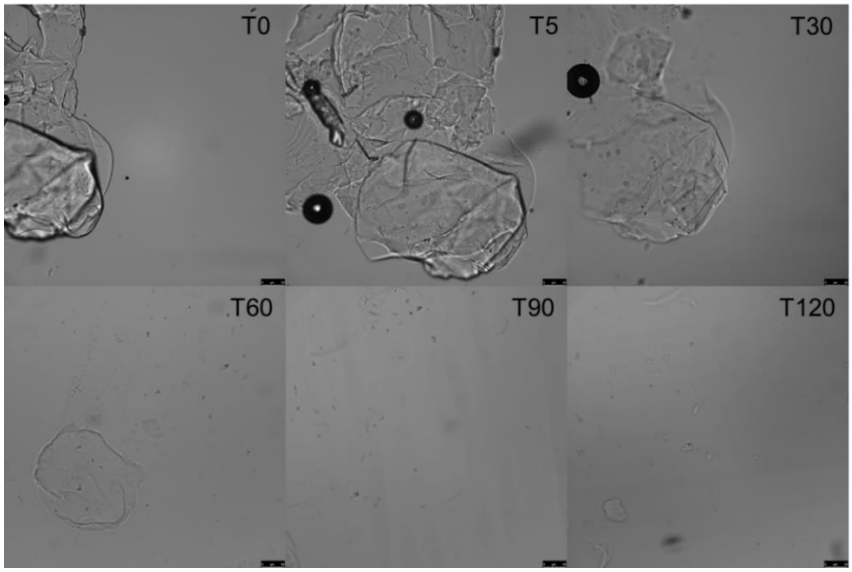

Figure 13: pH dependent 2 degradation oxD25 hydrogel incubated in 50mM phosphate buffer pH2 to investigate solution behaviour (scale 50 µm)

As shown in **figure 13** the hydrogel was fully degraded 90 minutes in phosphate buffer pH 2. This indicates that the hydrazone cleavage can be achieved at acidic pH. However, for the current application, cleavage at higher pH is more desirable. A plausible explanation is that the hydrazone bonds are very stable in this case and can be optimized further.

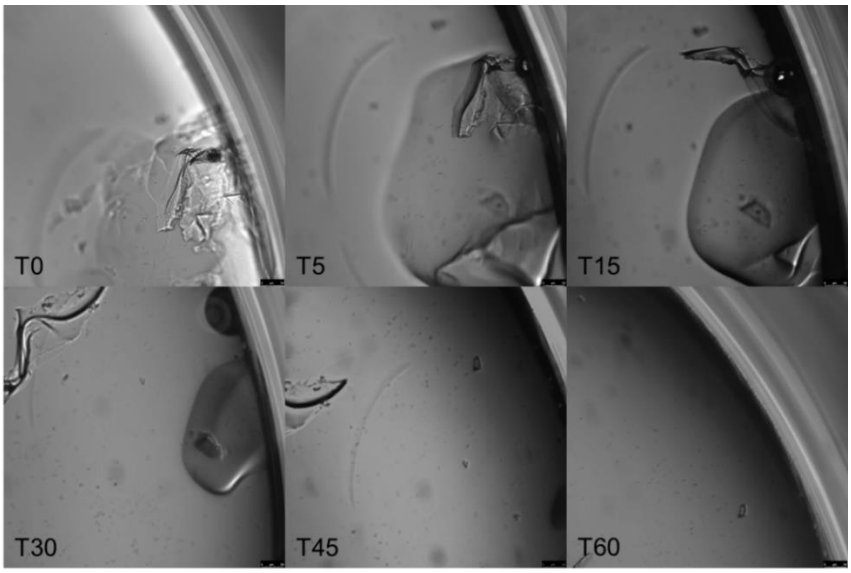

Figure 14 dextranase dependent degradation oxD25 – hydrogel incubated in 1.5 mg/ml dextranase solution at 37 °C over 60 minutes. (scale 50 µm)

The enzymatic degradation can be seen in **figure 14**. After 60 minutes incubation at 37 °C with dextranase, the hydrogel has disintegrated. As a control the hydrogel was incubated at 37 °C in water parallel to the samples (see Appendix).

The degradation experiments show, that degradation of the hydrogel is possible under acidic conditions or in the presence of dextranase. The pH value, which is necessary for the pH dependent degradation, will not be reached in the colon. To achieve a pH degradation at a colon specific pH value a further modification of the crosslinker or the polymer backbone have to be required. The dextranase dependent degradation is suitable for colon specific delivery.

4.2.5. Viability of Bacteria

The hydrogel has to fulfil a central requirement, which is the ability to encapsulate bacteria and build a stable 3D matrix while maintaining their viability. The model we are using in our lab is the *Escherichia coli* strain K12. The anaerobic bacteria used as model from our collaboration partner in Ulm is *Akkermansia muciniphila* (YL44).

Next, the hydrogel was applied to encapsulate bacteria and to investigate the viability of the bacteria. The colonies are counted, for the samples incubated 0, 1, 7, 14 days and the CFU per ml sample is calculated. As we can see in the graph below we have a stable encapsulation, till day 7. The results after 14 days show a degree of bacteria count. Right after encapsulation 44% of the bacteria are still alive, compared to the

control (see Appendix). This seems like the process of encapsulation with this hydrogel results in a high bacteria death. At day 7 there are 103% bacteria compared to day 0. After 14 days only 49% of the bacteria are still alive compared to the day 0, right after the encapsulation. This is a strong reduction compared to the surviving at day 7.

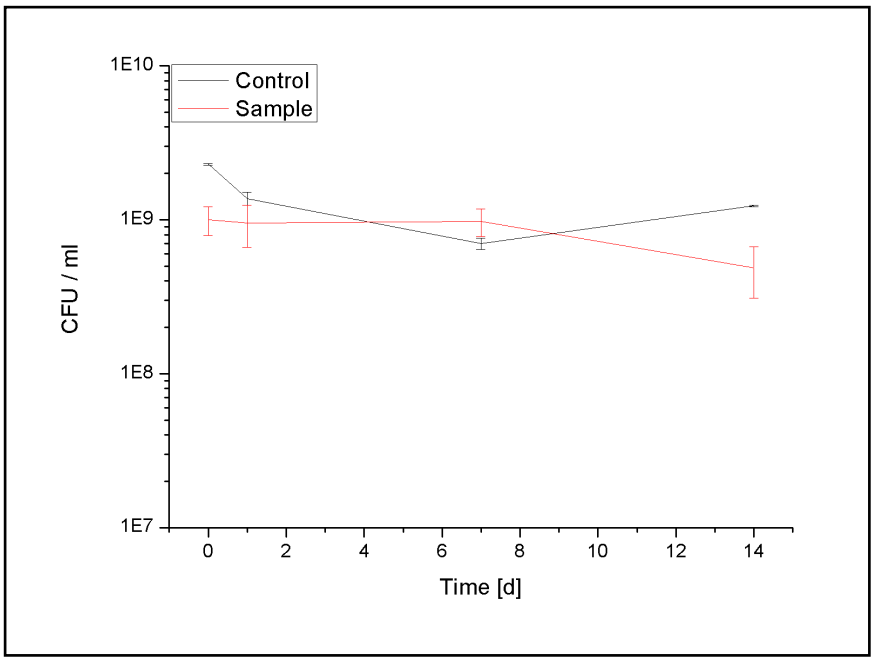

Figure 15: Colony formation of oxD25 – CFU /ml of E.coli samples encapsulated and stored for 0, 1, 7 and 14 days

Investigation of the hydrogels with anaerobic strains were conducted by collaboration partners in Ulm. The living anaerobic bacteria reduced to less than 10 % after 3 days (see Appendix)

Summarized it can be postulated, that the oxD25 hydrogel can form a stable 3D-culturematrix over a timeframe of 7 days for *E. coli*. After this time a degree of cell surviving can be observed. This may be caused by a lag of nutrition or too much death cells, which lead to intoxication. The results from Ulm show, that the hydrogel is not suitable for the project and further optimization of the design is required.

4.3. Summary and Outlook

In literature dextran hydrogels are described for many uses, like encapsulation of human cells and drug delivery. Encapsulation of bacteria is compared to the other usages a small field. This could be explained by the observed reduction of living bacteria through the process of encapsulation. In both experiments less than the half

of the bacteria survive the process of encapsulation. The target for the project is to design a hydrogel which encapsulates anaerobic bacteria over several weeks at a constant level. But after three days our anaerobic strains show a very low survival. A similar result was indicated after 14 days encapsulation of *E. coli*. The oxD25 could be a beneficial component for the hydrogels, but do not fit our requirements for the main component of a bacteria encapsulating hydrogel. The requirement of colon specific delivery and the observed ability of degradation through dextranase cleavage make oxD25 an interesting compound. Therefore oxD25 will be used as further crosslinker for protein based hydrogels.

5. cHSA-PEG-Hyd Dextran Hybrid Hydrogel

5.1. Introduction

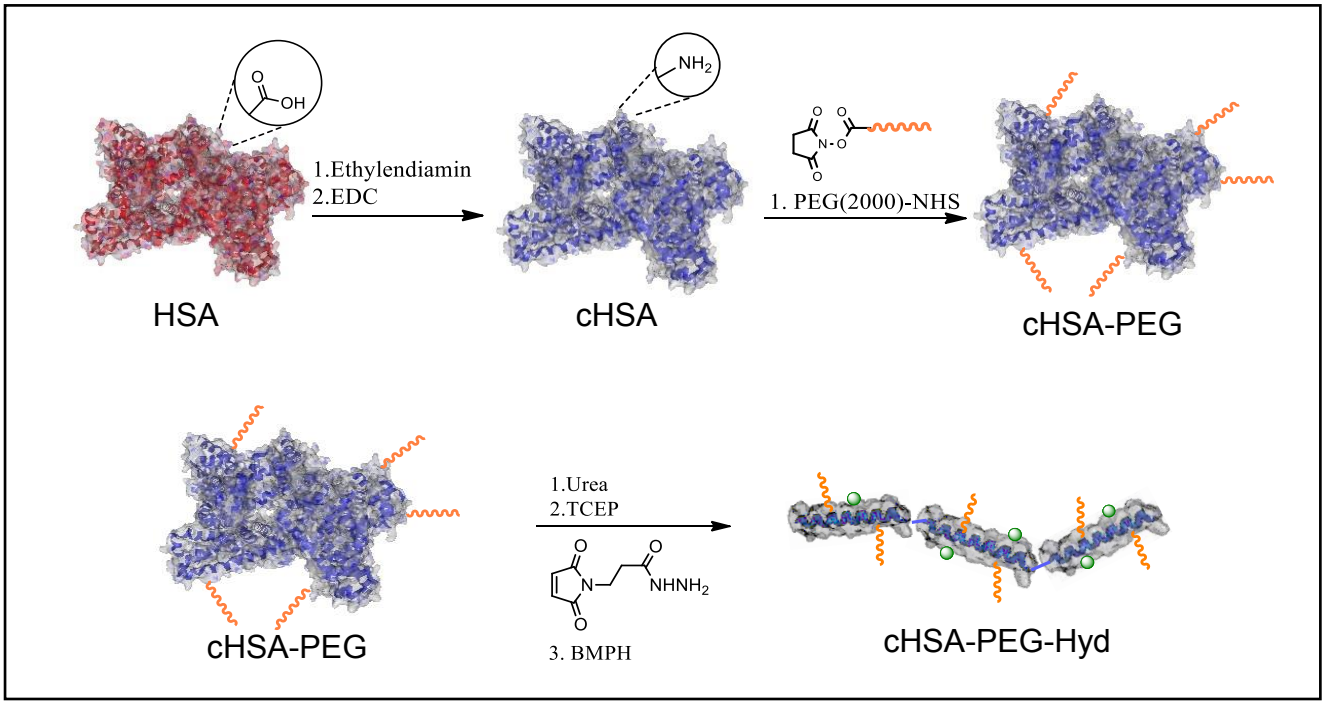

Figure 16: Synthesis cHSA-PEG-Hyd

Previous studies from J. F. Ng show, that the cationic charge leads to bacteria adhesion on materials coated with this material [38]. This ability could be beneficial for the bacteria culturing in hydrogels. Therefore a hybrid system between a protein derivative and a functionalized dextran based on a cationized albumin derivate is proposed for the encapsulation. To achieve a pH and enzyme depending degradation

a suitable crosslinker have to be implemented. The protein backbone can be combined with the hydrazone crosslinked dextran system which was developed in **chapter 4**. To incorporate the required components, the HSA have to be modified through several chemical steps. A hydrazide substituted maleimide, N-β-maleimidopropionic acid hydrazide (BMPH), was used to introduce the aldehyde responsive substitution. With a denaturation reaction, which contains the reduction of cysteine bridges followed by the reaction with maleimides, an explicit number of crosslinking points could be introduced to the protein. Further the solubility of the protein had to be increased, especially in its denaturated form. Therefore PEG(2000) chains were introduced by condensation reaction of MeO-PEG(2000)-NHS ester with the amine groups on the surface of the protein. After gelation the hydrogel were characterized before further studies were done. The bacteria viability was tested through colony formation assay to evaluate colony formation units per ml. First *E. coli* was encapsulated as an aerobic model. Later the anaerobic strains like *Akkermansia* are tested by collaboration partners.

5.2. Results and Discussion

5.2.1. Preparation of Hydrogels in Water and Media

Therefore cHSA-PEG-Hyd solution was mixed with oxD25 solution to achieve a ratio from aldehydes to hydrazides from 2:1. The hydrogel appeared to gel in seconds after mixing the two solutions. Then over several minutes it seems to harden. The hydrogel was colourless and homogenous and appears to be sticky, as shown in **figure 17**.

