Multifunctional Chitosans obtained via Polymer-Analogous Reaction with Functional Five-Membered Cyclic Carbonates

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„Stets findet Überraschung statt, da, wo man’s nicht erwartet hat.“ Wilhelm Busch

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List of Publications

Parts of this thesis are published, submitted to be published and presented at conferences.

Articles:

Carbonate Couplers and Functional Cyclic Carbonates from Amino Acids and Glucosamine, N. Fricke, H. Keul, M. Möller, Macromol. Chem. Phys. **2009**, *210*, 242-255.

Synthesis of chitosan surfactants, N. Fricke, H. Keul, M. Möller, *Macromol. Chem. Phys.* **2009**, *210*, 752-768.

Biocompatible chitosan biocides, N. Fricke, H. Keul, E. Heine, B. Cerda, C. Grandfils, M. Möller, **2009**, in preparation.

Posters:

Peptides as building blocks for the preparation of hybrid materials, N. Fricke, H.-G. Gattner, H. Keul, A. Körner, M. Möller*, BioForum **2004**,* Lüttich (Belgium)

Multiblock copolymers with Elastin-like peptide sequences*,* N. Fricke, H. Keul, M. Möller, *Bayreuth Polymer Symposium* **2007**, Bayreuth (Germany)

Chitosan derivatives for antimicrobial finishing*,* N. Fricke, H. Keul, E. Heine, M. Möller*, Aachen-Dresden International Textile Conference* **2007**, Aachen (Germany).

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List of Abbreviations

Amino acids are either described with the three letter- or the single letter code.

AAA	amino acid analysis
AlaOMe	alanine methyl ester
AMM	antimicrobial macromolecules
AMP	antimicrobial peptides
ATRP	atom transfer radical polymerisation
BSA	bovine serum albumine
CFU	colony forming units
cmc	critical micelle concentration
CycOMe	cystein methyl ester
DP	degree of polymerisation
Da	Dalton
DA	degree of acetylation
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DD	degree of deacetylation
DHB	2,5-dihydroxybenzoic acid
DIPEA	diisopropylamine
DLS	dynamic light scattering
DMAPA	3-dimethylamino-1-propylamine
DMAP	4-dimethylamino pyridine
DMF	dimethyl formamide
DPC	diphenyl carbonate
ELP	elastin-mimetic polymer
FT-IR	Fourier transform infrared spectroscopy
GC	glycol chitosan
GlyOMe	glycine methyl ester
GTMAC	glycidyltrimethylammonium chloride
HCCA	α-cyano-4-hydroxy cinnamic acid
HDP	host defense peptides
HOBt	1-Hydroxybenzotriazol
ITT	inverse temperature transition
LCST	lower critical solution temperature
LysOMe	lysine methyl ester
LMWS	low molecular weight standard
MALDI-TOF MS	matrix assisted laser desorption ionisation time of flight mass spectrometry
MIC	minimal inhibitor concentration
mPEG	methoxy poly(ethylene glycol)
MWCO	molecular weight cut-off
NMP	N-methyl-pyrrolidone
NMR	nuclear magnetic resonance
PBS	phosphate-buffered saline

PEG	poly(ethylene glycol)
PEI	poly(ethylene imine)
PES	poly ether sulfone
Poly(HEMA)	poly(hydroxyethyl methacrylate)
PNIPAM	poly(*N*-alkyl acrylamides)
ppm	parts per million
RBC	red blood cells
RGD	arginine-glycine-asparic acid
Rpm	rounds per minute
SA	sinapinic acid
SDS PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SPDP	3-(2-pyridyldithio)propionic acid N-hydroxy-succinimide ester
r.t.	room temperature
TCA	trichloric acid
TFA	trifluoric acid
TGA	thermogravimetric analysis
THF	tetrahydrofuran
ValOMe	valine methyl ester

Summary

This thesis deals with the preparation and characterisation of multi-functional chitosan derivatives. The resulting materials can be used as surfactants and as biocides. In the preparation of these compounds carbonate couplers play an important role. Therfore different carbonate couplers were developed and evaluated by reaction with amines.

Chitosan is a biocompatible natural polyamine, which is obtained from chitin - the second most abundant polysaccharide in nature - by deacetylation with sodium hydroxide. It is composed of glucosamine and N-acetyl-glucosamine units and is available in different molecular weights and degrees of deacetylation (DD). The solubility is influenced by these two properties; however, the majority of chitosan derivatives are only soluble at low pH values in aqueous solution. Only low-molecular weight chitosans, so-called chitosan oligosaccharides and modified chitosans like glycol chitosan are soluble in water at any pH.

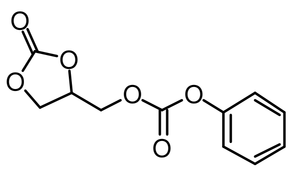
Chitosan surfactants, amphiphilic derivatives bearing hydrophilic and hydrophobic (alkylic) branches were prepared from glycol chitosan (GC) and chitosan oligosaccharides (COS) in a three-step synthesis. In the case of GC water-insoluble products were obtained, the functionalisation of which was proven via IR spectroscopy. With the oligomers a broad variety of products were obtained using a short (C3) or a long (C6) carbonate linker. The products vary in the degree of functionalisation, hydrophobic to hydrophilic ratio and length of the hydrophobic side group (C8, C12 and C14) and were characterised by NMR spectroscopy and partially by IR spectroscopy. Thermogravimetric analysis showed a higher thermal stability for the derivatives investigated and measurement of surface tensions resulted in a critical micelle concentration (cmc) being 0.01 mg/mL for a hydrophilic to hydrophobic ratio of 1:1 and a C12 alkyl chain.

The antibacterial activity of these chitosan surfactants was tested against *B. subtilis* and *E. coli*. Highest activity was determined for derivatives prepared with the C6 linker, dodecyl- (A12) branches and moderate (1:1, 2:3) ratios of cationic to A12 side chains. The hemocompatibility of the more potent antibacterial compounds was assessed based on their haemolytic action on human red blood cells (RBCs). All samples investigated were found harmless for blood cells.

Moreover, direct functionalisation of chitosan with ethylene carbonate or functional ethylene carbonates was studied. The results show that due to the low reactivity of the amino group in glucosamine repeating units this procedure is not viable.

The carbonate coupler is a bifunctional molecule bearing two amino-reactive groups, which can be addressed selectively: The linear carbonate unit reacts at low temperatures; the five-membered cyclic carbonate moiety reacts at elevated temperatures (> 40 °C).

On the basis of glycerol carbonate, four coupling reagents were prepared: (2-oxo-1,3-dioxolan-4-yl)methyl phenyl carbonate (**I**), (2-oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate (**II**), (2-oxo-1,3-dioxolan-4-yl)methyl 1-imidazole carbonate (**III**) and (2-oxo-1,3-dioxolan-4-yl)methyl 4-(propan-2-ylideneaminooxy) carbonate (**IV**).



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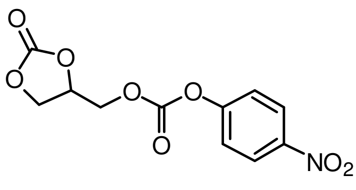

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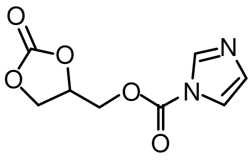

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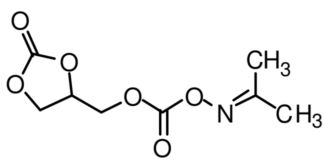


IV

Their synthesis and characterization, starting from glycerol carbonate is described. To evaluate the differences in reactivity these couplers were reacted with valine methyl ester and the results were compared with the already known coupler **I**. Reaction conditions influenced the conversion of the couplers **I-IV** containing -phenyl, -4-nitrophenyl, -imidazolyl and -acetone oxime leaving groups; highest conversion and fastest reaction rates were obtained

with 2-oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate (**II**). The coupling reagents **I**, and **II** were used for the synthesis of functional carbonate building blocks from amino acid methyl esters and glucosamine.

In an additional study amino acids were reacted with (2-oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate (**II**) and with glycerol carbonate chloroformiate. It could be shown that the desired products were formed; however the isolation of these amino acid couplers failed.

Zusammenfassung

Die vorliegende Arbeit behandelt die Synthese und Charakterisierung von multifunktionellen Chitosanderivaten. Die erhaltenen Materialien können einerseits als Tenside, andererseits als Biozide verwendet werden.

Chitosan ist ein natürliches, biokompatibles Polyamin, das aus Chitin – dem nach Cellulose häufigsten natürlich vorkommenden Polysaccharid - durch Deacetylierung mit Natriumhydroxid gewonnen wird. Chitosan ist aus Glucosamin und N-Glucosamineinheiten zusammengesetzt und ist in verschiedenen Molekulargewichten und Deacetylierungsgraden erhältlich. Das Molekulargewicht und der Deacetylierungsgrad sind für die Löslichkeit bestimmend. Der größte Teil der Chitosanderivate ist nur bei niedrigen pH-Werten löslich. Nur Derivate geringen Molekulargewichts, sogenannte Chitosanoligosaccharide, sowie Glykol Chitosan sind bei jedem pH-Wert in Wasser löslich.

Chitosantenside, also amphiphile Derivate mit hydrophilen und hydrophoben (Alkyl-) Seitenketten wurden ausgehend von Glykol Chitosan (GC) und Chitosanoligosacchariden (COS) in einer dreistufigen Synthese hergestellt. Im Falle der GC-Derivate wurden nur wasserunlösliche Produkte erhalten, deren Funktionalisierung aber mittels IR Spektroskopie eindeutig nachgewiesen wurde. Eine Vielzahl von Produkten wurde durch Modifizierung der Oligomere (COS) mit einem kurzen (C3) oder einem langen (C6) Carbonatkoppler erhalten. Die Produkte variieren im Funktionalisierungsgrad, im Verhältnis der hydrophoben zu hydrophilen Segmente und der Länge der hydrophoben Seitenkette (C8, C12, C14) und wurden mittels NMR Spektroskopie und teilweise durch IR Spektroskopie charakterisiert. Die thermogravimetrische Analyse zeigte, dass die untersuchten Derivate thermisch stabiler sind als das Ausgangsmaterial. Die Messung der Oberflächenspannung ergab für das Produkt mit

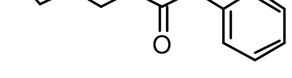
einem gleichen Verhältnis von hydrophilen zu hydrophoben Seitenketten und einer C12-Seitenkette eine kritische Mizellkonzentration von 0,01 mg/mL.

Die antibakterielle Aktivität dieser Chitosantenside wurde gegen *B. subtili* und *E. coli* getestet. Die höchste Aktivität wurde für Derivate bestimmt welche mit dem C6-Koppler hergestellt wurden und eine C12-Seitenkette tragen und ein moderates Verhältnis (1:1, 2:3) von kationischen zur Alkylseitenkette aufweisen. Die Hämokompatibilität der wirksamsten Chitosanbiozide wurde durch ihre Wechselwirkung mit Erythrozyten untersucht. Alle untersuchten Derivate erwiesen sich als nicht hämolytisch.

Zusätzlich wurde die direkte Funktionalisierung von Chitosan mit Ethylencarbonat oder funktionellen Ethylencarbonaten untersucht. Es zeigt sich, dass bedingt durch die geringe Nucleophilie der Aminogruppe der Glucosaminwiederholungseinheit dieser Syntheseweg keine Option war.

Carbonatkoppler sind bifunktionelle Verbindungen mit zwei aminoreaktiven Gruppen, welche selektiv umgesetzt werden können: Die lineare Carbonatgruppe reagiert schon bei niedrigen Temperaturen; die fünfgliedrige cyclische Carbonateinheit reagiert bei erhöhten Temperaturen (> 40 °C).

Ausgehend von Glycerincarbonat wurden vier Kopplungsreagenzien hergestellt: (2-Oxo-1,3-dioxolan-4-yl)methyl phenyl carbonat (**I**), (2-Oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonat (**II**), (2-Oxo-1,3-dioxolan-4-yl)methyl 1-imidazole carbonat (**III**) und (2-Oxo-1,3-dioxolan-4-yl)methyl 4-(propan-2-ylideneaminooxy) carbonat (**IV**).