Figure 17: cHSA-PEG-Hyd/oxD25 Gel

As shown in the figure below gelation occurred in all of the media used. Because of the chemical bond between the hydrazide and the oxidized dextran the media was not expected to show influence on the gelation process. The aldehyde moiety could only react with the amine group of the amino acids which are presented in the media used, resulting in the formation of an imine bond. Such bond formations are known to be formed in alkaline pH. Side reaction can be eliminated through strict control of the pH with selective formation of the desired hydrazine bonds

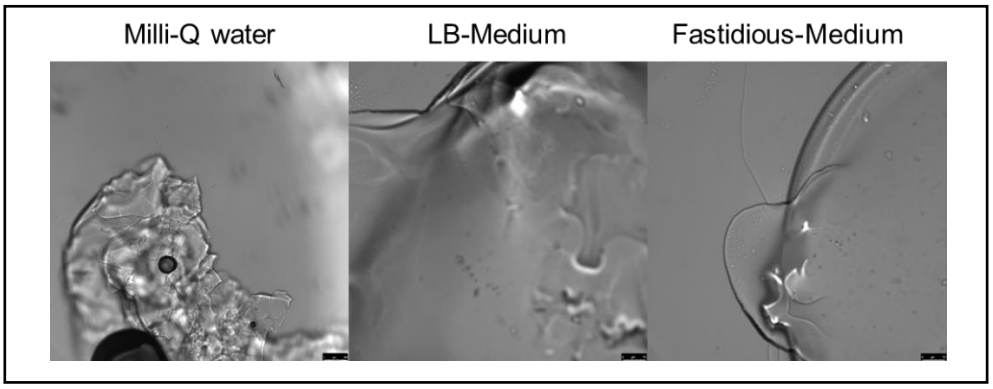

Figure 18: Gelation in different media - cHSA hydrogels prepared with LB and Fastidious medium as solvent. Milli-Q water was used as control (scale 50 µm)

5.2.2. Rheological Measurements

Time sweeps experiments and amplitude measurements were executed in triplicates. The results are summarized in the figures below

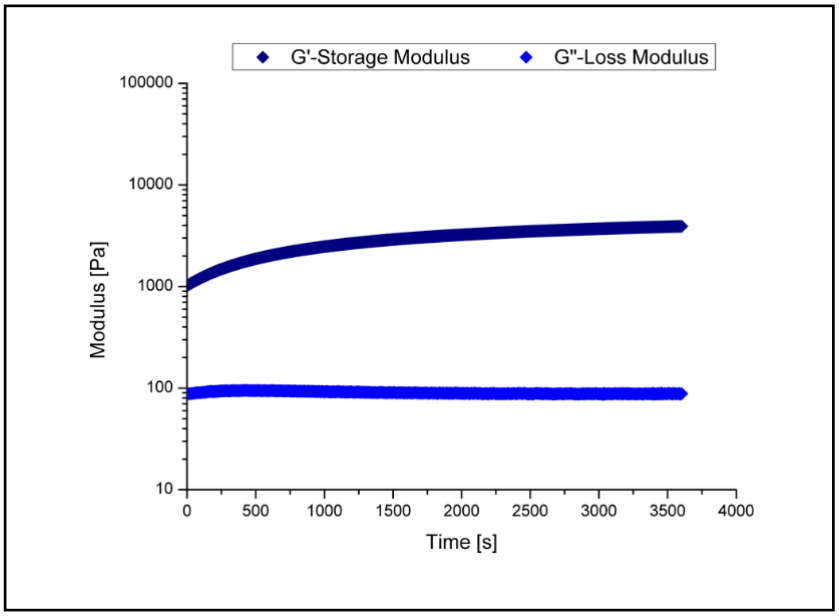

Figure 19: Time sweep cHSA-PEG-Hyd - time sweep performed with a strain of 1 % at 1 Hz to monitor the gelation time and moduli

In **figure 19** we can see the storage and loss modulus of the gel over time. After 20 minutes no further significant change of the moduli could be observed. It can be concluded that the gelation process is completed. The cHSA-PEG-Hyd/oxD25 hydrogel shows a storage modulus G' of 4140 Pa and a loss modulus G" of 90 Pa.

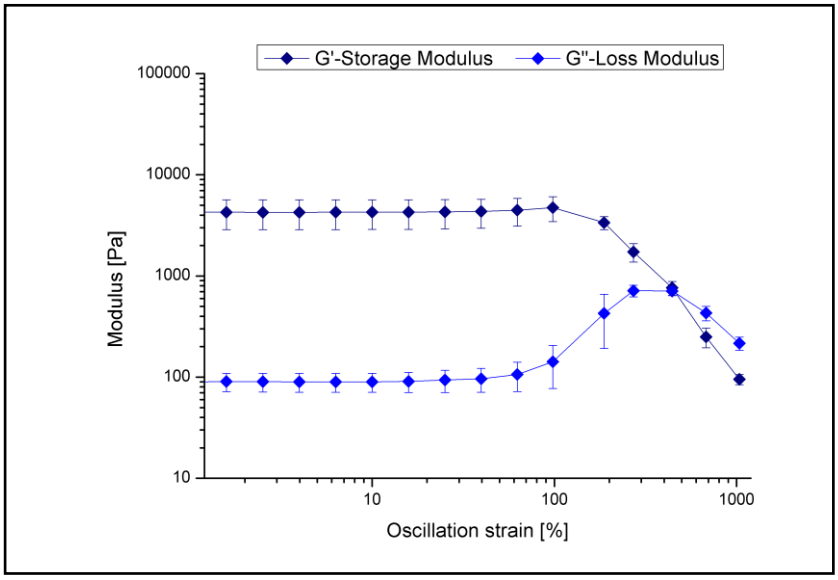

Figure 20: Amplitude sweep cHSA-PEG-Hyd – amplitude sweep to investigate the collapse point of the hydrogel

In **figure 20** we can see the storage and loss modulus over an increasing oscillation strain. The hydrogels show a crossover of the moduli at a strain of 465 % and a modulus of 669 Pa. After the collapse of the hydrogel, the following time sweep showed reversal of the hydrogel collapse (see Appendix).

Compared with other peptide hydrogels, which show a storage modulus G' of less than 1000 Pa, our hydrogels show more solid like behaviour [39]. This increase could be caused by the polysaccharide component and the covalent bonds.

5.2.3. Degradation Assays

The hydrogels provide two possible degradation mechanisms. Both cleavage processes are aimed to release the later encapsulated bacteria in the colon. The first mechanism is the degradation through a pH change. Here the hydrazone bond is cleaved with a change to a slightly acidic pH. The second mechanism is the cleavage of the polysaccharide chain or the protein through enzymes. The enzyme targeted for this degradation is dextranase for all four hydrogels and trypsin for the systems

containing protein. Dextranase is not produced by the human body itself but by bacteria living in the human gastro intestinal tract. Trypsin is an enzyme which digests peptides in the small intestine.

The results for the degradation assays are shown below. A pH responsive cleavage of hydrazone bonds would be expected at an acidic pH resulting in degradation of both samples at pH 2 and pH 4.

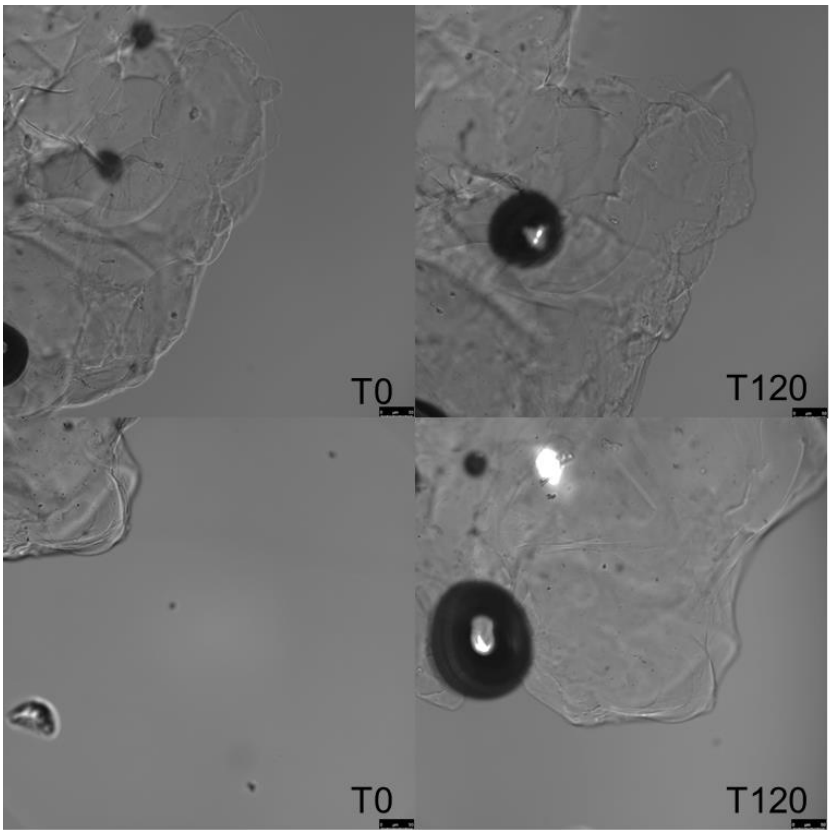

Figure 21: pH dependent degradation of cHSA-PEG-Hyd - hydrogel incubated in 50 mM phosphate buffer pH4 (top) and pH 2 (bottom) at 37 °C for 120 min. (scale 50 µm)

Nevertheless, as seen in **figure 21,** no observable disintegration over several minutes appeared. No changes are seen on the hydrogels and a plausible explanation is that the expected hydrazone bond was not formed. Instead, a cyclic structure as shown in **figure 22**, which has been documented and is not pH-cleavable, could have been formed [40,41].

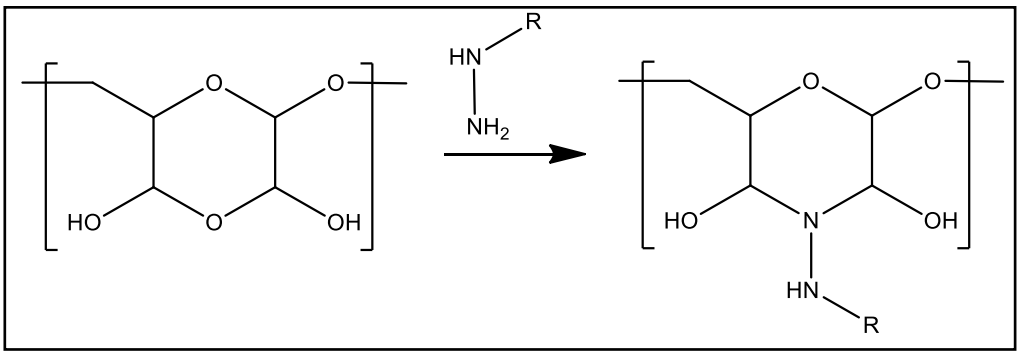

Figure 22: Cyclic reaction of oxidized dextran with hydrazides.

This leaves the question why the material in chapter 4 showed disintegration. This behaviour could be explained by the fact, that the residue if the hydrazide changed from SucHyd to cHSA.

For the enzymatic degradation we obtained a different result compared to the pH dependent experiments. Here a change in the form of the hydrogels and subsequent degradation is observed. As shown in **figure 23** the hydrogel is entirely degraded after five minutes.

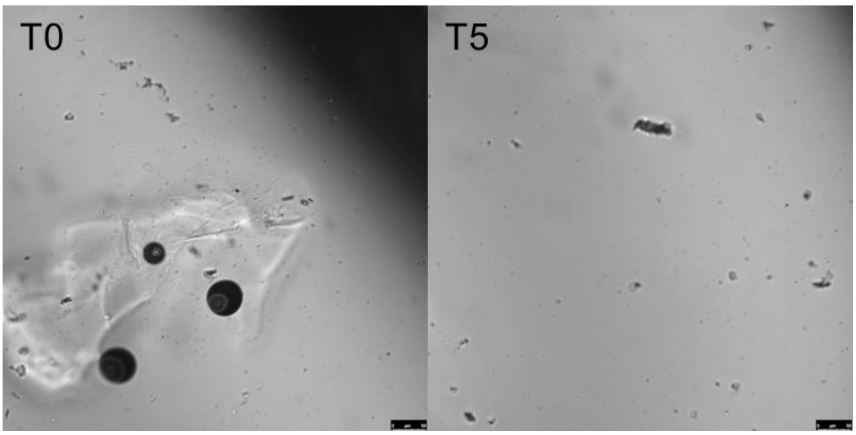

Figure 23: Trypsin dependent degradation cHSA-PEG-Hyd – hydrogel incubated at 37°C in 1mg/ml trypsin solution for 5 minutes. (scale 50 µm)

The degradation of the cHSA-PEG-Hyd is shown in **figure 24**. After 60 minutes incubation in dextranase solution full disintegration is observable.

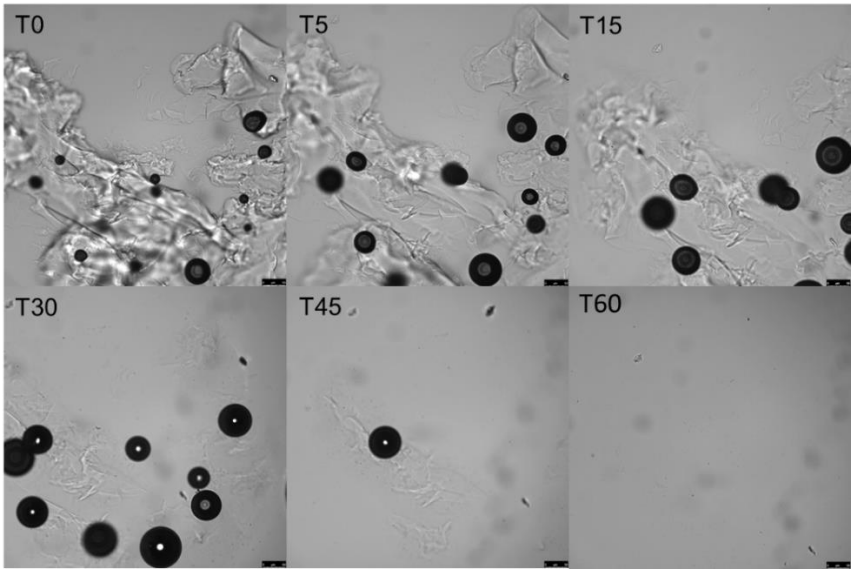

Figure 24: Dextranase dependent degradation cHSA-PEG-Hyd – hydrogel incubated in 1.5 mg/ml dextranase solution at 37 °C for 60 minutes (scale 50 µm)

Overall the degradation with enzymes is possible. This proves, that the modification of dextran does not block the dextranase activity on the polymer. Further the functionalisation with PEG and BMPH on the amino acid residues do not affect the Trypsin cleavage of the backbone. The time needed for the decomposition has to fit the timeframe the hydrogel is exposed to the enzymes. The minimal timeframe of 60 minutes the hydrogel have to stay inside the colon is given [42].

5.2.4. Viability of Bacteria

The viability of the bacteria was tested with a colony formation assay. As seen in the figure below, the hydrogel showed an antibacterial effect on *E. coli*. Even the hydrogels which were destroyed and analysed directly after the gelation showed no colony formation on the agar plates. A plausible reason could be explained by the Zeta potential due to the fact that cHSA-PEG-Hyd has a very positive Zeta potential of +20.6mV. This might have caused an adhesion of the negative surface of *E.coli* to the protein resulting in a disruption of the membrane which leads to cell death. To support the hypothesis and determine which of the components lead to bacteria death, a solution of 3 mg/ml *E.coli* was prepared. Different components were dissolved in similar conditions for the preparation of the hydrogels and the mixtures were plated on agar. In **figure 25** we can see cHSA-PEG-Hyd/oxD25 hydrogel a.), pure hydrazide crosslinker succinyldihydrazid b.), cHSA-PEG-Hyd c.), oxD25 d.), pure culture as

control e.). It can be observed, that the hydrogel a.) and the modified protein c.) show no colony formation in contrast to the other samples.