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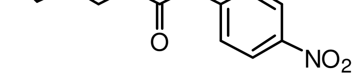

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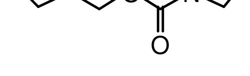

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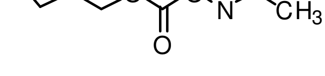


IV

Um die Reaktivitätsunterschiede näher zu untersuchen, wurden die Koppler mit Valinmethylester umgesetzt und die Ergebnisse mit dem bisher schon bekannten Koppler **I** verglichen. Durch die Reaktionsbedingungen wurde die Umsetzung der Koppler **I**-**IV** mit Phenyl-, 4-Nitrophenyl-, Imidazolyl- und Acetonoximabgangsgruppe entscheidend beeinflusst. Den höchsten Umsatz und die schnellste Reaktionsrate wurde für (2-Oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonat (**II**) ermittelt. Die Kopplungsreagenzien **I** und **II** wurden weiterhin für die Synthese funktioneller Carbonatbausteine aus Aminosäuremethylestern und Glucosamin eingesetzt.

In einer zusätzlichen Studie wurden Aminosäuren mit (2-Oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonat (**II**) und Glycerolcarbonat umgesetzt. Es konnte zwar gezeigt werden, dass die gewünschten Produkte gebildet wurden, allerdings konnten diese nicht isoliert werden.

Chapter 1 Introduction

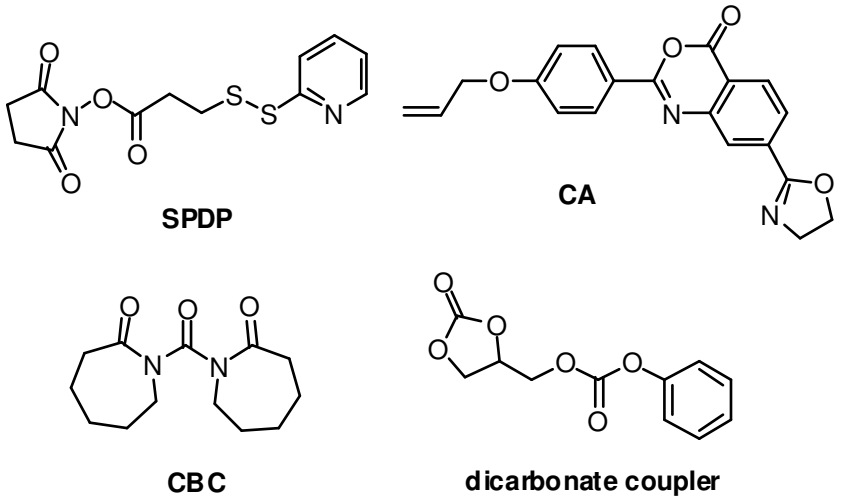
The versatility of macromolecular compounds is immense, including synthetic polymers as well as natural macromolecules. Applications range from food industry over technical aspects to cosmetics. The versatility can even be increased by combining different polymers, thereby gaining a synergistic effect by the introduction of additional properties. Amphiphilic polymers play a key role in such hybrid polymers due to their ability to self-assemble. Thus polymeric vesicles they are discussed for therapeutic applications in drug-delivery systems and pH- and temperature-sensitive polymers for biosensor applications.[1] These modern materials may also lead to artificial tissue or can be used as smart gels in chromatography.[2] Fields of application are broad, but what comes first is the preparation of such compounds. Since synthetic strategies are as broad as possible application areas, the focus within the next paragraph will be placed on (i) copolymer synthesis via coupling reaction, (ii) polymeric surfactants and (ii) polymeric biocides.

1.1 Copolymer synthesis via coupling reaction

Amphiphilic polymers combine segments which are usually incompatible. Covalent linking of such segments can be achieved by the application of suitable coupling agents, which activate a terminal group of one segment for the reaction with a functional group of the other segment. Said coupling agents are especially known in peptide chemistry, due the big variety of functional groups in natural amino acids.

Coupling agents have rarely been applied in the preparation of copolymers. Dicyclcocarbodiimide (DCC) which usually is used in peptide synthesis was used to prepare poly(methacrylic acid)-*graft*-methoxy poly(ethylene glycol) starting with the two polymers poly(methacrylic acid) and methoxy poly(ethylene glycol) (mPEG).[3] In this way products

with long mPEG chain (5000 Da) and randomly distributed side chains were obtained. Polymer-peptide conjugates from polyamines and polylysine and cysteine-containing polypeptides have been described using the heterobifunctional agent 3-(2-pyridyldithio)propionic acid N-hydroxy-succinimide ester (SPDP) (see Scheme 1).[4]

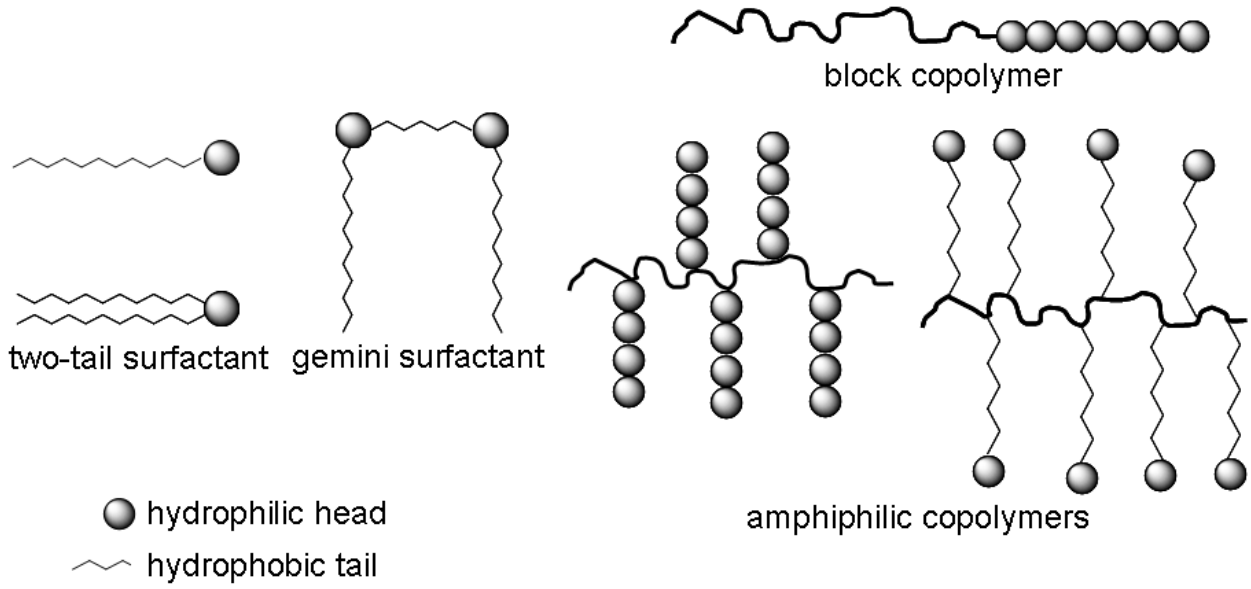


Scheme 1 coupling agents

Selective coupling of two compounds with different functionality is the advantage of heterobifunctional coupling agents or chain extenders compared to homobifunctional reagents, like terephthaloyl chloride and bisoxazolines.[5, 6] Combination of an oxazoline and oxazinone group in one molecule enables the covalent linking of polymers with carboxylic acid groups with polyamines or polyols.[7] In the preparation of polystyrene-COOH and poly(methylmethacrylate)-NH_2 blends the coupling agent (CA) cross-linked partially without catalyst and almost complete with catalyst. Blending of polyamines, e.g. jeffamines and polyols was achieved with carbonylbiscaprolactam (CBC).[8] Reaction proceeds via ring elimination (RE) with amines at 100 °C and via ring opening (RO) reaction with hydroxy groups in the presence of a catalyst. Amphiphilic polymers based on poly(ethylene imine) were prepared using the dicarbonate coupler, which enables the synthesis of functional carbonates for the modification of polyamines.[9]

1.2 Polymeric surfactants

Surfactants are compounds combining hydrophilic and hydrophobic segment. Such molecules can be monodisperse low-molecular substances or polydisperse macromolecules with different structural principles (see Scheme 2). Such amphiphilic structures are also found in nature where they are the matrix for biological membranes and therefore a crucial building block for living cells.[10]



Scheme 2 Structural principles of low-molecular (left) and polymeric surfactants (right)

Surfactants aggregate in solution to form supermolecular structures. These structures are influenced by the type of the hydrophilic and hydrophobic segment, the aspect ratio of the two segments, the temperature, the concentration of surfactant and the solvent. The ability to form aggregates is characterised by the *critical micelle concentration* (cmc), at which physical properties (e.g. surface tension, light scattering, osmotic pressure) of the surfactant solution change, due to the formation of micelles or vesicles. Common examples for low-molecular weight surfactants are sodium dodecyl sulphate (SDS; cmc = 8.1 x 10^{-3} mol/L), an anionic surfactant and the cationic cetyltrimethylammonium bromide (CTAB; cmc = 9.2 x 10^{-4} mol/L). Interesting structures can be found for “biosurfactants”. However, in

literature this title is not clearly defined and is either used for compounds which are produced by microbes or refers to substances bearing a biomimetic group, like DNA, phosphocholin and peptides.[11] *B. subtilis* produces several antimicrobial lipopeptides, in which a cyclic peptide is linked to a fatty acid chain.[12] The cmc was determined to be 20 mg/L and the material was used for enhanced microbial degradation of hydrocarbons from ship bilge wastes.[13] Beside such low-molecular weight detergents, nature also produces polymeric biosurfactants:[14] A prominent member of this class is *Alasan*, a complex of a polysaccharide (apo-alasan) containing covalently bound alanine and proteins with molecular masses of 16, 31 and 45 kDA.[15] The proteins were found to play an essential role in the emulsifying activity of *Alasan*. The structural variety of synthetic polymeric surfactants is not as high as in nature, but can also vary in the chemical constitution, length and and structure of the blocks and the already mentioned architecture (block, graft, star and multiblock copolymers). The outcome is the ability to produce tailor-made polymers and to trigger the cmc to much lower values as it is possible for low-molecular weight surfactants. Stimuli-responsive polymers are achievable by different pathways. Comb-type copolymers with a reversibly formed hydrophobic block are obtained by combining poly(methacrylic acid) and poly(methacrylic ester) with poly(ethylene glycol) grafts.[16, 17] Under acidic conditions hydrophobic chain segments are formed due to hydrogen-bonded complexation. Copolymers of the same chemical composition but completely grafted – poly[oligo(ethylene glycol)methacrylates] exhibit a lower critical solution temperature (LCST), comparable to poly(*N*-alkyl acrylamides) (PNIPAM) and elastin-like polymers (ELP, poly(VPGXG)).[18] The cloude point is greatly influenced by the length of the oligo(ethylene glycol) graft. Copolymers with very short grafts are not soluble in water at all, between 2 and 10 EO units the LCST increases with increasing length and copolymers with more than 10 EO units are also soluble at high temperatures. Amphiphilic glycopolymers gain an increasing interest during the last years,

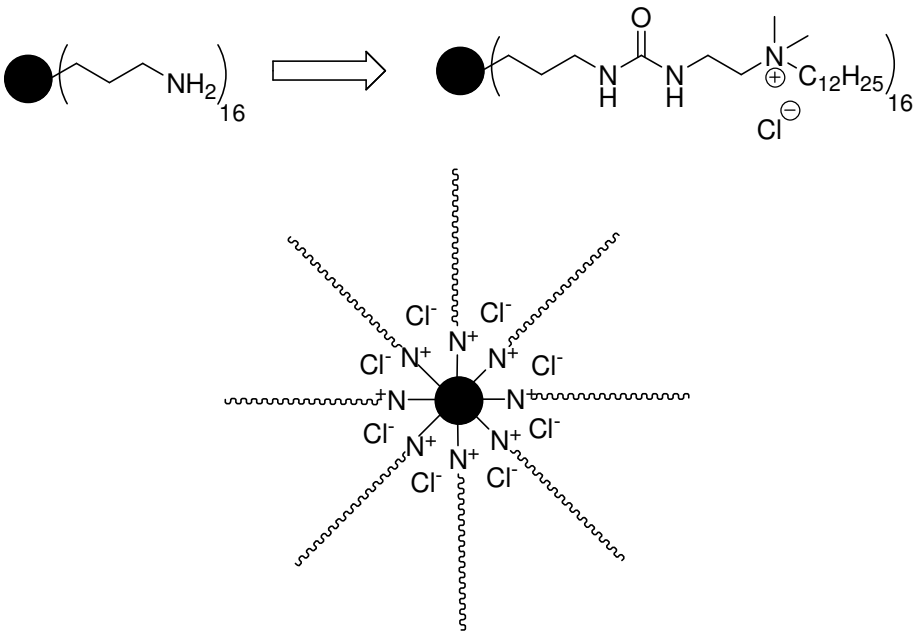
due their possible application in the field of medicine, biotechnology, sensors and separation science.[19] End-functionalised polystyrene with terminal mono- and oligosaccharide blocks were obtained via a post-polymerisation technique using chloromethylbenzylated saccharides and polystyryllithium.[20, 21] The authors observed the formation of reversible micelles with an increasing aggregation number correlated to an increasing number of introduced monosaccharides. Malto-oligosaccharide-carrying styrene monomers were homopolymerised and the supermolecular assembly in 0.1 M urea solution was identified as a molecular bottlebrush.[22] The same glycomonomer was used to prepare amphiphilic microgels and star microgels by cross-linking with divinylbezene.[23, 24]

1.3 Polymeric biocides

Antimicrobial polymers render surfaces insusceptible to biofilm formation and overcome problems with bacterial resistance. Additionally they do not exhibit cytotoxicity as low-molecular weight biocides do. Resistance formation is accounted to be of low risk in the case of polymeric biocides since their target is the cell membrane, which seems to be the Achilles heel of the bacteria.[25] Examples of mainly cationic polymers and their antimicrobial/antibacterial activity will be given in the following.