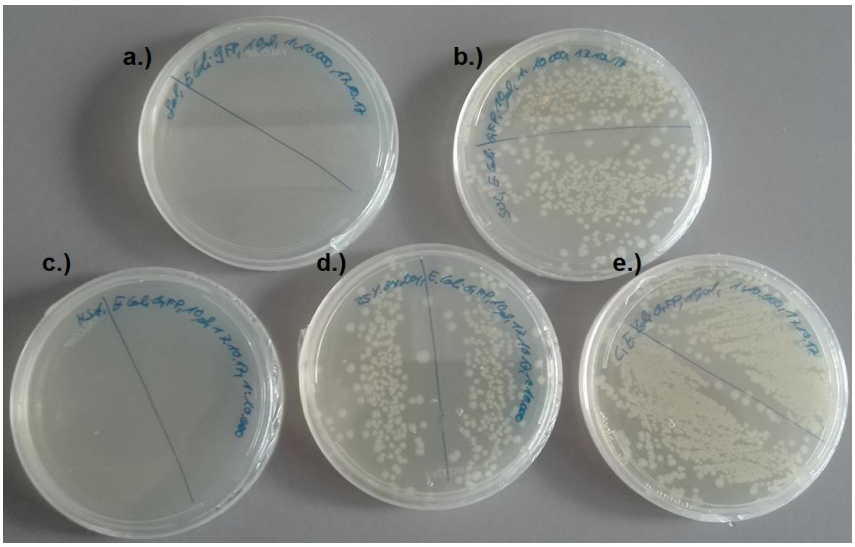

Figure 25: Viability test with different hydrogel components – solutions as used in hydrogel preparation incubated with E.coli and plated on agar for colony formation

Based on these results it can be postulated, that the protein compound may be the main problem. The hydrazide does not cause bacteria death like seen in b.). Taken together, the results support the postulation that the cationization of the protein results in the eradication of bacteria. In this context, the surface charges on the human serum albumin have to be changed.

5.3. Summary and Outlook

Hybrid hydrogels of proteins compared with polysaccharides are not mentioned in literature. This work opens up a new field of hydrogel design. Based on the previous work with oxD25 hydrogels a new material was developed using the cationized albumin. The gel components are easy to handle and the hydrogel formation allows several different gelation mechanics. The hydrogel showed fast gelation behaviour in Milli-Q water and was not affected by exposition of different factors in media. Furthermore the hydrogels show the ability to be cleaved by different enzymes which could lead to a colon specific delivery. To achieve a pH dependent degradation the crosslinker has to be improved. The protein as backbone polymer allows a wide range of modification reactions. The obtained cHSA-PEG-Hyd shows a negative effect of the bacteria viability. Through different modifications the surface and charge of the polymer

can be changed and the viability of the bacteria could be increased. This will be part of the next chapters.

The anti-microbiological property does not fit the targeted requirements but offers a new field of investigations. Over the last years multi-resistant bacteria are getting an increasingly challenging problem in our health system. A big focus of the biomaterial science in these days lies in the development of alternatives to the currently licensed antibiotics. Strains like *Staphylococcus aureus* and *Pseudomonas aureginosa* are a big problem in hospitals. They often infect wounds and can only be handled with complicate treatments. At the moment multivalent antibiotics are used to tread infections with such strains, causing damages on the natural microbiome. Our hydrogel may be an opportunity to deal with this questionability as a local wound dressing, which does not affect the whole organism.

6. HSA-PEG-Hyd Dextran Hybrid hydrogel

6.1. Introduction

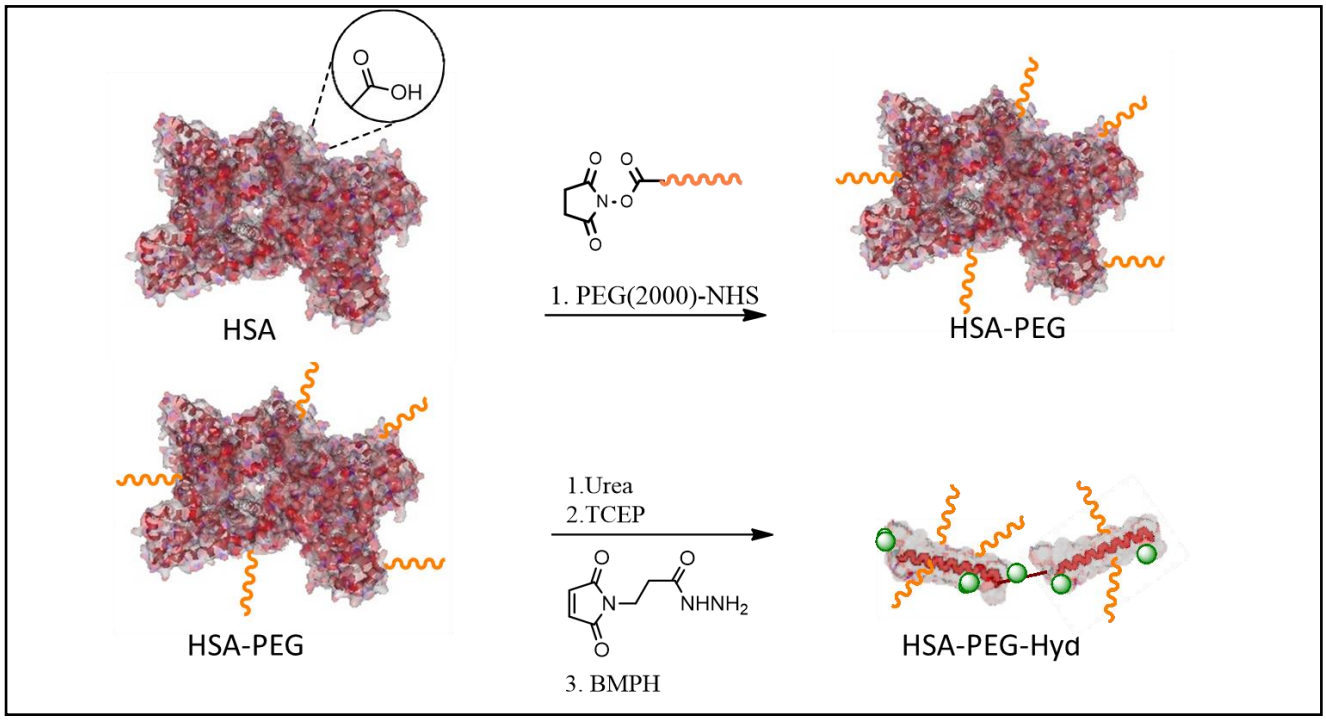

Figure 26: Synthesis of HSA-PEG-Hyd

The experiments done in the previous chapter showed that the surface charge has to be modulated to fit the requirements for application. Therefore, the design was

changed to use native HSA, which is negatively charged under physiological conditions, as the backbone. For systematic evaluation the other component oxD25 and hydrazide as a crosslinker were kept constant in the design. Here the degradation with trypsin and dextranase in the colon should be retained. Considering the usage of the same cross linker a pH dependent degradation is possible and has to be checked experimentally. The hydrazide group is introduced over the denaturation process again. Therefore BMPH is used. So the amount of hydrazide groups is still limited by the cysteine. To increase the solubility we introduce PEG(2000) chains by using a MeO-PEG(2000)-NHS ester. In the native form less amino groups are accessible than in the cationized form. The resultant hydrogel material will be evaluated in a similar fashion as described for the cHSA-based hydrogel. The hydrogel has to be characterised over rheological measurements. Additionally the aptitude for specific delivery in form of degradability has to be tested. Due to the components used for the hydrogel enzymatic and pH dependent degradation have to be considered. The essential test will be the colony formation assay to see if the obtained hydrogels show better bacteria viability.

6.2. Results and Discussion

6.2.1. Preparation of Hydrogels in Water and Bacteria Media

During the preparation, difficulty in handling arose in that the protein solution has to be handled really quickly because its gels by itself without crosslinking agent. It seems, that the protein does not dissolve completely and aggregates through hydrophobic interactions. When handled quickly and mixed directly a colourless hybrid hydrogel can be achieved (**see figure 27**). The material showed a very sticky behaviour.

Figure 27: HSA-PEG-Hyd/oxD25 hydrogel

In **figure 28** the gelation in different Media is monitored.

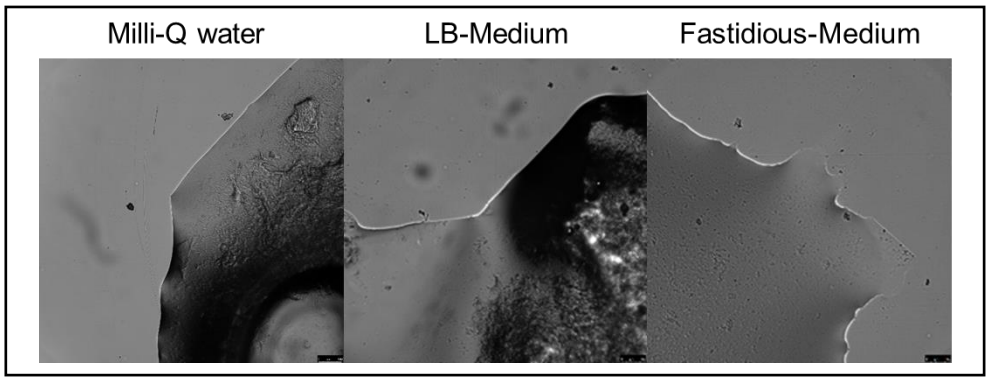

Figure 28: Gelation in different Media – HSA-PEG-Hyd hydrogels prepared with LB and Fastidious medium as solvent. Milli-Q water was used as control (scale 50 µm)

In both, LB and Fastidious medium, gelation can be observed. As expected the components of the media don't seem to influence the hydrazone bonds. The hydrophobic interactions do not seem to be affected by the media. In both pictures aggregates can be seen. The hydrogel in general is comparable to the previous synthesised systems.

6.2.2. Degradation Assays

The results for the degradation assays are shown in **figure 30**. There is no effect of the pH to the hydrazone bound and this might be caused by the same effect which is postulated in the chapter before. The substructure formed through oxidation reacts with the hydrazide to form the morpholino-like structure which does not react to a pH change (**see figure 29**)

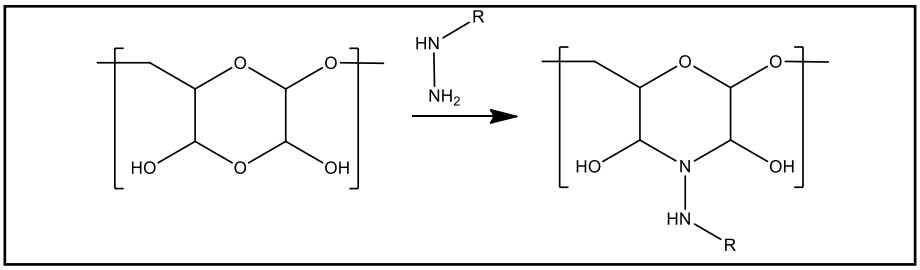

Figure 29: Reaction of oxidized dextran with hydrazide components

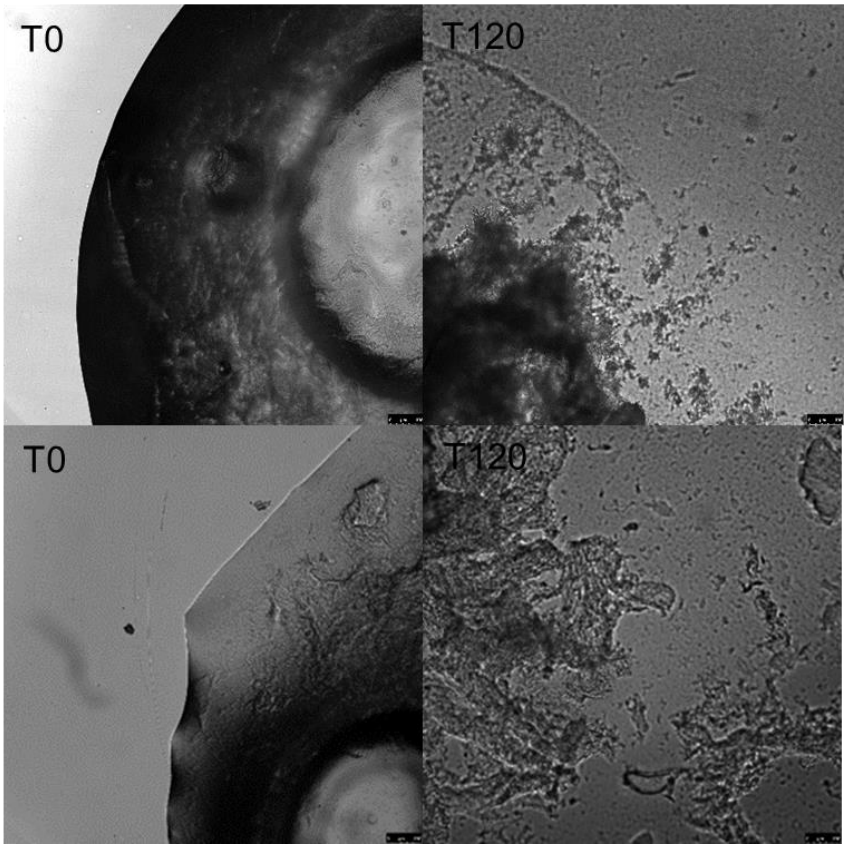

Figure 30: pH dependent degradation HSA-PEG-Hyd - hydrogel incubated in 50mM phosphate buffer pH4 (top) and pH 2 (bottom) at 37 °C for 120 min. (scale 50 µm)

For the enzyme degradation a different result compared to pH responsive cleavage can be observed like seen in **figure 31**. As expected trypsin degrades the whole hydrogel within 5 minutes. The timeframe the degradation is done is the same compared to the cHSA based hydrogel, which degrades in 5 minutes too. This approves, that the PEGylation does not impede the enzymatic digestion.

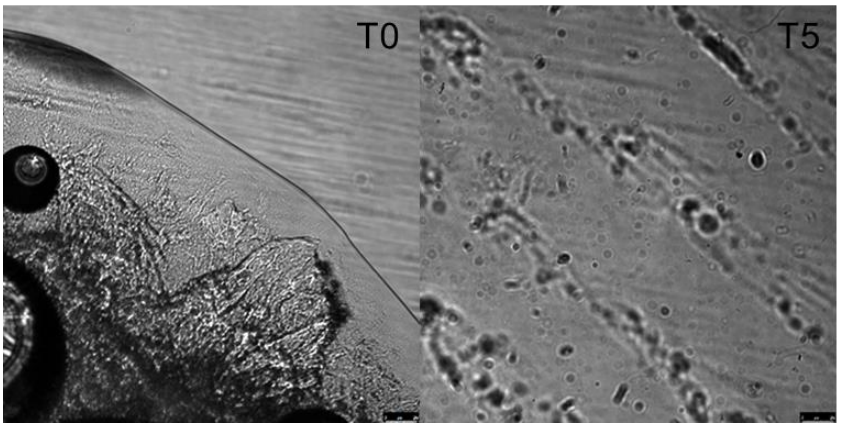

Figure 31: Trypsin dependent degradation HSA-PEG-Hyd – hydrogel incubated at 37 °C in 1 mg/ml trypsin solution for 5 min (scale 50 µm)

The second degradation method is the colon specific degradation with dextranase. The results for this experiment are shown in **figure 32**.