The effect off the molecular weight on the antimicrobial activity was tested already in the 1980s with homopolymers of polyacrylates and poly(meth acrylates) with biguanide side groups and the corresponding copolymers with acrylamide.[26, 27] It was shown that the antimicrobial activity increased up to a M_w of 5 x 10^4 and decreased from a M_w of 1.2 x 10^5. A similar tendency was also found for other polymeric biocides.[28] Interestingly dendritic biocides of poly(propylene imine) with quaternary ammonium groups (see Scheme 3) showed a parabolic dependence on molecular weight.[29] It was also observed that the antimicrobial

activity was influenced by the counter ion. The derivative with bromide as counter ion showed a higher activity against *E. coli* as the dendritic biocide with chloride as counter ion.



Scheme 3 Dendritic biocide with quaternary ammonium groups

However, the effect of the counter ion in polycationic biocides on the antimicrobial action is not fully understood, since there are also examples where no effect upon change of the counter ion was detected.[30] The correlation seems to be more an effect of solubility of the corresponding derivatives.

One of the few block copolymers with antibacterial efficacy is a copolymer composed of poly(ethylene-*co*-butylene) as hydrophobic block and poly(dimethylamino)ethylmethacrylate) as the cationic block, which was synthesised via ATRP.[31] An effective antibacterial action was already observed at a concentration of 0.15 mg/mL. A thermo-responsive antibacterial polymer was realised by polymerising a pyridine-containing methacrylamide and subsequent polymerisation of *N*-isopropyl acrylamide.[32] Antibacterial activity was achieved with this polymer by quaternisation of the pyridine unit with different alkylbromides. The LCST of the polymers was in the range of T=30 °C to T = 42 °C and increased with a decreasing alkyl chain attached to the pyridine group. All products showed good antibacterial activity. A

comparison of a polymeric biocide and host defense peptides was published in 2004:[33] The antibacterial action of polystyrene bearing 4-(dimethylaminomethyl) side group or a 4-(dimethylamino-methyl) side group were compared to the activity of [Ala]-magainin II amide, a α-peptide with potent antimicrobial activity.[34] It was observed that the polymer with biocidal activity upon N-protonation exhibited similar antibacterial, but also similar haemolytic activity as compared to the peptide toxin [Ala]-magainin II amide. The monomer was not active and the corresponding polymer with quaternary ammonium group was less active against *E. coli*, *B. subtilis*, *S. aureus* and *E. faecium*. The authors investigated the mode of action, too and discovered that the activity did not arose from a detergent-like membrane disruption since the polymer did not show any surfactant properties, like it is presumed for amphiphilic polymers.[35] However, a mode of action for this compound was not suggested. In order to prepare self-disinfecting surfaces it is necessary that the antimicrobial activity of a polymer in solution is retained upon immobilisation. Techniques to prepare such surfaces include simple "spray coating" of polystyrene-*b*-poly(4-vinyl-*N*-alkylpyridinium bromides,[36] "painting-like" coating of *N*-alkyl-PEIs,[37] casting of polymer particles with the antimicrobial copolymer block poly(4-vinyl-*N*-methylpyridinium iodide) onto glass slides,[38] electrografting for the preparation of antibacterial stainless steel surfaces and "grafting onto" and "grafting from" approaches of a broad variety of biocidal polymers.[39-41] All the described surfaces exhibited antibacterial activity against *Staph. aureus* within a reasonable time scale. That antimicrobial polymers retain their activity upon immobilisation was shown with poly(butyl methacrylate-*co*-aminoethylmethacrylate hydrochloride).[42] This polymer efficiently kills bacteria in solution and its hemolytic activity can be triggered by the hydrophobic to hydrophilic ratio and the molecular weight.[43] Via a "grafting from" technique using a covalently linked initiator the polymer was attached to silicon wafers and glass surfaces. The antibacterial effect was independently of layer thickness and layer density and it

could be shown that bacterial membrane disruption occurred although the layer thickness was only 3 nm which is much less than the cell wall of *Staph. aureus* (~30-70 nm).

The presented examples show the versatility of antimicrobial macromolecules (AMM) and the possibilities to chemically influence bacterial growth.

1.4 Content of this thesis

This thesis is concerned with the preparation of different compounds using activated carbonate chemistry, especially cyclic carbonates for the synthesis of antibacterial chitosan, chitosan surfactants, functional carbonates from amino acids and glucosamine and functional polymers with amino acid and peptide segments.

Chapter 1 gives a short introduction to some aspects of the synthesis of amphiphilic polymers and an overview of polymeric surfactants and polymeric biocides.

Chapter 2 deals with the synthesis and the characterisation of chitosan surfactants from glycol chitosan (GC) and chitosan oligosaccharides (COS). These chitosan surfactants have been prepared using a chloroformiate with a cyclic carbonate moiety prepared from glycerol and 1,2,6-hexanetriol and subsequent addition of amines to this cyclic carbonate. A cationic side group and and a hydrophobic alkyl side chain of twelve carbon atoms were introduced via this carbonate coupler approach. Details on synthesis and physical properties of the COS derivatives (TGA, cmc) are presented

Chapter 3 presents results with respect to the MIC of chitosan biocides from chitosan oligosaccharides with a cationic side group and hydrophobic alkyl side chains. Synthesis was performed according to the method described in chapter 2. However, the presented chitosan biocides vary in the ratio of hydrophobic to cationic side groups, the length of the hydrophobic alkyl chain (C_8, C_{12}, C_{14}) and the fraction of unmodified amino groups on the chitosan backbone. The haemolytic activity of samples with good antibacterial properties was

tested. Dynamic light scattering experiments were performed to determine solution properties of the chitosan biocides.

Chapter 4 describes the synthesis of functional couplers with a cyclic carbonate moiety of amino acid methyl esters and glucosamine. Three new carbonate couplers have been prepared to compare their reactivity towards different amines, including less nucleophilic natural amines.

Chapter 5 discusses possibilities to prepare conjugates from amino acid derivatives and different polymers. The range of amino acid derivatives is from simple amino acids, to tripeptides, an enzyme and elastin-like polymer (ELP). Applied polymers are PEG and modified polyacrylates, which were either activated previous to the coupling reaction or in situ using activated esters or carbonates.

Appendix A deals with the reaction of high-molecular weight chitosan and glycol chitosan with ethylene glycol and a quaternary ammonium coupler. Ethylene glycol displays a model for functional couplers and the quaternary ammonium coupler was used to introduce cationic groups. However, the aimed products were not obtained due to low nucleophilicity of the chitosans.

Appendix B covers the preparation of functional couplers in addition to chapter 4 with amino acids and peptides from silk fibroin. Moreover reaction with aliphatic amines and jeffamines will be presented.

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Chapter 2 Synthesis of chitosan surfactants

2.1 Introduction

Chitosan is a polysaccharide, composed of β–[1-4] linked glucosamine and N-acetyl glucosamine units and is derived from chitin, the second most abundant polysaccharide in nature. The main sources for this biopolymer are shrimps and shells of crabs which are deacetylated with sodium hydroxide up to a degree of 95 %. Alternatively chitosan can be obtained through fermentation of chitosan-containing fungi (e.g. Agaricus bisporus),[1] but up to now this method is only rarely applied.

The term chitosan is related to a heterogeneous group of macromolecules, which is characterized by its molecular weight and its degree of deacetylation (DD). The DD is the average molar ratio of D-glucosamine units within the macromolecular chain.

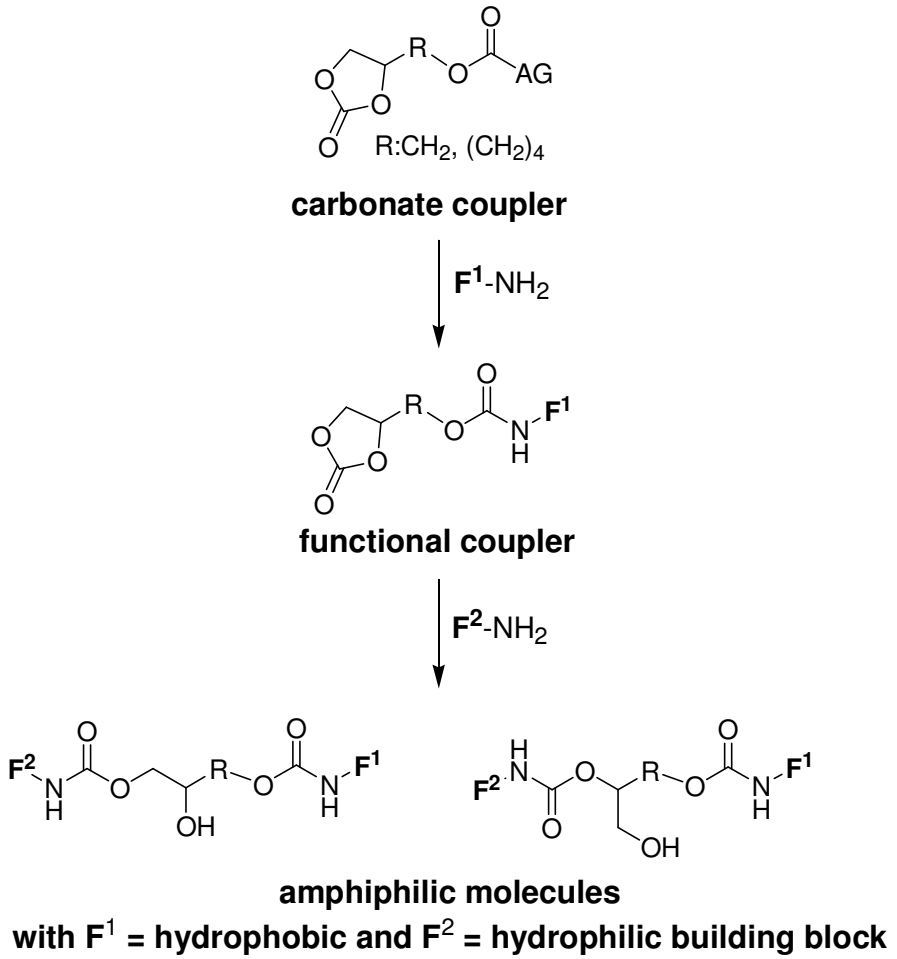
Chitosan is an interesting candidate for applications in the field of medicine and life science, due to its special biological and physical properties. It is a biocompatible material with a lethal dose as high as 16g/kg in mice. Furthermore it is biodegradable and promotes wound healing, shows hemostatic activity, immune enhancement and antimicrobial activity.[2] In neutral and acidic conditions chitosan is a polycation with a pK_a value of 6.0.[3]
Because of its versatile properties, chitosan has found industrial application. It is used in food processing[4, 5], pharmaceutical industry[6], personal care[7] and textile industry. [8]

Nevertheless applications are limited due to the poor solubility of chitosan at alkaline and neutral pH and in common solvents. The solubility is highly affected by the molecular weight and the DD. Water-soluble products up to a pH of 9 are obtained with a DD ~ 50 %. On the other hand low-molecular weight chitosan up to a DD of 50 % is soluble at any pH.[9]

In order to solve the solubility problem, chemical modification of chitosan has been studied. For this purpose the free amino group was quaternised with methyl iodide or grafted with glycidyltrimethylammonium chloride (GTMAC) to get water-soluble products.[10, 11] The same was achieved through grafting of either poly(ethylene glycol) chains or oligosaccharide fragments to the amino function.[12, 13] Carboxymethylation, either at C-6 hydroxy group with monochloroacetic acid or selective N-carboxymethylation with glyoxylic acid and $NaBH_3CN$ by reductive alkylation results in derivatives that are soluble in aqueous alkali.[14, 15]

Our aim was to prepare derivatives of chitosan, which have a cationic, thus ionic and hydrophobic side chains. Such amphiphilic structures are found in nature and exhibit important functionalities, like in host defense peptides (HDP) or biosurfactants.[16, 17] Beside these natural materials, synthetic polycations or associative polymers are a class of polymers that have found a widespread use in different applications. They are used in cosmetics as hair conditioner and washing components, as softening detergent, for waste-water treatment, as antimicrobial agents and are also discussed as gene delivery agents due to their interaction with the DNA.[18-24]

In the case of chitosan the introduction of quaternary ammonium salts leads to derivates with higher water solubility.[10, 25] Using the carbonate coupler approach quaternary ammonium groups were already successfully introduced into poly(ethylene imine) (PEI).[26] This means, that a carbonate coupler bearing a five-membered cyclic carbonate and a linear carbonate with an activating group (AG, see Scheme 4) is reacted in the first step with a primary amine leading to a functional coupler.