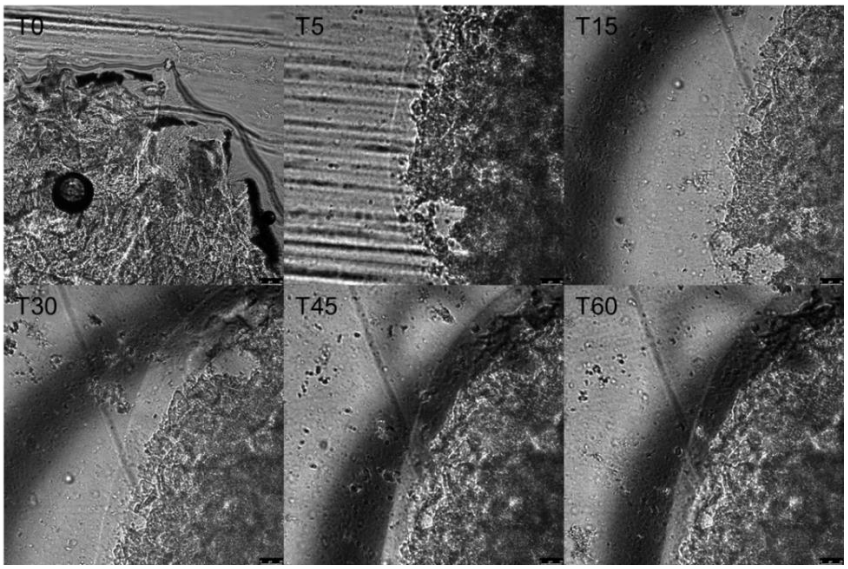

Figure 32: Dextranase dependent degradation HSA-PEG-Hyd – hydrogel incubated at 37 °C in 1.5 mg/ml dextranase solution for 5 min (scale 50 µm)

As depicted in the images no degradation appears. After 60 minutes the previously designed cHSA based hydrogel was completely dissolved. A possible explanation would be the gelation through hydrophobic interaction in combination with the hydrazone crosslinking. Even if the dextranase digests the polysaccharide, the hydrophobic interactions seem to hold the dimensional matrix together. This behaviour will cause problems for the application later on. The colon specific degradation through dextranase to release the bacteria is not possible

6.2.3. Viability of Bacteria

The viability of the bacteria is monitored through a colony formation assay. The CFU/ml is calculated after 0, 1, 7 and 14 days (see **figure 33**)

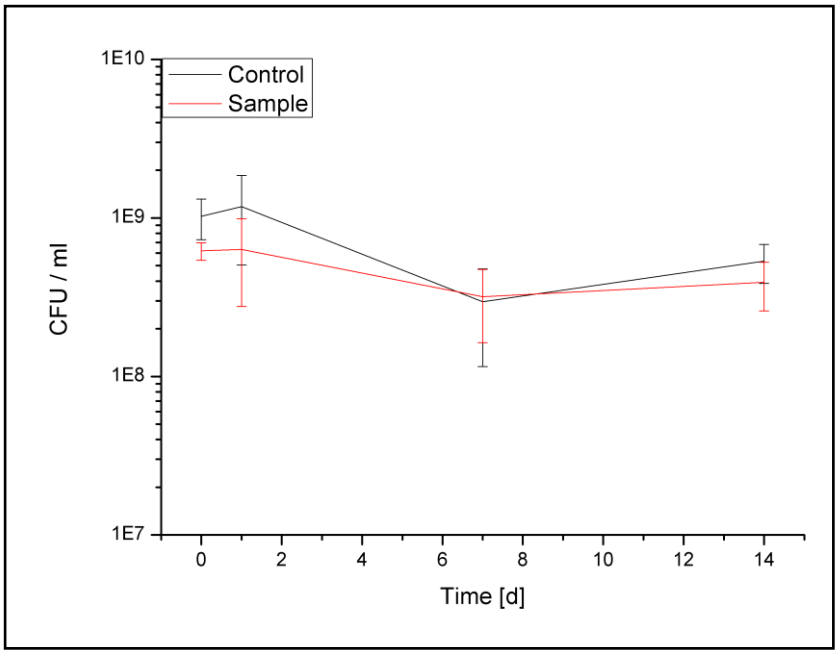

Figure 33: Colony formation assay HSA-PEG-Hyd - CFU /ml of E.coli samples encapsulated and stored for 0, 1, 7 and 14 days

The results from the colony formation assay shows that the bacteria survive inside the hydrogel. The *E.coli* seems to be compatible with the environment given in the gel. By switching the backbone from the positively charged cHSA to the negatively charged HSA, the bacteria viability is significantly improved and could be promising for the intended application. The Zeta potential of the components seems to have a big impact on the viability. After seven days a drop of CFU/ml can be seen. This drop is seen in the control as well, so it can be postulate, that other factors than the gel are responsible for the drop. Aspects like temperature in the lab, time until plating or sunlight irradiation could cause this drop. As control a fresh solution of 3 mg bacteria pellet in 1 ml medium is used The CFU at day zero compared to the control gives a survival rate of 60% right after encapsulation. After 14 days of incubation 63% of the bacteria are still alive, compared to day zero. If we compare the control with the sample after 14 days the surviving rate is increased to 74%. Summarized the gel show a stable culturing of *E.coli*. The studies with the anaerobic cultures are incomplete but the first results from the collaboration partner are promising with the survival of the *Akkermansia* strain and this material could be further evaluated and optimized.

6.3. Summary and Outlook

In general this hydrogel could be an option for 3D bacteria culturing. The surviving rate after 14 days is sufficient and the feedback from the colaboration partners is encouraging. For later application a higher solubility has to be reached. An instable protein solution can not be manufactured as a medicine later if the solution gelates spontaneously.

If we look at the amount of PEG chains indroduced to the protein we only have a little percentage compared to the cHSA compound. This results in the hydrophobic interactions and the incomplete dissolving. In native HSA not more than four PEG chains can be introduced over NHS ester. A PEGylation after denaturation could modify the hydrazide groups and block the crosslinking process. Therefore other possibilities to indroduce PEG chains must be considered. A possibility could be the modification of HSA over the asparaginic acid and glutaminic acid endgroups. A coupling with EDC or another synthesis route could introduce more PEG chains and result in a better solution stability. It has to be faced that a higher PEGylation degree changes the Zeta potential, for example cHSA (33.4 mV) and cHSA-PEG (17.8 mV), and the environment the bacteria are encapsulated in. The results here can be taken as a basis for further investigations.

7. cHydHSA-PEG Dextran hybrid hydrogel

7.1. Introduction

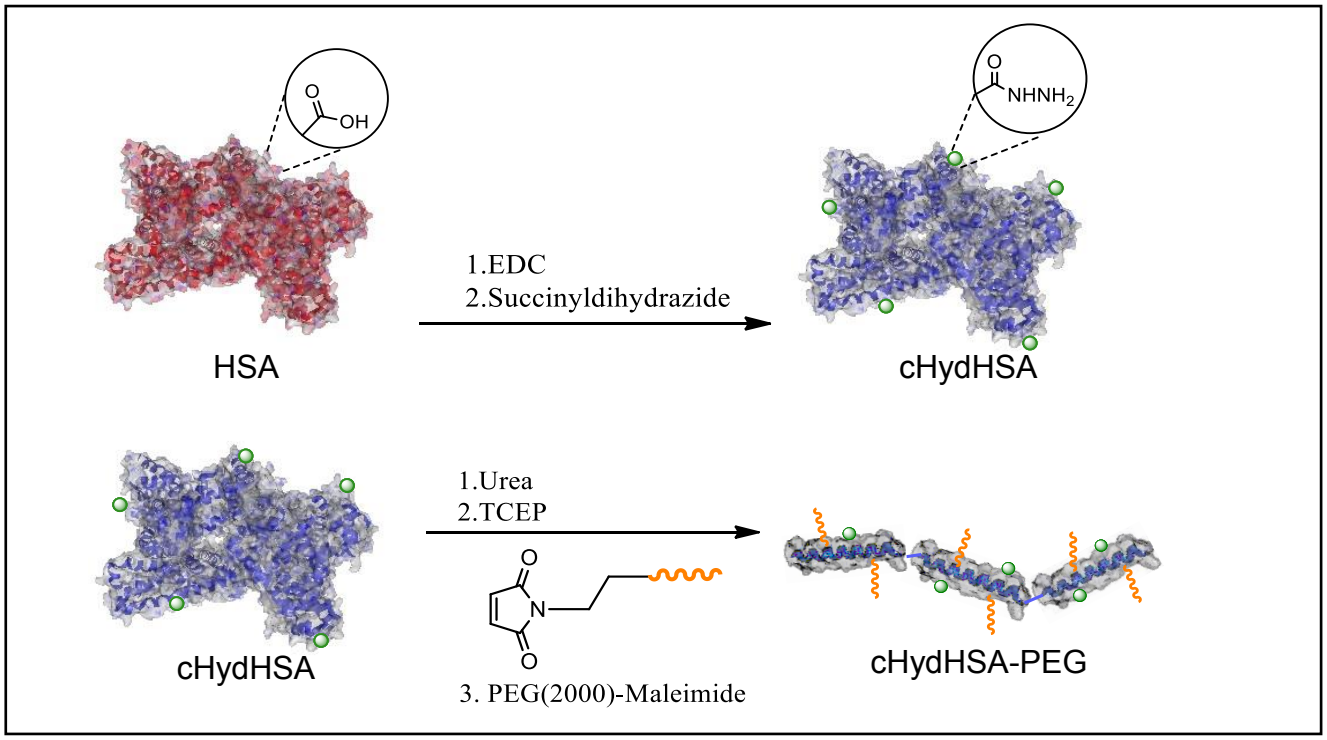

Figure 34: Synthesis of cHydHSA-PEG

A different modification pathway for HSA was adopted to prepare the following hybrid hydrogel. The synthetic schemes are already known through the previous work but the order of modification is different. Based on the previous work the degradation through pH and enzymatic cleavage is targeted. For consistency, similar crosslinking groups and modifications are applied. This hydrazide groups were introduced again to be able to use the oxidized dextran as the crosslinker of choice. In contrast to the cHSA hydrogel the hydrazide was introduced over EDC activated carboxylic acids for this synthesis. Therefore the chemical crosslinker succinicdihydrazide is used. This reaction method allows to use the denaturation reaction to introduce the PEG chains. For this reason a PEG-maleimide is used. Where fourteen MeO-PEG chains were introduced over NHS ester, it is now possible to introduce up to thirtyseven PEG chains. More PEG chains should lead to a negatively charge and better solubility. With this modification way it is planned to receive a compound with the desired degradation behaviour in the colon and good encapsulation properties.

7.2. Results and Discussion

7.2.1. Preparation of Hydrogels in Water and Media

Figure 35: cHydHSA-PEG/oxD25 hydrogel

After mixing the two components the hydrogel gelation appears while inverting the mixture. After several minutes it seems like stiffness does not change anymore. The hydrogel appears as an orange hydrogel which is very sticky and soft. An optic observable self-healing after tearing the gel apart could not be observed. The concentration of the protein compound has to be higher than in the other gels. When preparation was performed with a concentration of 1 mM and mixed with the equivalent amount of oxD25 the gelation did not appear like expected. The resulting fluid seems to be a rather viscous liquid than a hydrogel. This may be due to the higher degree of PEG functionalization. In **figure 36** the gelation in different media is monitored.

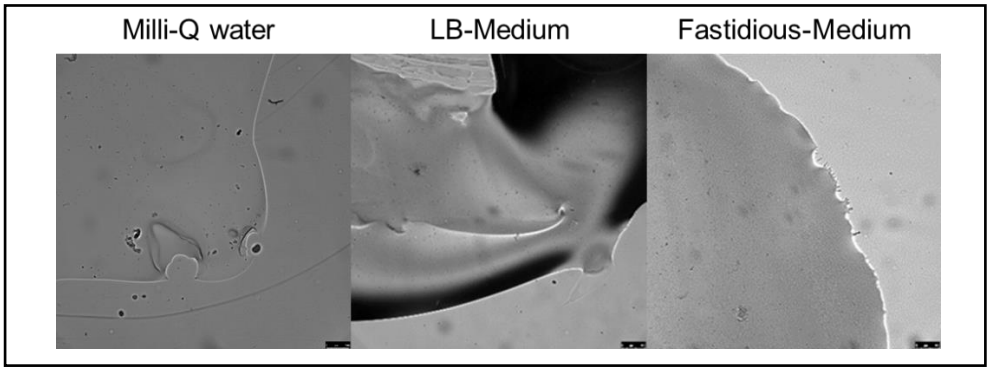

Figure 36: Gelation in different media cHydHSA hydrogels prepared with LB and Fastidious medium as solvent. Milli-Q water was used as control (scale 50 µm)

It can be seen that gelation in both media appears like expected. The environmental factor presented through the media did not affect the gelation.

Compared to the previous chapters the hydrogel showed optical similarities to the cHSA hydrogel. Insoluble residues, which aggregate through hydrophobic interference, like seen in the HSA hydrogels, do not exist. This result proves, that a certain level of PEG chains have to be introduced to receive a soluble polymer backbone.

7.2.2. Rheological Measurements

The rheological experiments were performed in triplicates. The obtained data is summarized in the figures below.

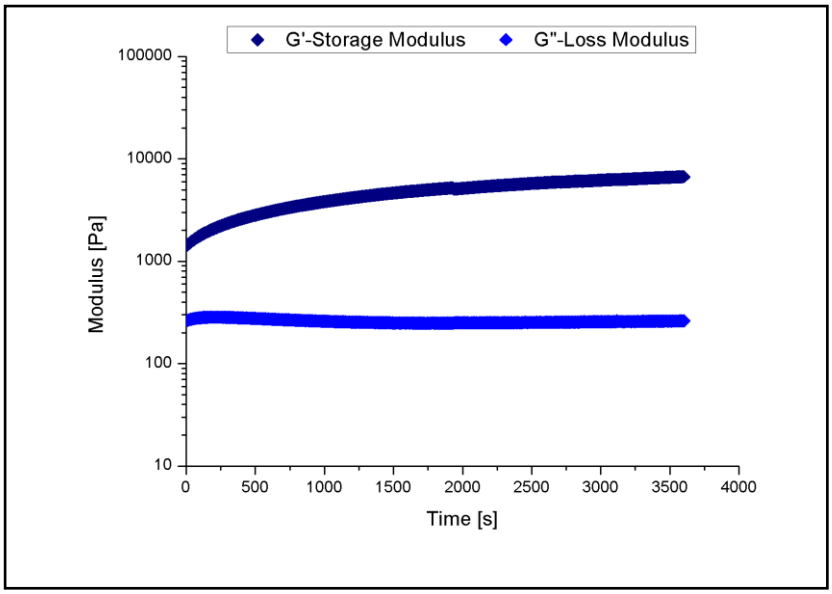

Figure 37: Time sweep cHydHSA-PEG - time sweep performed with a strain of 1 % at 1 Hz to monitor the gelation time and moduli

In **figure 37** we can observe the storage and loss modulus of the gel over a timeframe of 1 h. After 25 minutes the moduli reached a plateau. It can be concluded that the gelation process has reached its end. The cHydHSA-PEG/oxD25 hydrogel shows a storage modulus G' of 7403 Pa and a loss modulus G" of 291 Pa. Compared to the cHSA-PEG-Hyd/oxD25 hydrogel this gel appears to be more stiff. This observation may be caused by the higher concentration of protein backbone inside the hydrogel. Furthermore the crosslinking points are distributed in a different pattern on the protein caused by the changed design.

In **figure 38** we can see the storage and loss modulus over an increasing oscillation strain. The hydrogel has a crossover point at a strain of 366 % and a modulus of

864 Pa. After the crossover of the moduli a time sweep was performed to see, if a self-healing effect appears. The collapse of the hydrogel could not be reversed (see Appendix).

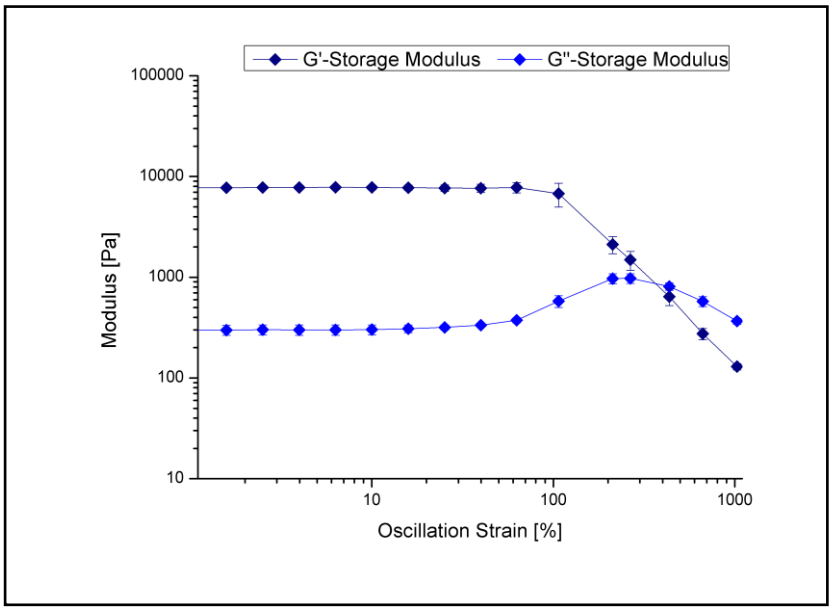

Figure 38: Amplitude sweep cHydHSA-PEG – amplitude sweep to investigate the collapse point of the hydrogel

7.2.3. Degradation Assays

The results for the degradation assays are shown below. It can be seen that the hydrogel does not dissolve like expected. Like the other hydrogels before this may be caused through cyclic side reactions like described in the previous chapters. This behaviour could not change through the changed synthesis. The hydrazide bound does not seem to be cleaved like expected. The modification with succinic dihydrazide should show similar results than the oxD25 hydrogel described in the second chapter. But like showed in **figure 39** the hydrogels reveal no degradation in weather pH 2 or pH 4. Based on these results the enzymatic degradation becomes the only targetable mechanism besides mechanical forces for this hydrogel.

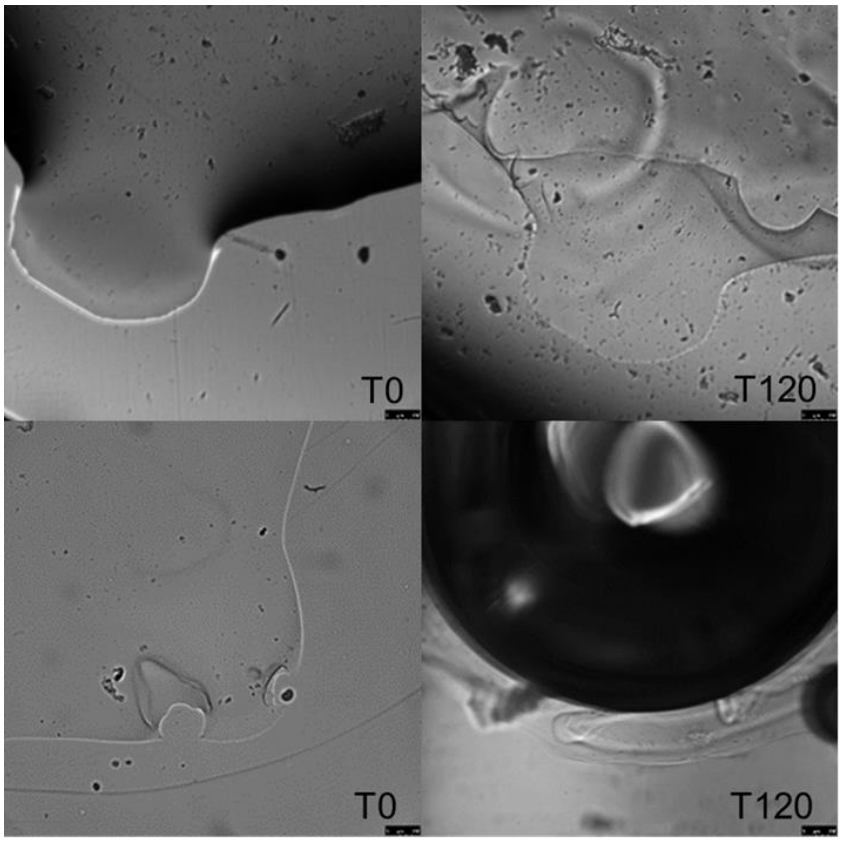

Figure 39: pH dependent degradation cHydHSA-PEG - hydrogel incubated in 50 mM phosphate buffer pH4 (top) and pH 2 (bottom) at 37 °C for 120 min. (scale 50 µm)

Like shown in **figure 40** trypsin degraded the sample within five minutes of incubation at physiological conditions. This result coincides with the observations received in the experiments before.

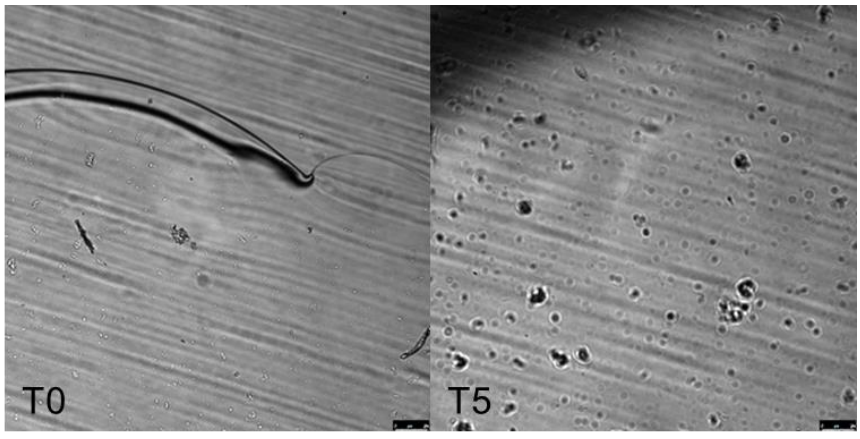

Figure 40: Trypsin dependent degradation cHydHSA-PEG – hydrogel incubated at 37 °C in 1 mg/ml trypsin solution for 5 min (scale 50 µm)

The second degradation method is the colon specific degradation by dextranase. The results for this experiment are shown in the **figure 41**. Here the results are unexpected.

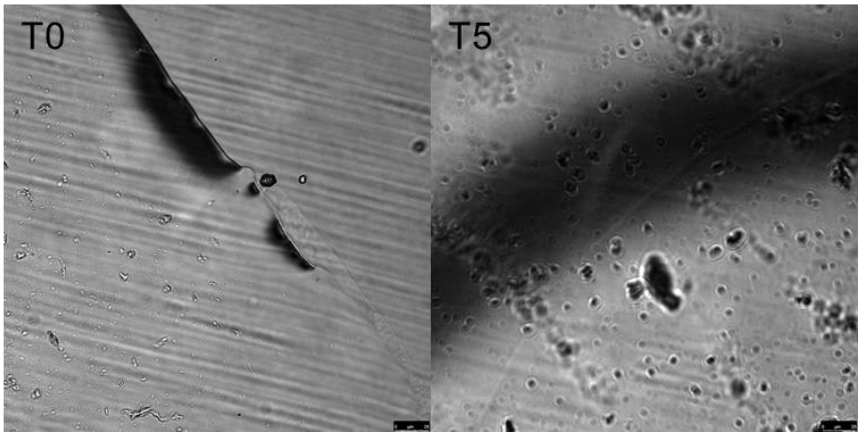

Figure 41: Dextranase dependent degradation cHydHSA-PEG – hydrogel incubated at 37 °C in 1 mg/ml trypsin solution for 5 min (scale 50 µm)

A degradation seems to appear within 5 minutes similar to the trypsin experiments. But compared to the other hydrogels the time needed for digestion of the hydrogel is shorter. This observation could be explained by different effects. The first could be, that through the process of modification not all hydrazides are available for hydrazone bonds. This would result in a faster degradation through dextran cleavage. Through the denaturation the amino acids are better accessible for the enzymatic digestion, leading to a faster degradation.

7.2.4. Bacteria Surviving

The surviving of the bacteria was tested with a colony formation unit assay, seen in the **figure 42**. The assay shows, that the bacteria exhibit a constant slightly increasing viability through the storage of 14 days. The graph shows, that the control is not stable for this assay. The sample on the other hand is stable and shows a small increase after 14 days. If we compare the CFU/ml of the sample and the control at day zero we see, that 85 % of the bacteria survive the process of encapsulation. After an incubation time over 14 days the bacteria population increases to 129 %.

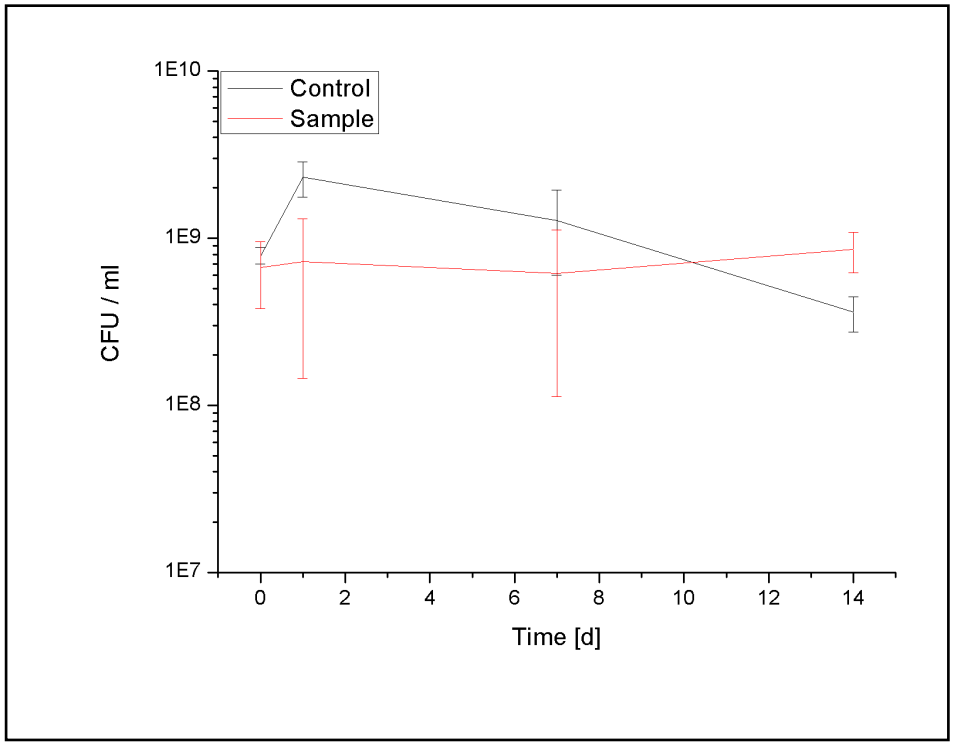

Figure 42: Colony formation assay cHydHSA-PEG - CFU /ml of E.coli samples encapsulated and stored for 0, 1, 7 and 14 days.

Summarized the gel shows stable viability through the process of cultivation of *E.coli* inside the hydrogel. The studies with the anaerobic cultures are incomplete, but the first information (data not received) from the collaboration partner show no surviving of *Akkermansia* inside the hydrogel.

7.3. Summary and Outlook

The cHydHSA-PEG/oxD25 shows interesting properties. In the process of hydrogel design several changes were done. In the previous chapters the hydrogel synthesis was changed stepwise in different ways to achieve optimal toxicity and mechanical properties. The result of this optimization procedure is presented in this chapter. The obtained hydrogel show good gelation properties independent of the solvent used. Furthermore the colon specific delivery, which is targeted in this work, can be achieved through enzymatic cleavage of the polymer backbone, although a pH depend degradation was not realized. The viability of bacteria was successfully investigated with *E.coli*. Through the gelation process 85 % of the encapsulated bacteria stay alive. Over 14 days the strain shows an increase to 129 %. These results make the hydrogel a suitable material for the encapsulation of *E.coli* for 3D cultures. Nevertheless, preliminary studies show, that the anaerobic strains targeted do not survive in the

hydrogels. To validate this problem further systematic experiments have to be done, to obtain information for redesigning the components.

8. Summary and Outlook

The systematic design of hydrogels for the encapsulation of bacteria for application towards the treatment of Alzheimer's disease is presented in this thesis. Specifically, four different hydrogels were prepared and the gelation process in two application relevant media was investigated. Our results indicate that gelation was achieved in all the media tested. The characteristics and properties of the resulting hydrogel materials were systematically evaluated using rheology measurements. Further the viability of the encapsulated bacteria was monitored.

The results of the rheology are summarized in the table above. In the case of the HSA-PEG-Hyd-/oxD25 hydrogel, rheology characterization was not possible due to self-gelation of the protein components.