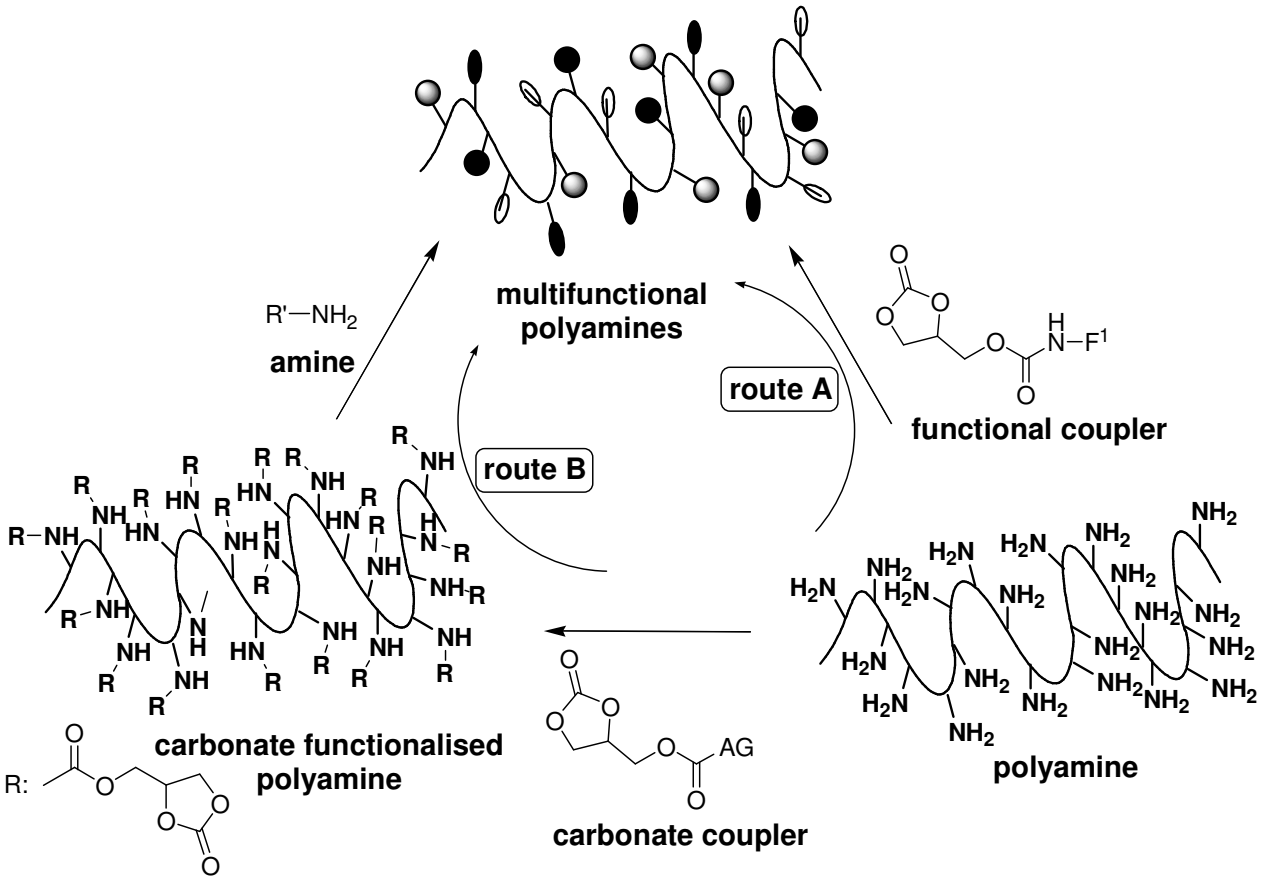
Scheme 4 Carbonate coupler approach (AG: activating group, e.g.: Cl, OPh, OPh-pNO_2) for the preparation of amphiphilic molecules

The linear carbonate moiety is more reactive towards amines than the ethylene carbonate and can be addressed selectively. In the second step another amine reacts with the cyclic carbonate moiety under ring-opening. In the case of PEI modification different functional couplers (F^1: hydrophobic or ionic moieties) were prepared and used for the functionalization of the primary amino groups of PEI (F^2: PEI).

This paper presents our results with respect to the modification of glycol chitosan (GC) and chitosan oligosaccharides (COS) using the carbonate coupler approach.

2.2 Results and Discussion

The carbonate coupler approach comprises two steps in which two amines, e.g. a ionic and a hydrophobic amine are selectively, consecutively combined within one molecule resulting in an amphiphilic molecule (Scheme 4). Alternatively hydrophilic, hydrophobic and ionic amines are attached to a polyamine resulting in a multifunctional polymer. The last approach can be performed in two alternative procedures: In the first the amines are linked to the carbonate coupler and than a mixture of functional couplers reacts with the polyamine (Scheme 5, route A),[26] in the second approach the coupler is first reacted with the polyamine and then with a mixture of hydrophobic and ionic amines (Scheme 5, route B).



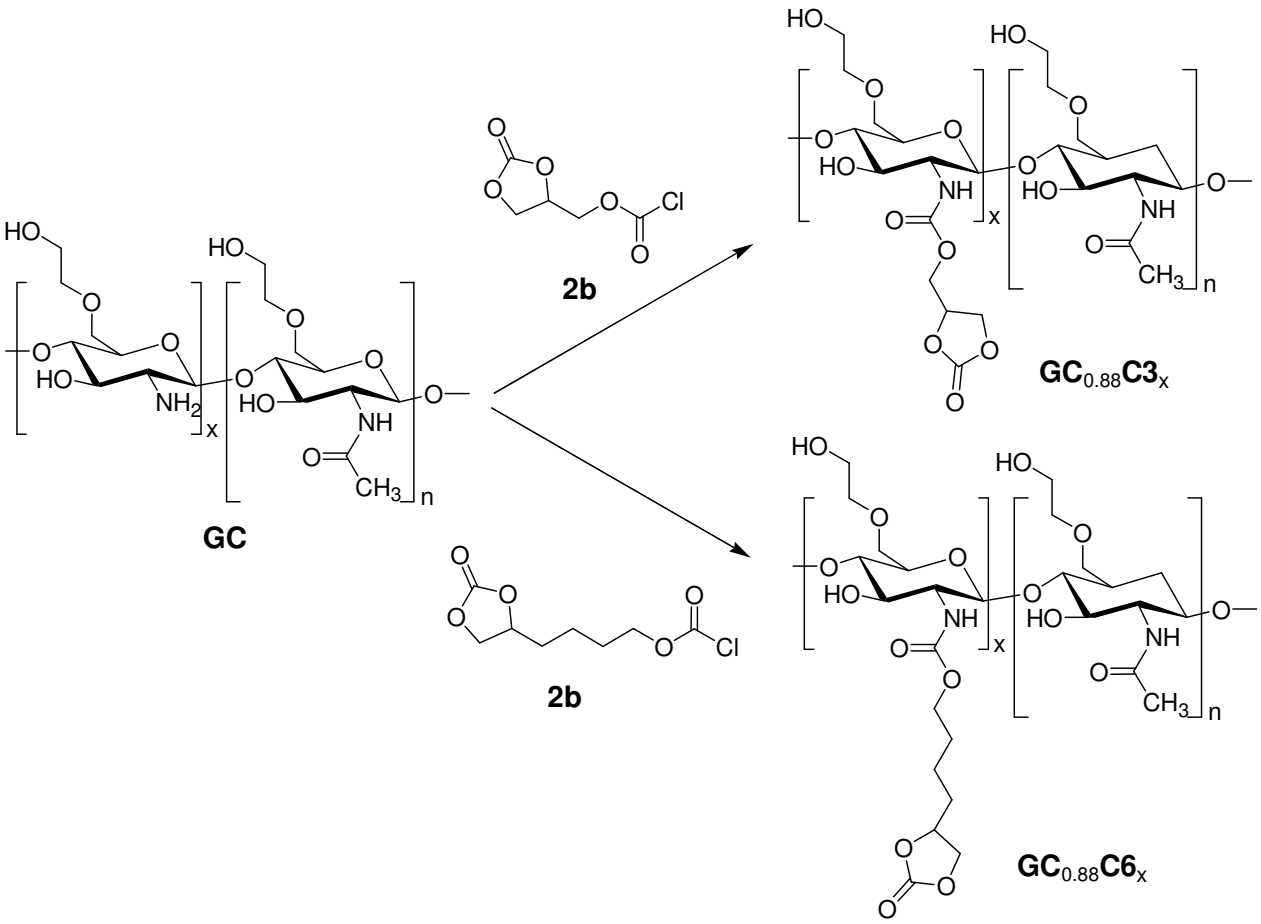
Scheme 5 Pathways for the synthesis of multifunctional polyamines via the carbonate coupler approach

Which of these procedures will be selected depends on the reactivity of the amines. In a previous paper we could show that glucosamine has a low reactivity towards the carbonate coupler.[27] Therefore the carbonate coupler approach was adapted to the low nucleophilicity of chitosan: In the first step free amino groups of chitosan are reacted with the carbonate coupler, having a chloroformate as activating group, to yield carbonate functionalised chitosan, which is further functionalised in the second step with appropriate amines. Since the reaction needs an alkaline medium, this approach was applied to glycol chitosan (GC) and chitosan oligosaccharides (COS). Both are chitosan derivatives with high solubility at any pH. Glycol chitosan (GC) is a commercially available chitosan derivative (poly (6-hydroxyethyl)glucosamine)) with a degree of polymerisation (DP) higher than 400 and a degree of deacetylation of 88 %, as determined by ^{1}H-NMR spectroscopy.[28] On the other hand, chitosan oligosaccharides (COS) are low-molecular weight derivatives with a DP around 15 and a DD of 84 %, which are obtained by chemical or enzyme catalyzed depolymerisation of chitosan.

First, the modification of glycol chitosan will be presented and afterwards the derivatisation of chitosan oligosaccharides.

2.2.1 Synthesis of N-(2-oxo-1,3-dioxolan-4-yl)alkyl carbamate glycol chitosan

According to the method of Amit[29] for the protection of amino groups in glucosamine, glycol chitosan **1** was reacted under Schotten-Baumann conditions with (2-oxo-1,3-dioxolan-4-yl)methyl chloroformate **2a**, hence named as C3 linker or with 4-(2-oxo-1,3-dioxolan-4-yl)butyl chloroformate **2b**, hence named as C6 linker (see Scheme 6). During addition of the glycol chitosan to the chloroformate solution, the chitosan derivative became insoluble in water and a suspension was formed.