Table 2: summary of rheological data

	oxD25	cHSA-PEG-Hyd	HSA-PEG-Hyd	cHydHSA-PEG
G'-StorageModulus [Pa]	12524	4140	-	7403
G"-Loss Modulus [Pa]	63	90	-	291
Crossover [%]	200	465	-	366
Crossover [Pa]	693	669	-	864

The oxD25 hydrogel, crosslinked by the bivalent crosslinker succinic dihydrazide, shows a higher storage modulus (G') than the protein gels. This could be caused by the rather short crosslinker and the amount of the crosslinking points at the polymer backbone. In oxD25 every 6th saccharide ring is modified and can bind to a crosslinker molecule. The two protein hydrogels reach a higher oscillation strain at the crossover point which might be caused by the more flexible system. After characterisation through rheological measurements, several degradation mechanisms, to achieve a

release of the encapsulated bacteria in the colon, were tested. All four hydrogels showed no degradation at pH 4 and only the oxD25 hydrogel showed a cleavage at pH 2. Overall the pH responsive degradation at pH 2 is too low to be suitable for a colon specific cleavage of the hydrogel scaffold. Further design and improvement of the backbone and the crosslinker have to be done to achieve the targeted behaviour. The enzymatic degradation of the hydrogels on the other hand is able to release the encapsulated bacteria in the colon. The three protein hydrogels degraded in less than 5 minutes with trypsin under experimental conditions. The cleavage with dextranase showed degradation for the oxD25, cHSA-PEG-Hyd and cHydHSA-PEG hydrogels. The HSA-PEG-Hyd hydrogel did not degrade under experimental conditions within 120 minutes. After a colon specific delivery was possible for all four hydrogels the viabillity of the bacteria cells was tested. The CFU assays showed that *E.coli* was able to survive in oxD25, cHydHSA-PEG and HSA-PEG-Hyd. The cHSA-PEG-Hyd hydrogel showed antimicrobial behaviour.

In conclusion, our results show that dextran and HSA represent a versatile natural platform which allows us to rationally design a hybrid hydrogel material for the intended application. These materials offer the advantage of biocompatibility and huge optimization potential through rheology and bacteria culture experiments. The results taken from the experiments show that the surface charge has to be tailored to maximize the viability of the bacteria. Further the modification with PEG chains can be optimized to adjust the solubility.

List of Abbreviations

Aβ	Amyloid β
AD	Alzheimer´s disease
APP	Amyloid precursor protein
BMPH	N-β-maleimidopropionic acid hydrazide
CFU	colony formation unit
cHSA	cationized HSA
cHydHSA	hydrazide modified HSA
CONVR	conventional raised
DMSO	dimethyl sulfoxide
E.coli	Escherichia coli
EDC	1 ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
EGDMA	ethylene glycol dimethacrylat
ENS	enteric nervous system
FDA	US Food and Drug Administration
GBA	Gut brain axis
GF	Germ free
HSA	human serum albumin
HEMA	hydroxyethylmethacrylate
HSQC	heteronuclear single quantum coherence
LB	lysogeny broth
MALDI	Matrix-assisted Laser Desorption/Ionization
MeO	Methoxy
MWCO	Molecular weight cut off
NHS	N-Hydroxysuccinimid
NFT´s	neurofibrillary tangles
NMR	nuclear magnetic resonance
OxD25	oxidized dextran 25% theoretical oxidation
pHEMA	polyhydroxyethylmethacrylat
PEG	polyethyleneglycol
RT	room temperature (25 °C)
SucHyd	succinic dihydrazide
TCEP	tris(2-carboxyethyl)phosphine
ToF	Time of Flight