Scheme 6 Synthesis of carbonate functionalized glycol chitosan with C3 **2a** or C6 **2b** linker, respectively

The products of these reactions could not be analyzed via NMR spectroscopy due to the insolubility of the products, thus the degree of functionalization could not be determined. As a consequence in the name of these compounds x denotes this unavailable (missing) information: $GC_{0.88}C_{3x}$ and $GC_{0.88}C_{6x}$. Whether this insolubility is due to cross-linking or due to an amphiphilic character of the biopolymer is unclear at this stage. However, analysis via IR spectroscopy clearly reveals the structural modification of the polymer: Signals for the cyclic carbonate group can be detected. Cross linking might occur if both reactive groups of the coupler react either with two amino groups or with one amino group and one alcohol group. The IR spectrum (Figure 1) clearly shows absorption signals for the cyclic carbonate (**3a**: 1801 cm^{-1}; **3b**: 1795 cm^{-1}) and no absorption bands of linear carbonates. Furthermore, the

two signals for carbamate groups belonging to the amid I and II vibration can be detected (**3a**: 1705 cm^{-1} and 1552 cm^{-1}; **3b**: 1697 cm^{-1} and 1552 cm^{-1}).

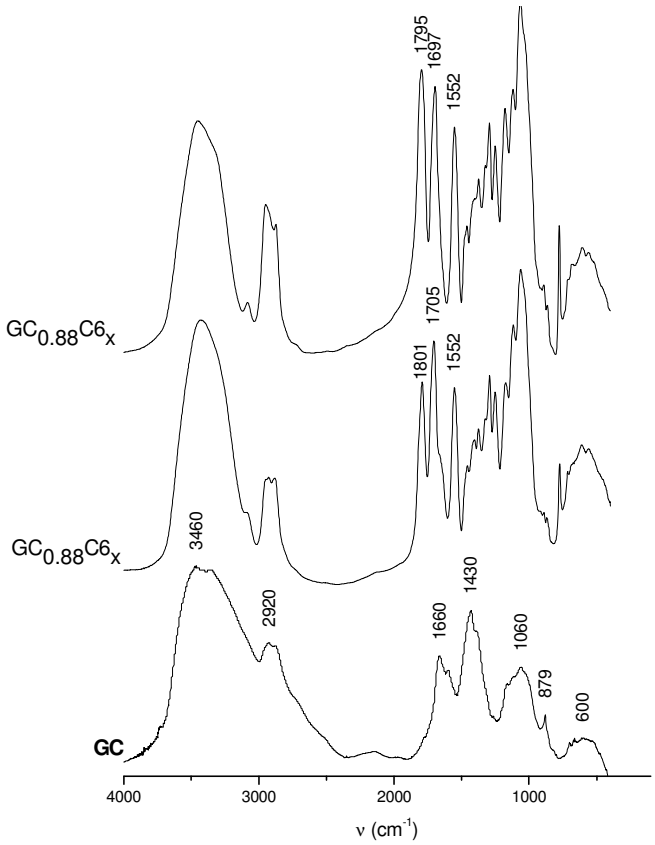
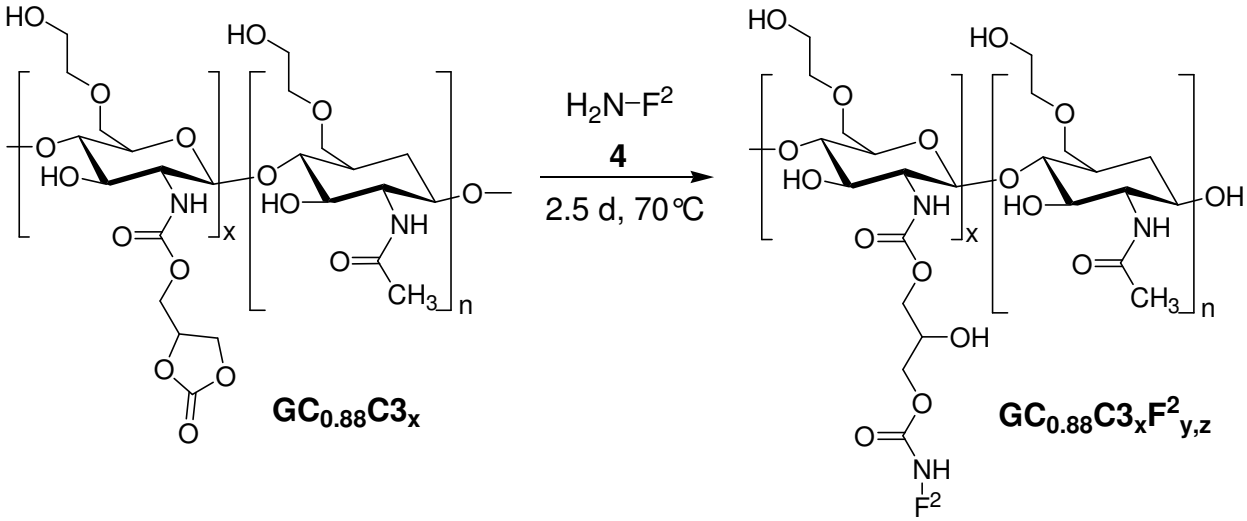


Figure 1 FT-IR spectra of glycol chitosan (**GC**) and of carbonate functionalized glycol chitosan

The absence of the bands for linear carbonate groups proofs that alcohol groups do not react with ethylene carbonate under the reaction conditions. Since it cannot be excluded that a second amine group reacts with the ethylene carbonate unit of the carbonate functionalised glycol chitosan producing a cross linked product a model experiment was performed, in which glycol chitosan was treated with a functional carbonate coupler. In this reaction no conversion was observed.

2.2.2 Reaction of amines with cyclic carbonate glycol chitosan

The second step in the preparation of multifunctional chitosan is the addition of amines to the cyclic carbonate with formation of a urethane and a hydroxyl group in α-position (Scheme 7):



Scheme 7 Reaction of carbonate functionalized glycol chitosan with amine **4**, e.g. DMAPA or 1-dodecylamine; the same procedure was applied for $\mathbf{GC_{0.88}C6_{0.88}}$

Functionalised glycol chitosans were obtained either starting from carbonate functionalised chitosan $\mathbf{GC_{0.88}C3_x}$ or $\mathbf{GC_{0.88}C6_x}$ by treatment with one amine or a mixture of two amines or were prepared in a one-pot-two-step reaction starting from glycol chitosan which was consecutively treated with the carbonate coupler **2a** or **2b** and the amine.

According to the last procedure product $\mathbf{GC_{0.88}C6_xQIp_x}$ was obtained by adding DMAPA to the mixture of carbonate functionalised glycol chitosan $\mathbf{GC_{0.88}C6_x}$, $NaHCO_3$, 1-4-dioxane and H_2O. This mixture was stirred at 60 °C for 3 days; the product $\mathbf{GC_{0.88}C6_xQIp_x}$ was then purified by dialysis after extraction of soluble components with chloroform. This modification did not enhance the solubility of the derivatives, thus characterization was restricted to analysis via IR spectroscopy, too. The absorption band of the cyclic carbonate disappears and only the amide I and amide II vibrations of the carbamate are still present.

In order to proof that the disappearance of characteristic bands of the cyclic carbonate are a consequence of the nucleophilic addition of the amine and not of hydrolysis two experiments were performed and the IR spectra of the products were compared with the product obtained in aqueous suspension (see Figure 2). In the first experiment the aqueous suspension of carbonate functionalised glycol chitosan was heated without the amine and in the second experiment the reaction was performed in dry NMP. In all cases, with and without amine, the band of the cyclic carbonate disappeared. However, in the case of the reaction in aqueous suspension signals with low intensity (2700-2850 cm^{-1}) were present, which suggest the presence of the terminal dimethylaminopropyl group in the chitosan derivative **$GC_{0.88}C6_xQIp_x$**. In the IR spectrum of the product obtained by reaction in NMP, characteristic signals of the tertiary amino group are clearly detectable (see Figure 2, III):

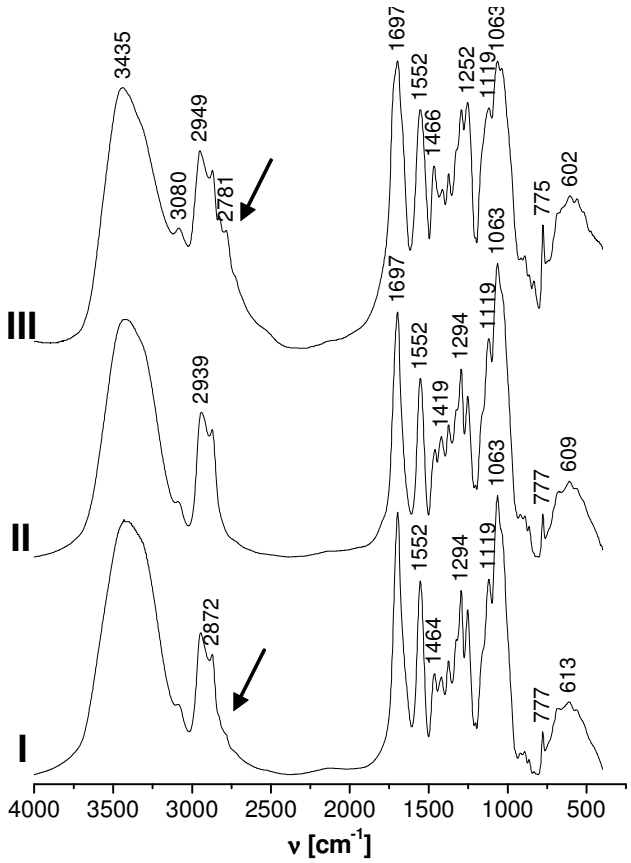


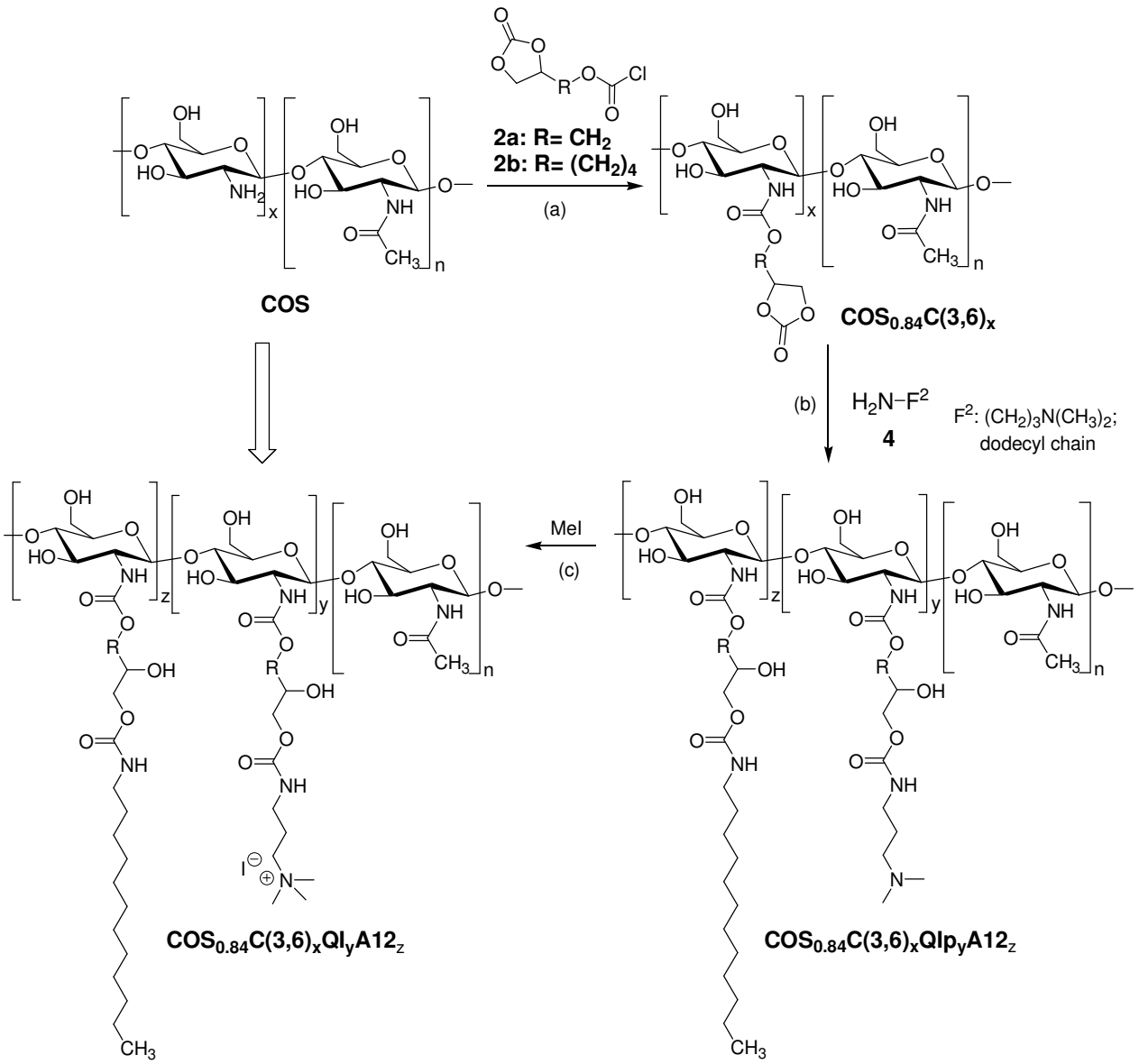
Figure 2 FT-IR spectra of glycol chitosan **$GC_{0.88}C6_xQIp_x$** after nucleophilic addition of DMAPA; I: in aqueous suspension, II without DMAPA in aqueous suspension, III: in dry NMP

At a wave number of 2781 cm^{-1} the band of the CH_2-group adjacent to the tertiary amino group appears. The same signals were observed in the IR spectrum of the product **$GC_{0.88}C6_xQIp_{0.7x}A12_{0.3x}$**, synthesised in dry NMP. The low intensity in the spectrum of the **$GC_{0.88}C6_xQIp_x$**, obtained via reaction in aqueous suspension (I) might occur due to a lower concentration of the CH_2-$N(CH_3)_3$-group, which is caused by the simultaneous hydrolysis of the cyclic carbonate moiety in water.