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Appendix

1. NMR of modified oxD25 for oxidation degree analysis

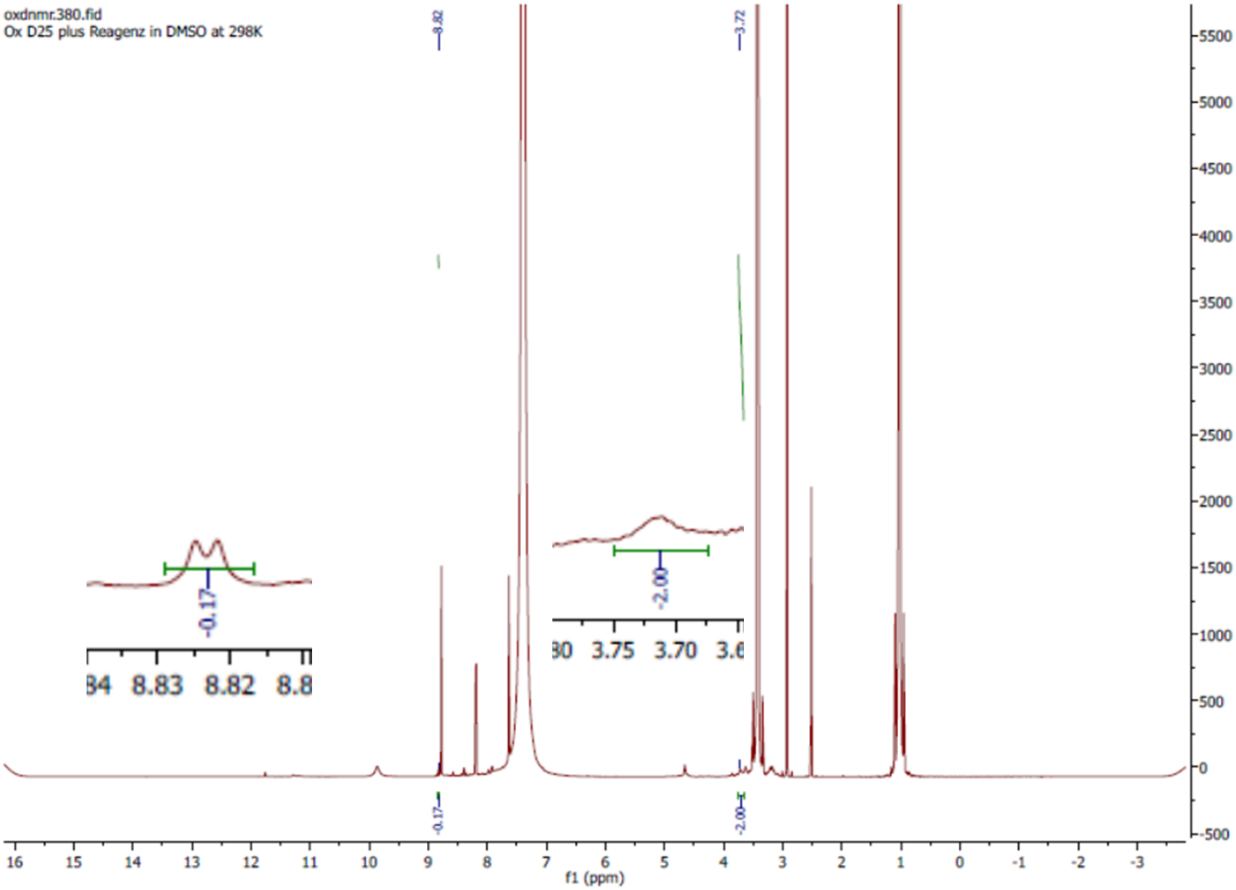

Figure 43: 850 Hz NMR of modified Dextran to investigate oxidation degree

2. Viability of anaerobic bacteria in oxD25 hydrogels

Time	CFU/ml	C
0	444,33	1097,33
1	114,67	1486,67
3	42,00	942,33

Figure 44: CFU of Akkermansia in oxD25 after 0, 1, 3 days

3. MALDI ToF Spectrum cHSA

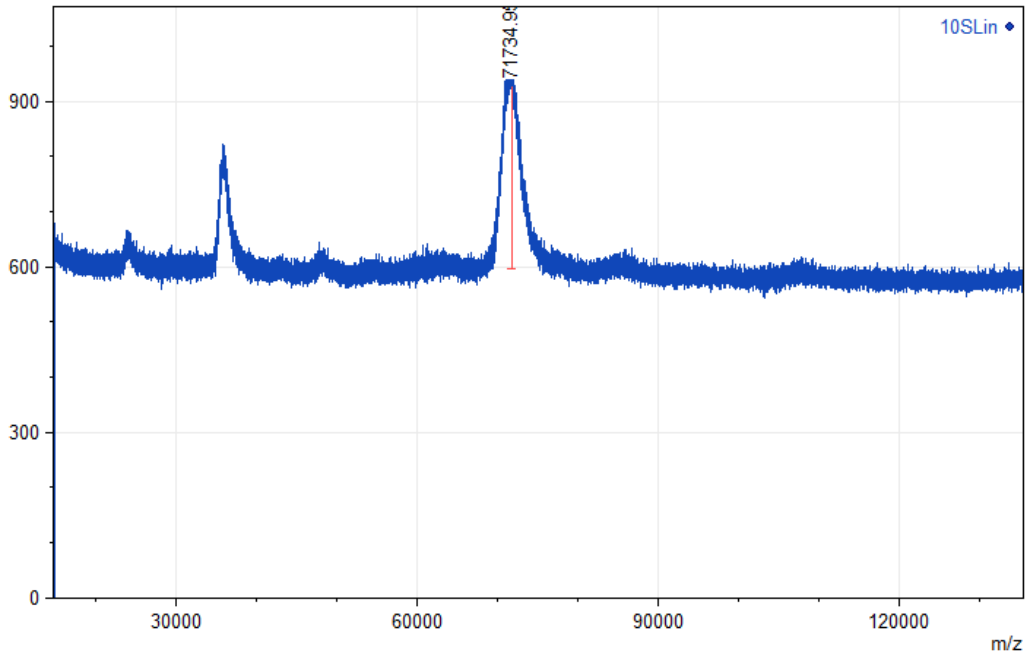

Figure 45: MALDI-ToF spectrum of cHSA

4. MALDI ToF Spectrum cHSA-PEG

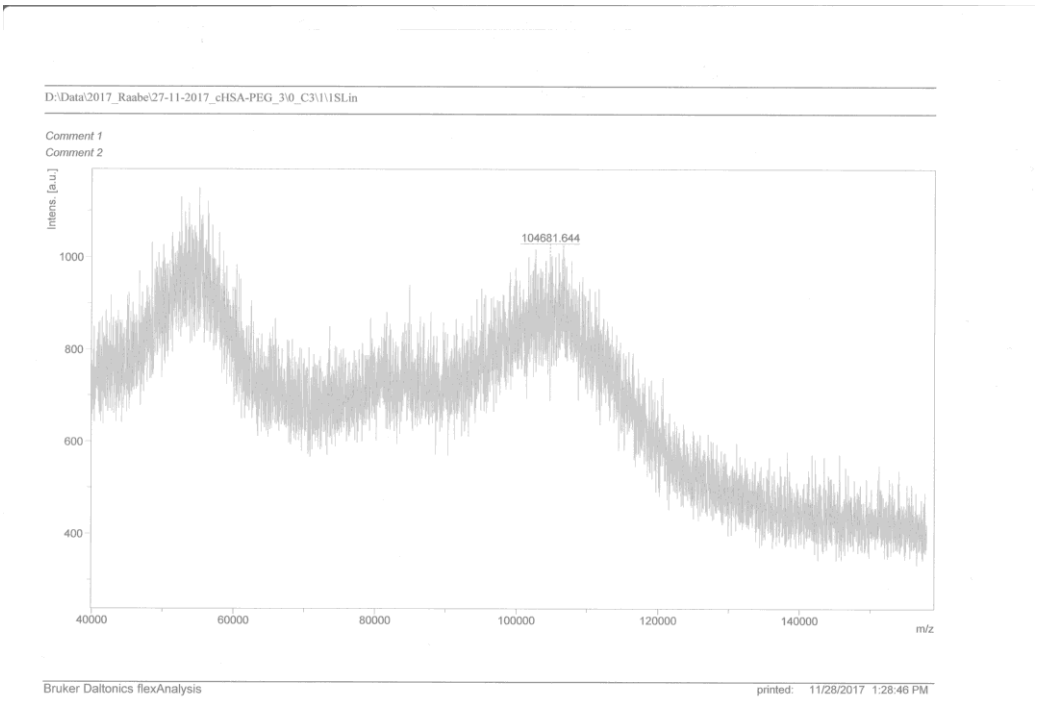

Figure 46: MALDI-ToF spectrum of cHSA-PEG to investigate the amount of PEG

5. MALDI ToF Spectrum of HSA-PEG

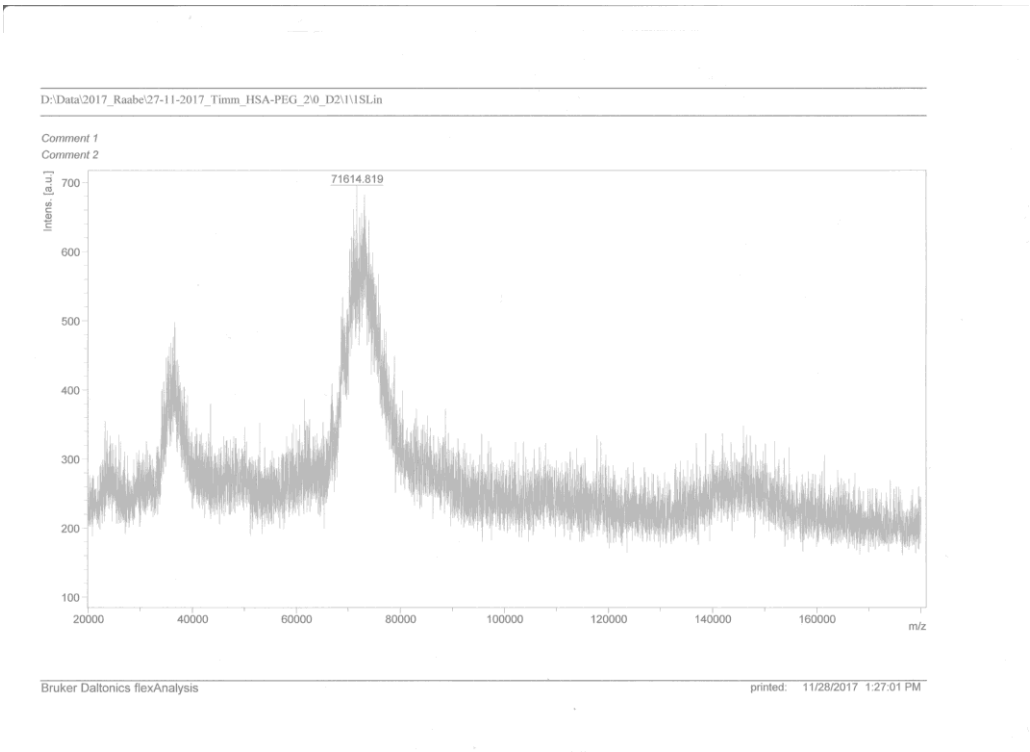

Figure 47: MALDI-ToF spectrum of HSA-PEG to investigate the amount of PEG

6. MALDI ToF Spectrum of cHydHSA

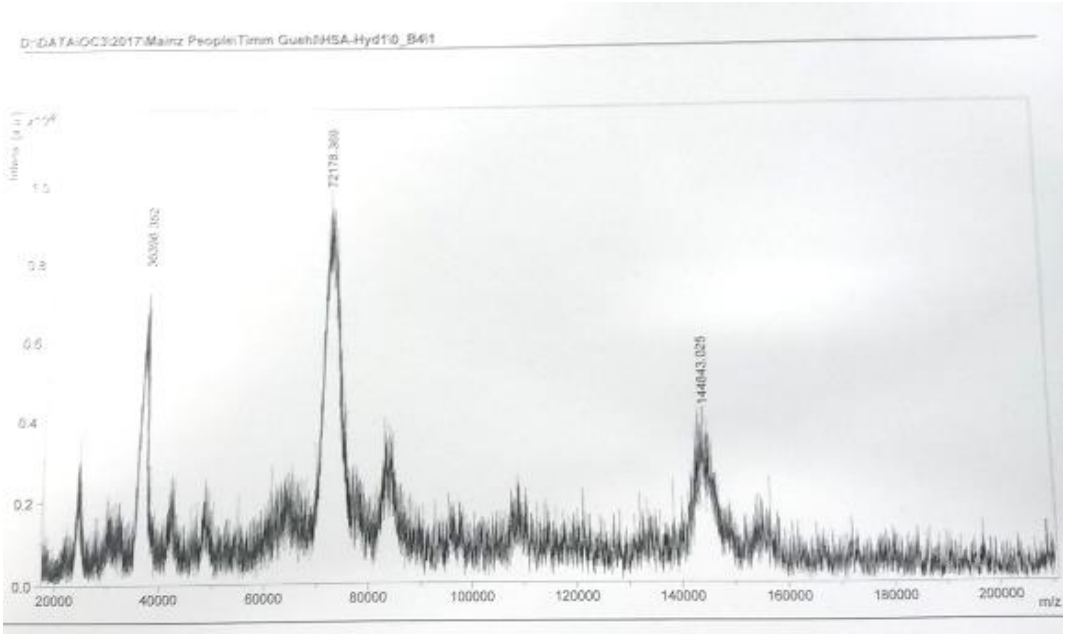

Figure 48: MALDI-ToF spectrum of cHydHSA to investigate the amount of Hyd

7. Zeta Potential cHSA

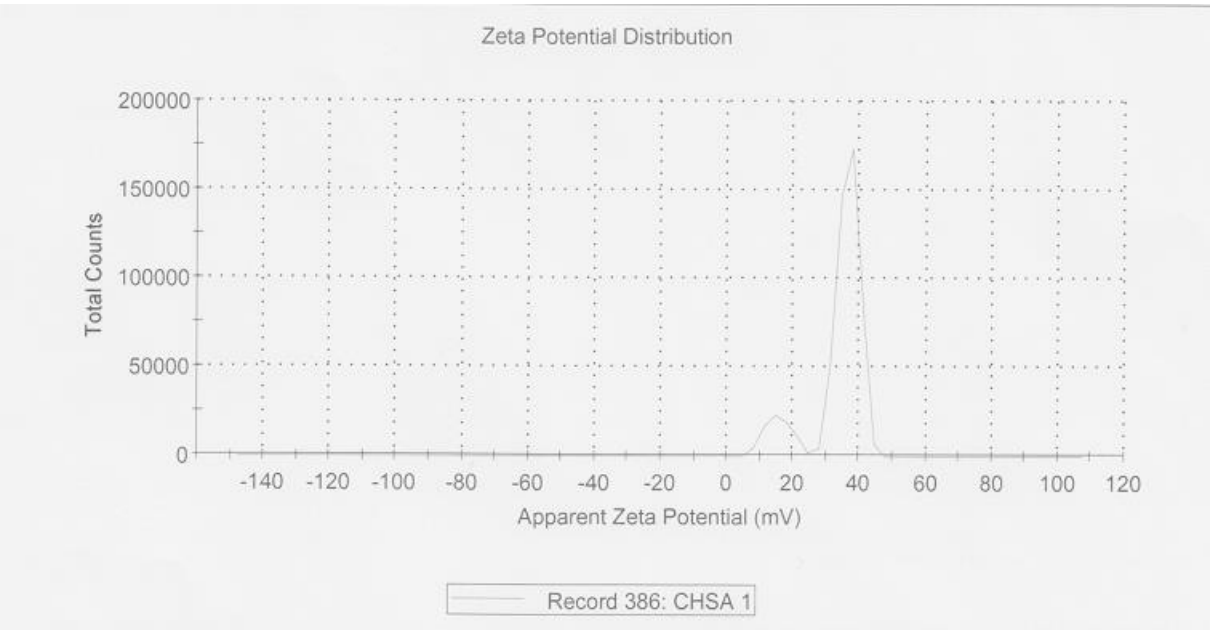

Figure 49: Zeta potential cHSA

8. Zeta Potential cHSA-PEG

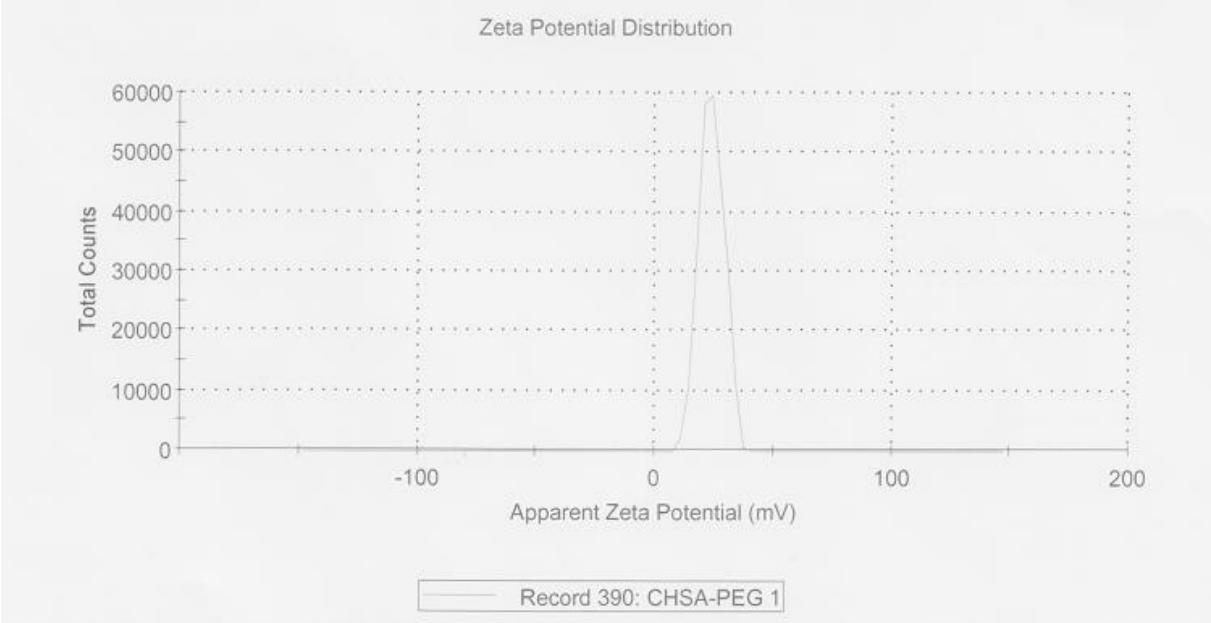

Figure 50: Zeta potential of cHSA-PEG

9. Zeta Potential cHSA-PEG-Hyd

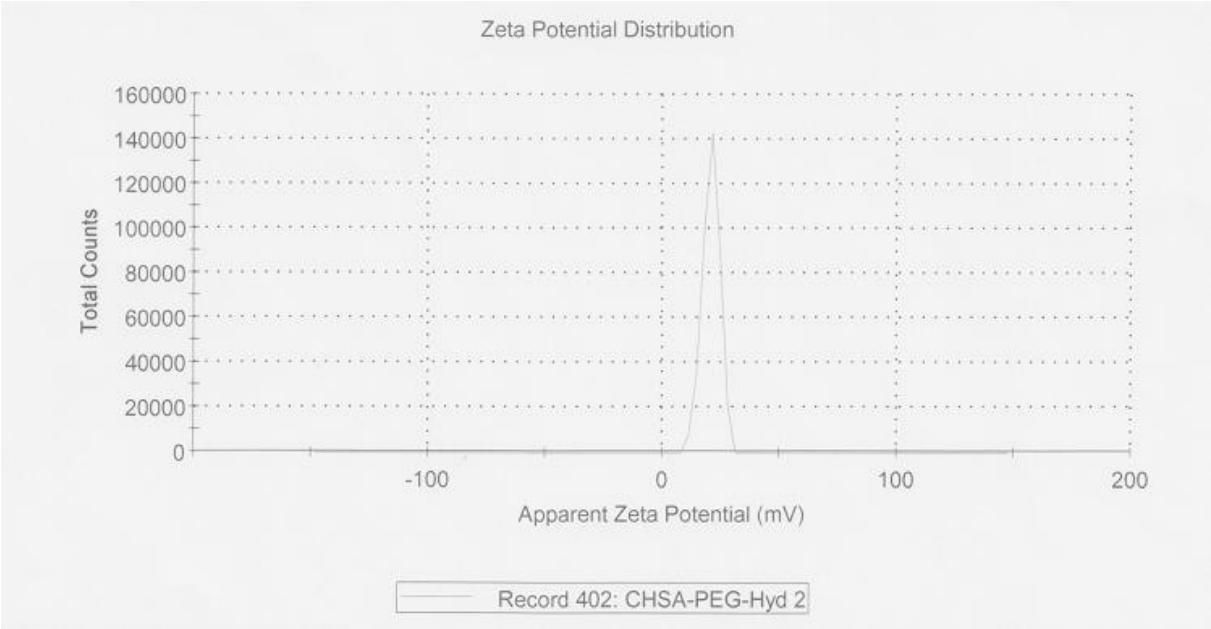

Figure 51: Zeta potential of cHSA-PEG-Hyd

10. Zeta Potential HSA-PEG

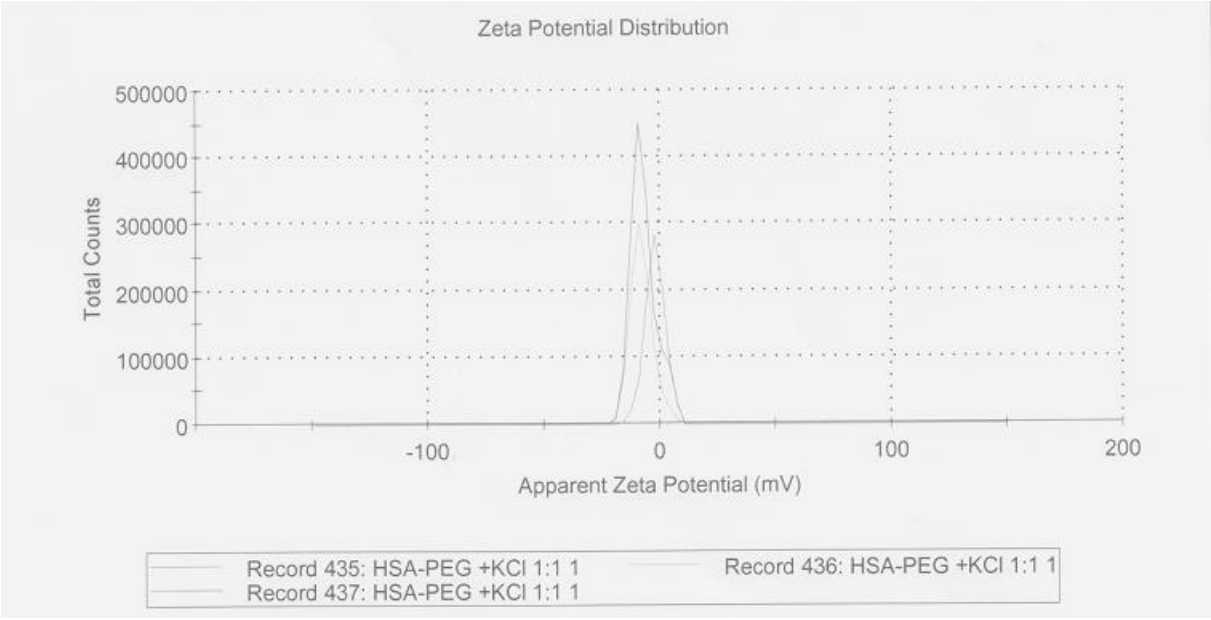

Figure 52: Zeta potential of HSA-PEG

11. Zeta Potential HSA-PEG-Hyd

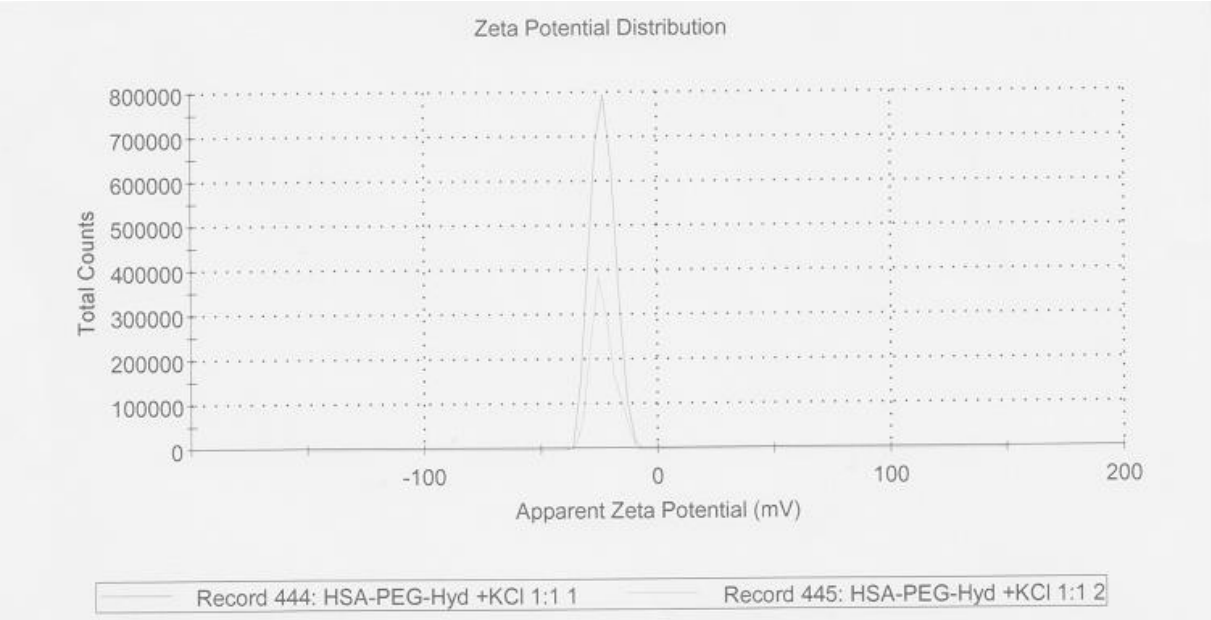

Figure 53: Zeta potential of HSA-PEG-Hyd

12. Zeta Potential cHydHSA

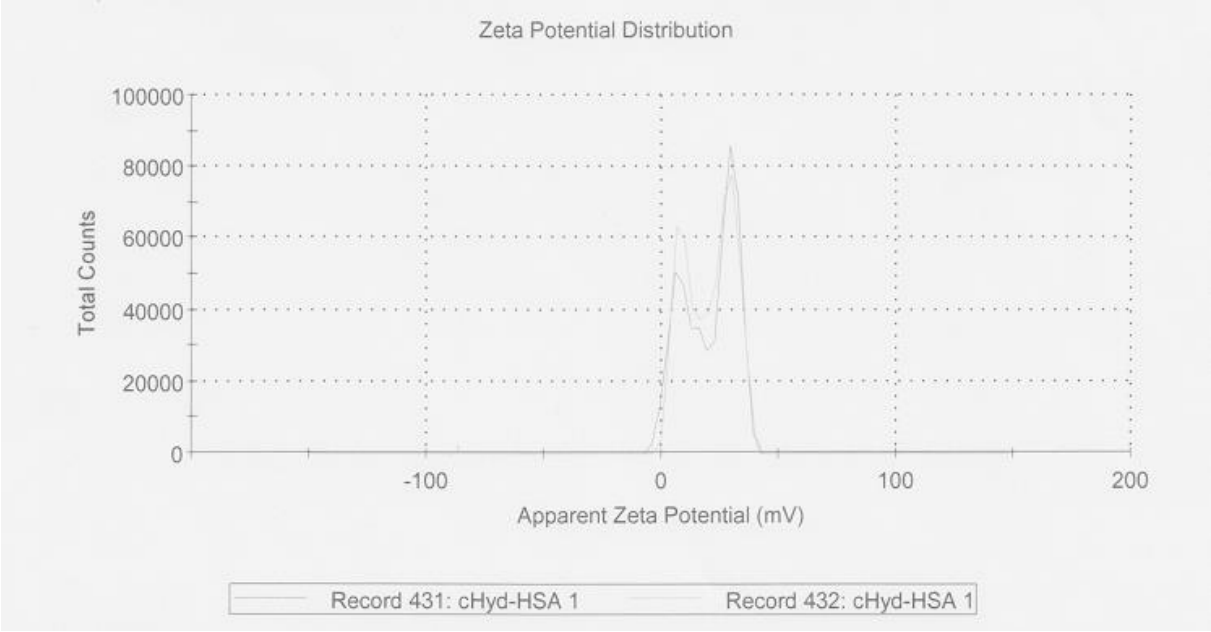

Figure 54:Zeta potential of cHydHSA

13. Zeta Potential cHydHSA-PEG

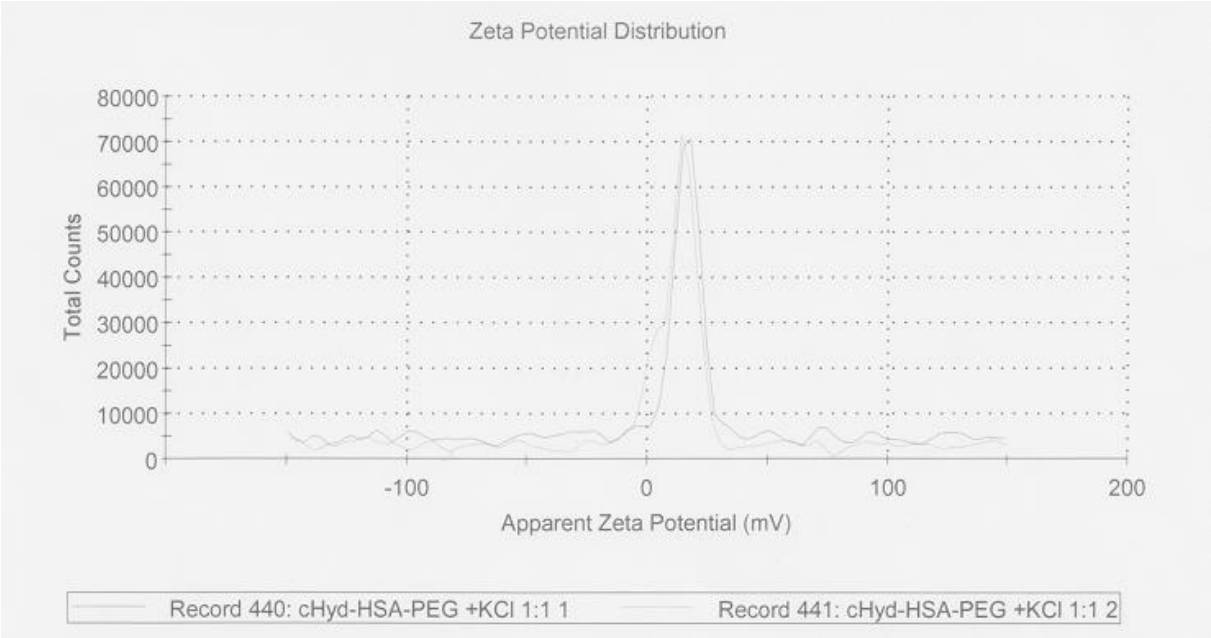

Figure 55: Zeta potential of cHydHSA-PEG

14. Test of self-healing behaviour (oxD25/SucHyd)

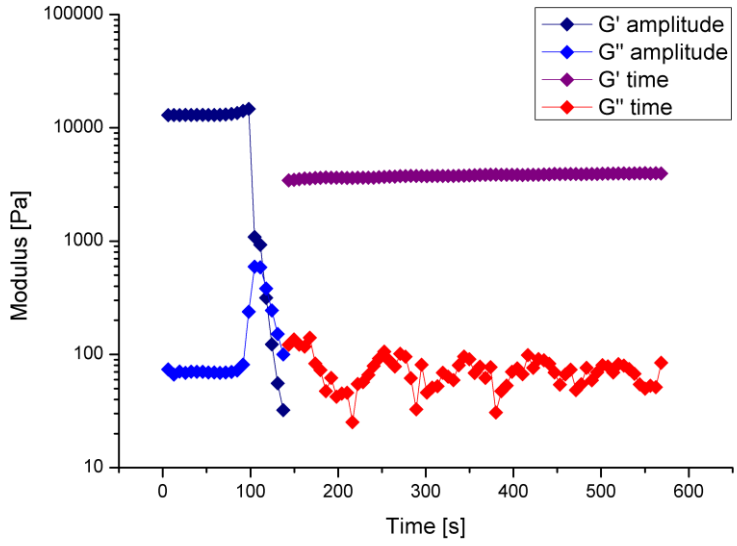

Figure 56: Amplitude sweep followed by time sweep to investigate self-healing behaviour

15. Test of self-healing behaviour (cHSA-PEG-Hyd./oxD25)