To complete the modification of glycol chitosan, quaternisation with methyl iodide was performed in a heterogeneous reaction in NMP. The glycol chitosan derivative **$GC_{0.88}C6_xQIp_{0.7x}A12_{0.3x}$** was suspended in NMP at room temperature, an excess of methyl iodide was added and the mixture was stirred for 17 h. The product **$GC_{0.88}C6_xQI_{0.7x}A12_{0.3x}$** was treated with diethyl ether and washed after filtration to remove any impurities. Again, this modification did not enhance the solubility. Hence, analysis was again performed by IR spectroscopy. Even though no macroscopic effect concerning the solubility was observed, the IR spectrum showed the disappearance of the signal at 2781 cm^{-1}, which is an indicator for the tertiary amino group.

2.2.3 Synthesis of carbonate functionalised chitosan oligosaccharide

According to the carbonate coupler approach and the procedure developed for the modification of glycol chitosan, chitosan oligosaccharide (COS) was functionalised in a similar way using the C3 and the C6 linker **2a** and **2b** (see Scheme 8). Additionally, a more convenient method was established.



Scheme 8 Synthetic pathway for the preparation of chitosan surfactants from **COS**

At first we attempted a complete conversion of free amino groups of COS into the corresponding carbamate $\mathbf{COS_{0.84}C3_{0.84}}$ and $\mathbf{COS_{0.84}C6_{0.84}}$. Since the reaction was performed in aqueous medium on one hand hydrolysis of the chloroformate has to be considered, on the other hand the low reactivity of the amino group in COS must be taken into consideration. Having this in mind **COS** was reacted with the C3 linker **2a** in three different ratios and the efficiency of functionalisation was determined via ^{1}HNMR-spectroscopy (see Table 1).

Table 1 Evaluation of necessary amount of C3 chloroformate **2a** for complete conversion of glucosamine units in **COS**

Entry	Ratio C3:COS	Relative number of functional groups [%]		
		$-NH_2$	-NHC3	-NHAc
1	1.02 : 1	22	62	16
2	1.5 : 1	2	82	16
3	2 : 1	0	84	16

To assure a full conversion of the glucosamine unit into the carbonate functionalised unit, the chloroformates were applied in excess. Via ^{1}H-NMR spectroscopy the full conversion can easily be detected from the signal of the proton at C2, which shifts from $\delta = 2.90$ ppm into the multiplett region with $\delta > 3.40$ ppm (Figure 3).

Due to the purification via dialysis the preparation procedure is very time-consuming and low-molecular weight fractions get lost, so that only yields of 48 % were achieved. Another aspect is the hydrolysis of the carbonate unit, which has to be considered for the reaction in water. In the beginning the synthesis was performed as described for the modification of glycol chitosan: Water and 1,4-dioxane served as solvent and $NaHCO_3$ as acid scavenger. Later the synthesis was optimised and a mixture of water and acetonitrile with triethylamine as acid scavenger was used. Acetonitrile made it easier to cool the reaction because of its lower freezing temperature than 1,4-dioxane, but is still easy to remove by distillation. By using Et_3N instead of $NaHCO_3$, it was possible to isolate the product by precipitation which is an advantage compared to dialysis. With this purification method, low-molecular weight fractions also get lost and the product contains impurities of triethylamine hydrochloride. But the procedure is more time-saving and the yield is higher.

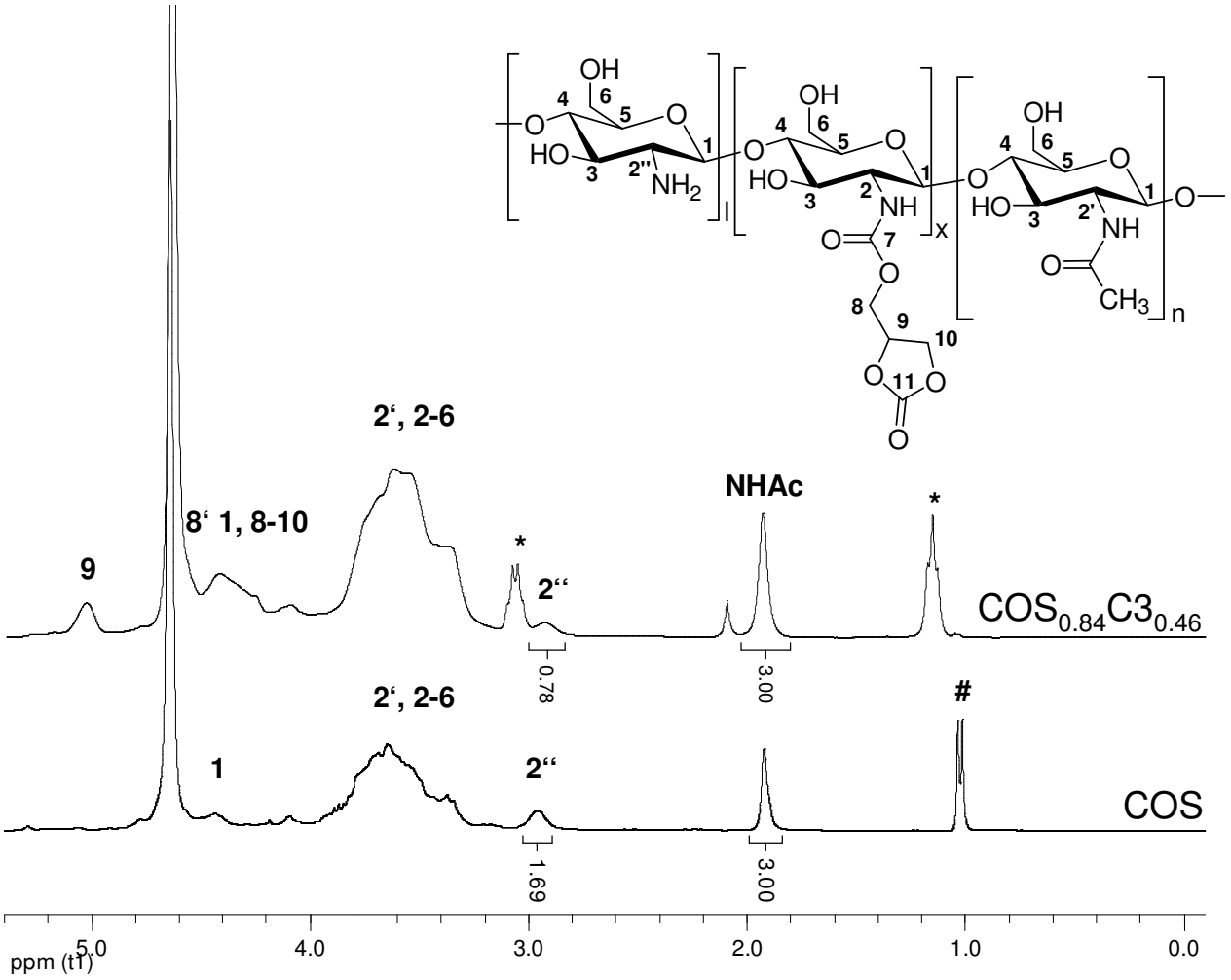


Figure 3 ^{1}H-NMR spectrum of C3 functionalized chitosan oligosaccharide **$COS_{0.84}C3_{0.46}$** with free amino group, compared to the starting material **COS** (spectrum below; # iso-propanol, * Et_3N hydrochloride))

It should be mentioned that the solubility of carbonate functionalised **COS** is dependent on the linker used: The product **$COS_{0.84}C6_{0.84}$** was found to be less soluble than the product **$COS_{0.84}C3_{0.84}$**.

If partially carbonate functionalisation of **COS** is wanted, in order to have free amino groups available for the attachment of i.e. active compounds, the share of free amino groups can be adjusted by the amount of added chloroformate linker.

Such products are of interest since nucleophilic groups are still available and further functionalisation can be performed. The degree of free amino groups can again easily be determined from ^{1}H-NMR spectroscopy (Figure 3): Therefore the ratio of the signal intensity

of the proton adjacent to the amino group **H-2''** ($I_{H2''}$, δ = 2.93 ppm) in the product and the starting material is determined.

The share of free amino groups (SAG) is calculated by the equation (1):

$$SAG = (I_{H2''}(product)/I_{H2''}(educt)) \times DD = (0.78/1.69) \times 84\ \% = 39\ \% \quad (1)$$

The SAG of the shown product is 39 %. Depending on the amount of chloroformate applied, products with SAG from 15 % to 50 % were achieved.

The NMR spectrum (see Figure 3) shows the signal of the remaining free amino groups (δ = 2.93 ppm), beside the typical signal of the cyclic carbonate (H-9) at δ = 5.04 ppm.

2.2.4 Preparation of multifunctional chitosan oligosaccharides

An amphiphilic structure was realised by introducing cationic groups which increase hydrophilicity and alkyl chains which are known to be hydrophobic. The cationic groups were introduced in two steps: First, a precursor polymer was prepared via nucleophilic addition of DMAPA to the carbonate functionalised **COS**, followed by quaternisation of the tertiary amino group with methyl iodide. For the hydrophobic side chains, an alkyl amine with a chain length of 12 carbon atoms (further termed as A12) was used. Different ratios of the ionic and hydrophobic units were realised, each for C3 and C6-functionalised **COS**. By this way different derivatives were obtained (Table 2).

Table 2 Multifunctional COS derivatives obtained via aminolysis of C3- and C6-functionalised $\mathbf{COS_{0.84}C(3,6)_x}$

QI:A12	C3	C6
1:0	$COS_{0.84}C3_{0.84}QI_{0.84}$	$COS_{0.84}C3_{0.84}QI_{0.84}$
4:1	$COS_{0.84}C3_{0.84}QI_{0.67}A12_{0.17}$	$COS_{0.84}C6_{0.84}QI_{0.67}A12_{0.17}$
3:2	$COS_{0.84}C3_{0.84}QI_{0.50}A12_{0.34}$	$COS_{0.84}C6_{0.84}QI_{0.50}A12_{0.34}$
1:1	$COS_{0.84}C3_{0.84}QI_{0.42}A12_{0.42}$	$COS_{0.84}C6_{0.84}QI_{0.42}A12_{0.42}$
	$COS_{0.84}C3_{0.46}QI_{0.23}A12_{0.23}$	$COS_{0.84}C6_{0.69}QI_{0.35}A12_{0.35}$
2:3	$COS_{0.84}C3_{0.84}QI_{0.34}A12_{0.50}$	$COS_{0.84}C6_{0.84}QI_{0.34}A12_{0.50}$
1:4	$COS_{0.84}C3_{0.84}QI_{0.17}A12_{0.67}$	$COS_{0.84}C6_{0.84}QI_{0.17}A12_{0.67}$

As mentioned before for the preparation of functionalised glycol chitosans, functionalised **COS** derivatives were obtained either starting from carbonate functionalised $\mathbf{COS_{0.84}C3_{0.84}}$ or $\mathbf{COS_{0.84}C6_{0.84}}$ by treatment with one amine or a mixture of two amines in aqueous solution or in dry NMP. It would be desirable to perform the reaction in an aqueous medium; however, in the presence of water hydrolysis of the cyclic carbonate might occur. In order to evaluate the efficiency of functionalisation versus hydrolysis $\mathbf{COS_{0.84}C3_{0.84}}$ was reacted with DMAPA**.** At first the one-pot-two-step approach, which means adding the amine to the reaction mixture of the carbonate functionalised **COS** and heating for 3d at 60 °C was performed. Alternatively the reaction was performed under water-free conditions at 70 °C and 2.5 d in NMP, in which starting materials and products were soluble. The efficiency of functionalisation was determined by comparing the ^{1}H-NMR spectra (see Figure 4).

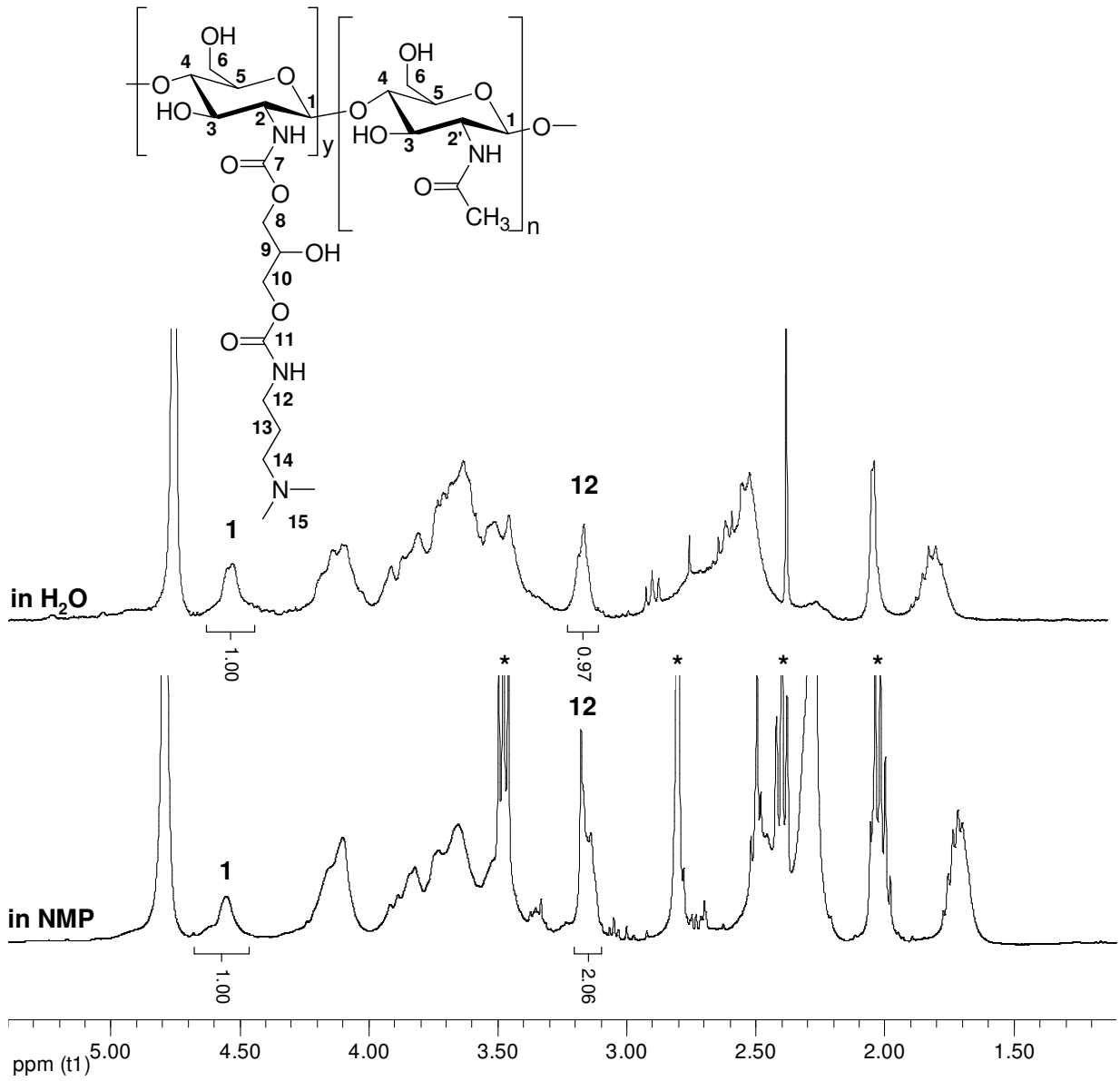


Figure 4 Comparison of reaction conditions by ^{1}H-NMR spectrum (* NMP) for the preparation of $\mathbf{COS_{0.84}C3_{0.84}QIp_{0.84}}$

The intensity of the peak of the methylene group 12, using the intensity of proton 1 as reference, showed that in NMP quantitative conversion is observed while in water only 50 % is obtained. The reason for this is that in water hydrolysis of the cyclic carbonate occurs as side reaction. To have high functionalisation efficiency, the following reactions were performed in NMP. Since NMP was also used as solvent in the following quaternisation step, no extra effort was made to remove all NMP.

The quaternisation was achieved by converting the tertiary amino group into the quaternary ammonium group by reaction with methyl iodide at room temperature over night, according to the direct quaternisation of chitosan.[10] The chitosan surfactants were isolated by precipitation in cold diethyl ether, filtration and subsequent washing with acetone to remove NMP residues. Figure 5 shows the structure of a product with a ratio of QI:A12 of 2:3. The ratio between the side chains (QI:A) was determined by the ratio of the amines in the feed and corresponds to the ratio of grafts in the copolymer as determined from ^{1}H-NMR spectroscopy. The ratio was determined from the signal intensity of H-16 to H-38 in the final product (see Figure 6, b).

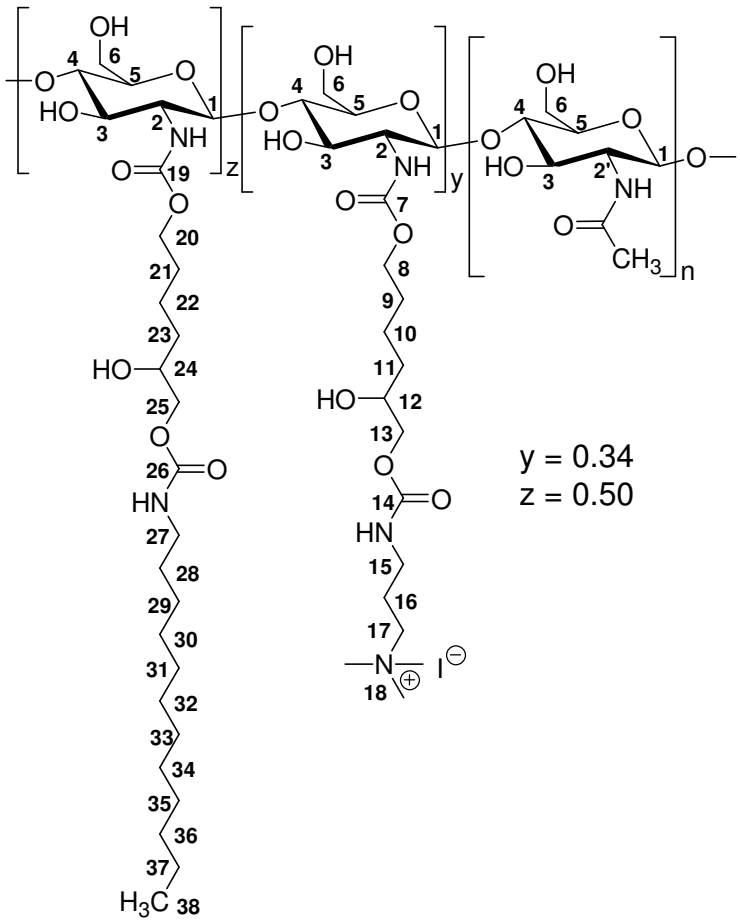


Figure 5 Structure of the chitosan surfactant $COS_{0.84}C6_{0.84}QI_{0.34}A12_{0.50}$

The chitosan surfactants were characterised by means of ^{1}H-NMR-spectroscopy and in some cases by IR spectroscopy. The chitosan surfactants are cream-white coloured powders, soluble in water and are surface active compounds due to their amphiphilic character.

The NMR spectrum (see Figure 6) of **$COS_{0.84}C6_{0.84}QI_{0.34}A12_{0.50}$** clearly reveals the insertion of the two grafts and the conversion of the tertiary amino groups (spectrum a) into the quaternary ammonium groups (spectrum b). The characteristic signal of the cyclic carbonate unit at $\delta = 5.05$ ppm (shown in Figure 3) disappeared. Signals of the chitosan backbone appear in the region from $\delta = 3.2$ to $\delta = 4.6$ ppm. Upon reaction with MeI the signals of the 3-dimethylaminopropyl group are shifted to lower field (see signal H-16, H-17, H-18).

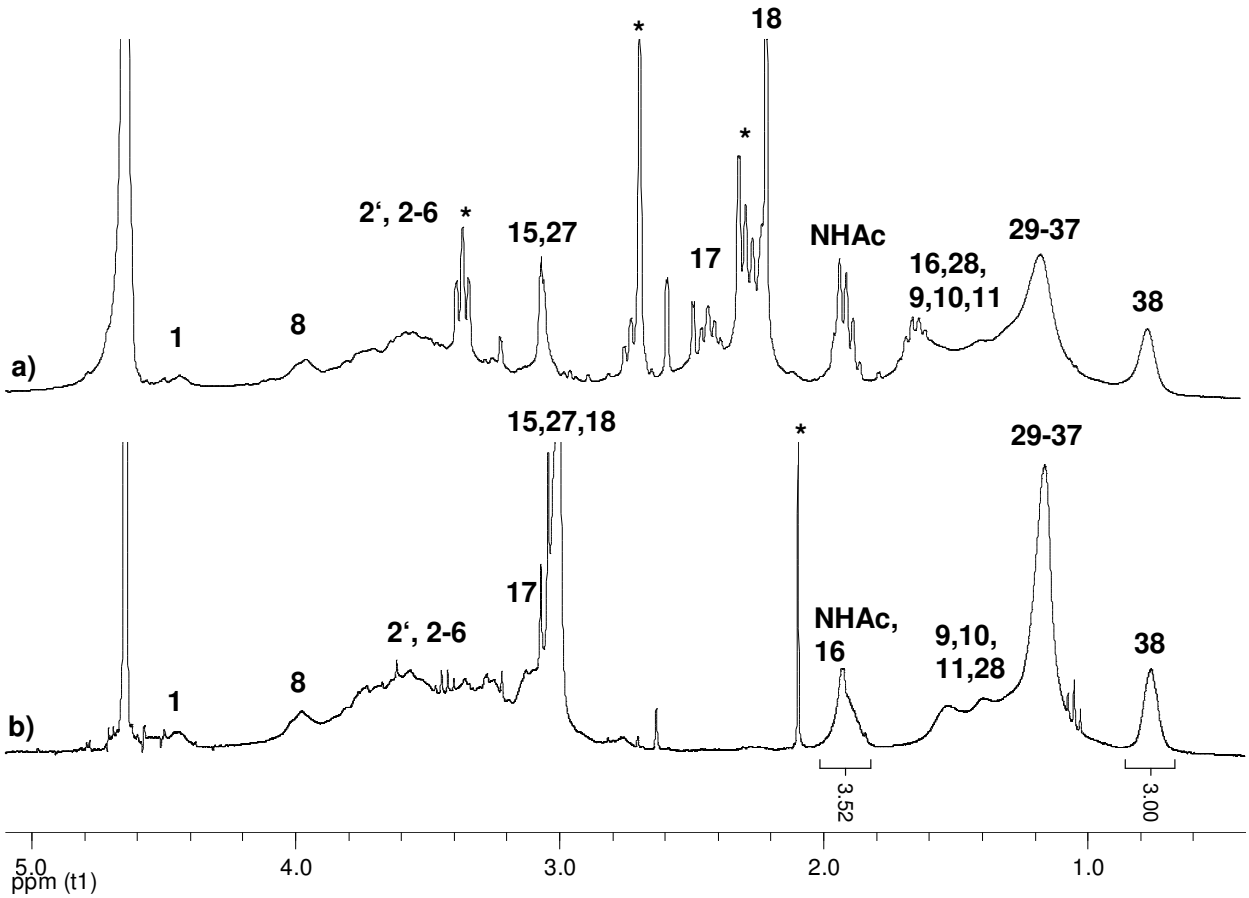


Figure 6 ^{1}H-NMR spectrum of chitosan surfactant **$COS_{0.84}C6_{0.84}QI_{0.34}A12_{0.50}$**, before (a) and after (b) quaternisation of the tertiary amino group (signal H-18)

It was already shown that a partial carbonate functionalisation is possible (see Figure 3). Two of these precursors, **$COS_{0.84}C3_{0.46}$** and **$COS_{0.84}C6_{0.69}$** have been used to prepare chitosan surfactants with a 1:1 ratio of QI:A12 and trimethyl ammonium groups attached to the backbone (QI*): **$COS_{0.84}C3_{0.46}QI_{0.23}A12_{0.23}QI^*_{0.39}$** and **$COS_{0.84}C6_{0.69}QI_{0.35}A12_{0.35}QI^*_{0.15}$**. Again, the quaternisation of the tertiary amino groups with methyl iodide was the last step. This means in the case of partially functionalised precursors, that the free amino groups would also react with methyl iodide. From the literature it is known that reaction of **COS** with methyl iodide leads to **COS** substituted with a mixture of trimethyl ammonium and dimethylamino groups.[30] In our experiment we found also the two groups in a ratio of 8.1 : 1 which confirms the results obtained in literature (see Figure 7).