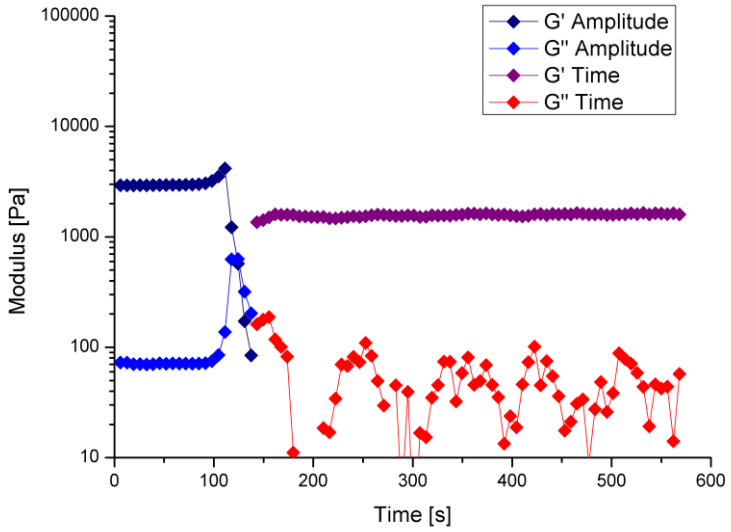

Figure 57: Amplitude sweep followed by time sweep to investigate self-healing behaviour

16. Test of self-healing behaviour (cHydHSA/oxD25)

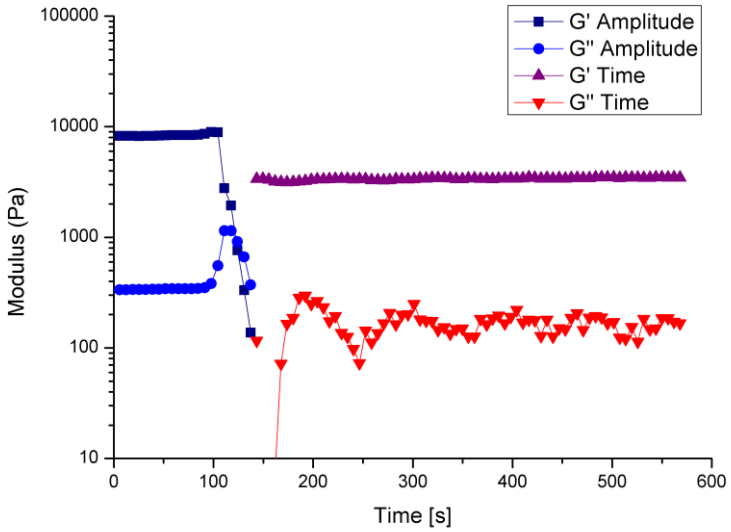

Figure 58: Amplitude sweep followed by time sweep to investigate self-healing behaviour

17. Colony formation assay oxD25

day	CFU/ml control	ΔCFU/ml control	CFU/ml	ΔCFU/ml
0	2291575000	34019330	999439188	209826966
1	1374945000	136077320	951524438	287343793
7	697888750	59533827	974961000	197495030
14	1229117500	17009665	487897150	178712146

Figure 59: Data of colony formation assay (oxD25)

18. Colony formation assay HSA-PEG-Hyd

day	CFU/ml control	ΔCFU/ml control	CFU/ml	ΔCFU/ml
0	1022250775	293557469	619246063	77897023,4
1	1180230567	675529346	632117571	356185275
7	296446475	180974938	318216438	155051751
14	533520325	146238215	392484300	132861926

Figure 60: Data of colony formation assay (HSA-PEG-Hyd)

19. Colony formation assay cHydHSA

day	CFU/ml control	ΔCFU/ml control	CFU/ml	ΔCFU/ml
0	787468500	89826493	665077563	286675273
1	2310324250	546567943	723929375	579510135
7	1272865750	670299082	617944031	505091976
14	360402250	86015775	855174125	232321800

Figure 61: Data of colony formation assay (cHydHSA-PEG)

20. Control Degradation Assay oxD25

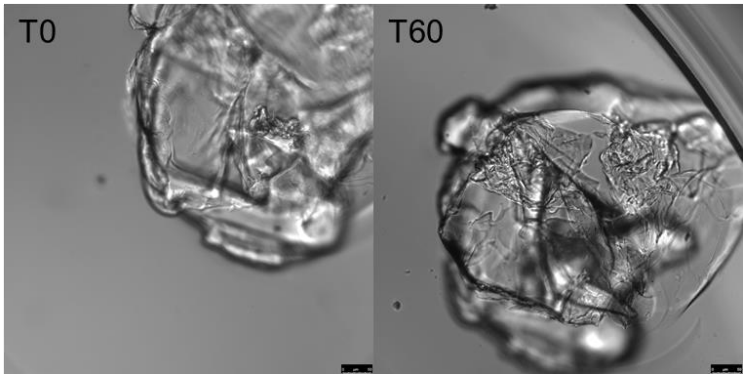

Figure 62: Hydrogel incubated in Milli-Q water at 37 °C for 0 and 60 minutes (scale 50 µm)

21. Control Degradation cHSA-PEG-Hyd

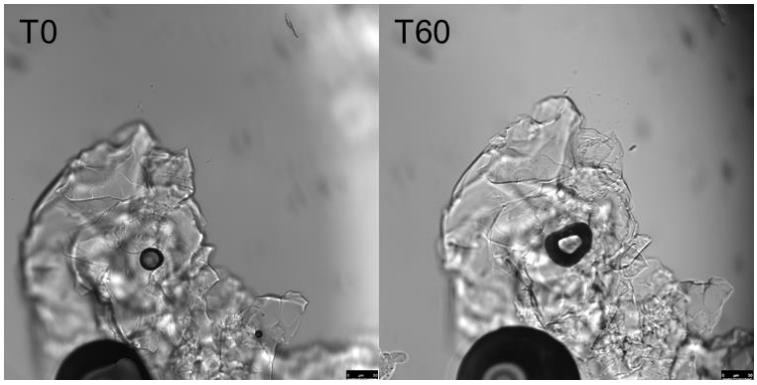

Figure 63: Hydrogel incubated in Milli-Q water at 37 °C for 0 and 60 minutes (scale 50 µm)

22. Control Degradation HSA-PEG-Hyd

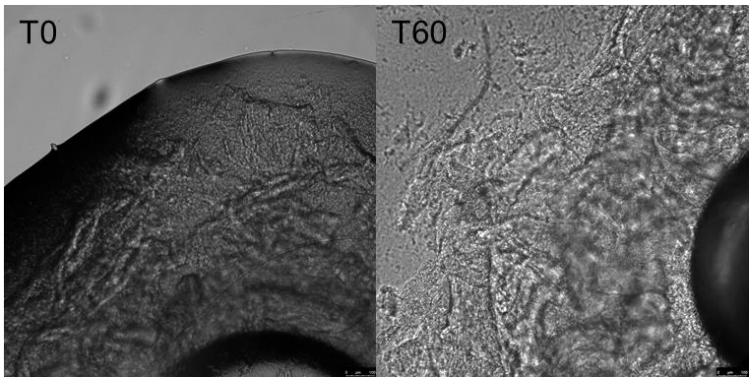

Figure 64: Hydrogel incubated in Milli-Q water at 37 °C for 0 and 60 minutes (scale 50 µm)

23. Control Degradation cHydHSA-PEG

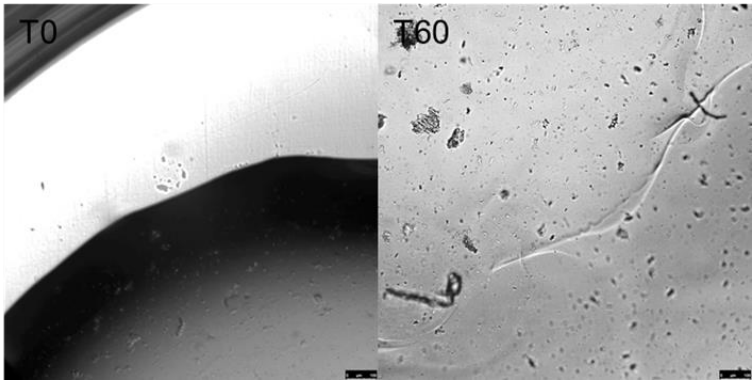

Figure 65: Hydrogel incubated in Milli-Q water at 37 °C for 0 and 60 minutes (scale 50 µm)