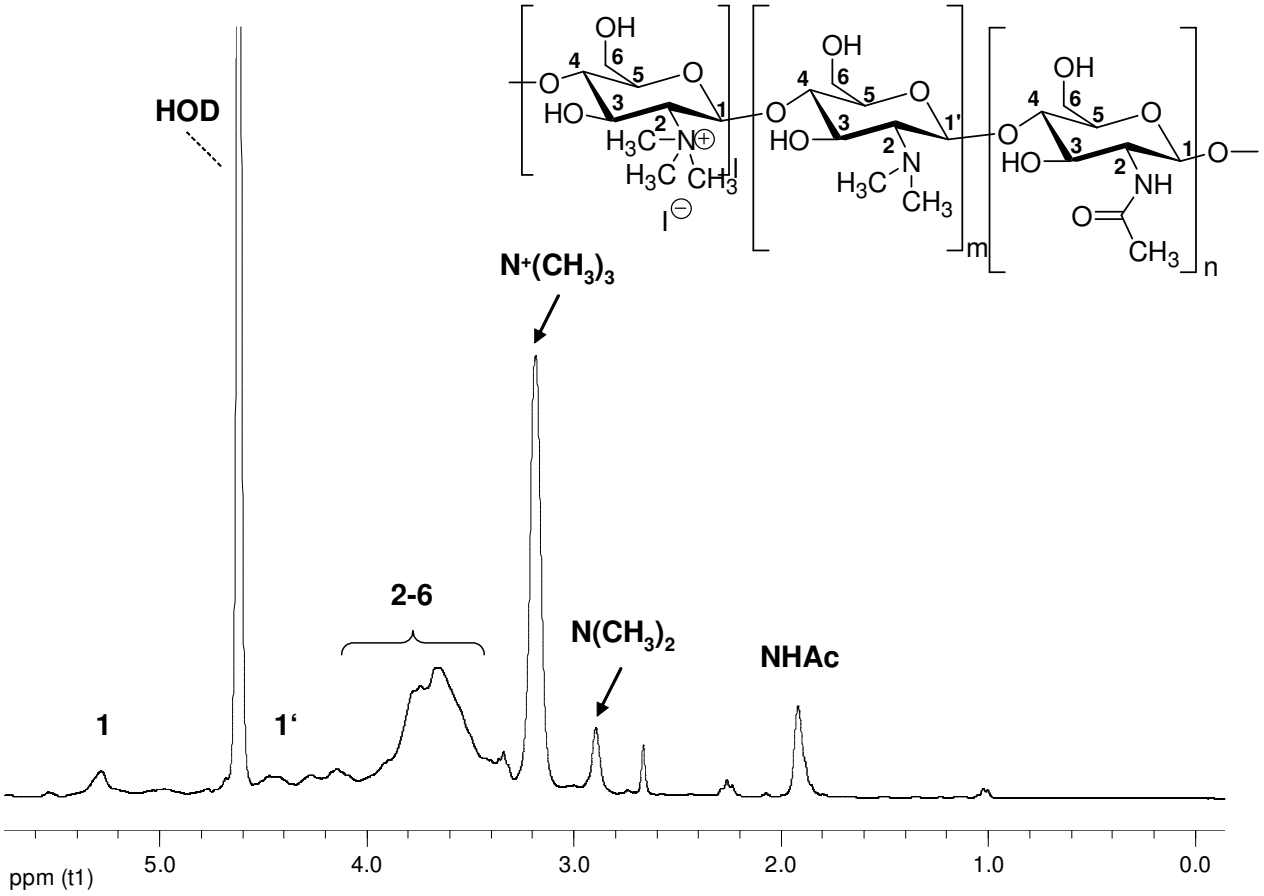


Figure 7 ^{1}H-NMR spectrum of methylated chitosan oligosaccharide **5**

If any further functionalisation at the free amino group is wanted, this reaction has to be done before the quaternisation step; either after reaction with the carbonate coupler, or after the nucleophilic addition of the amine.

2.2.5 Thermal and solution properties of selected chitosan surfactants

The influence of structural parameters on the thermal degradation and on surface tension has been investigated: Variable parameters were the length of the linker – C3 or C6 linker – and the ratio of hydrophobic and ionic grafts.

Thermogravimetric analysis (TGA) of COS and the derivatives based on the C3 linker $\mathbf{COS_{0.84}C3_{0.84}QI_yA12_z}$ performed under nitrogen are shown in Figure 8. The first weight loss is probably related to water or residual solvents and the second corresponds to the thermal degradation.[31] The maximum of the differential curve is T = 246.1 °C for COS and is in the range of 258.3 °C < 267.7 °C for the $\mathbf{COS_{0.84}C3_{0.84}QI_yA12_z}$. The highest value was observed for a ratio of 1:1 of ionic and A12 side chain ($\mathbf{COS_{0.84}C3_{0.84}QI_{0.42}A12_{0.42}}$).

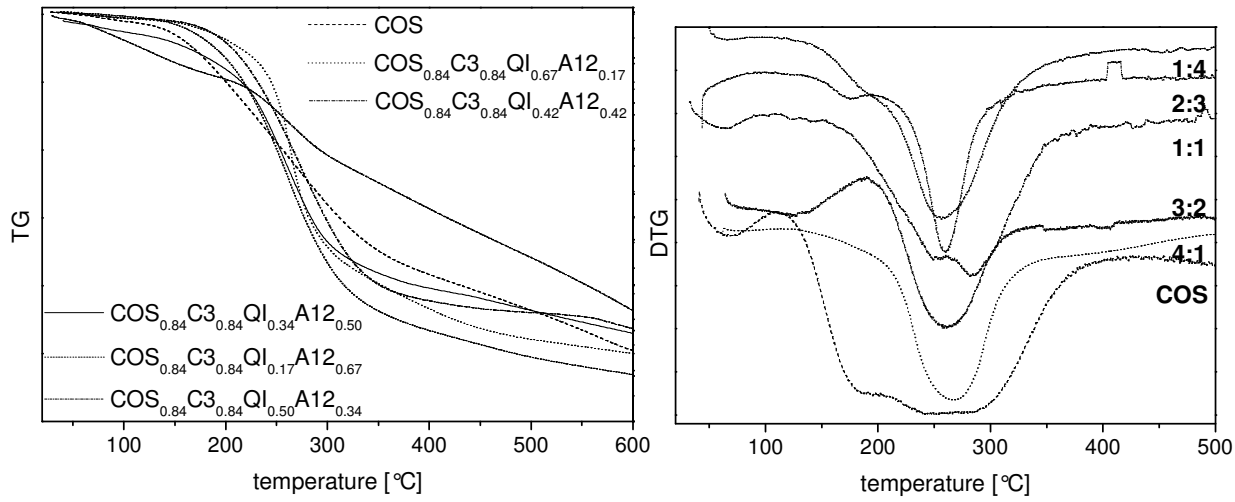


Figure 8 Thermogravimetric analysis of COS and COS-C3 derivatives $COS_{0.84}C3_{0.84}QI_yA12_z$

Concerning the C6-linker derivatives $\mathbf{COS_{0.84}C6_{0.84}QI_yA12_z}$, the sample with a ratio of 1:1 showed the highest temperature (T = 284.4 °C, $\mathbf{COS_{0.84}C6_{0.84}QI_{0.42}A12_{0.42}}$), too and the

thermal stability of all derivatives is similar to **COS** (see Figure 9). The temperature range of the C6 derivatives $\mathbf{COS_{0.84}C6_{0.84}QI_yA12_z}$ is between 251.7 °C < 284.4 °C.

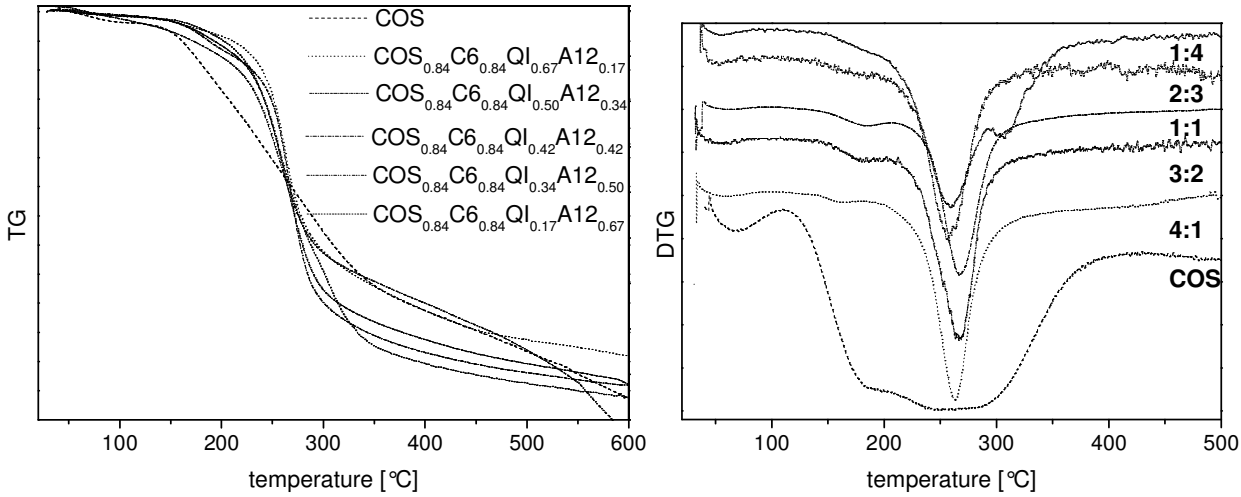


Figure 9 Thermogravimetric analysis of COS and COS-C6 derivatives $COS_{0.84}C6_{0.84}QI_yA12_z$

At first view, these results are surprising, because derivatisation of chitosan often causes a lower thermal stability of the resulting compounds.[31, 32] In the literature this decrease is explained with the disruption of the crystalline structure by the incorporation of flexible units into the rigid polysaccharide chain or the easy thermal degradation of the side chains. From our results on the thermal stability of **COS** we observe a decrease in the stability of the oligomers compared to high molecular weight chitosan (M_w = 70000): The maximum degradation of **COS** is T = 246.1 °C, while that of high molecular weight chitosan is T = 304 °C. This corresponds to the lower crystallinity of **COS**. We must assume that the stability of the grafts is similar to that of **COS**.

The solution properties of the $\mathbf{COS_{0.84}C6_{0.84}QI_yA12_z}$ derivatives were determined by measuring the surface tension at different concentrations using a *Wilhelmy* balance. The determination of the cmc value from the plot of the surface tension vs. concentration is exemplarily shown in Figure 10 a.

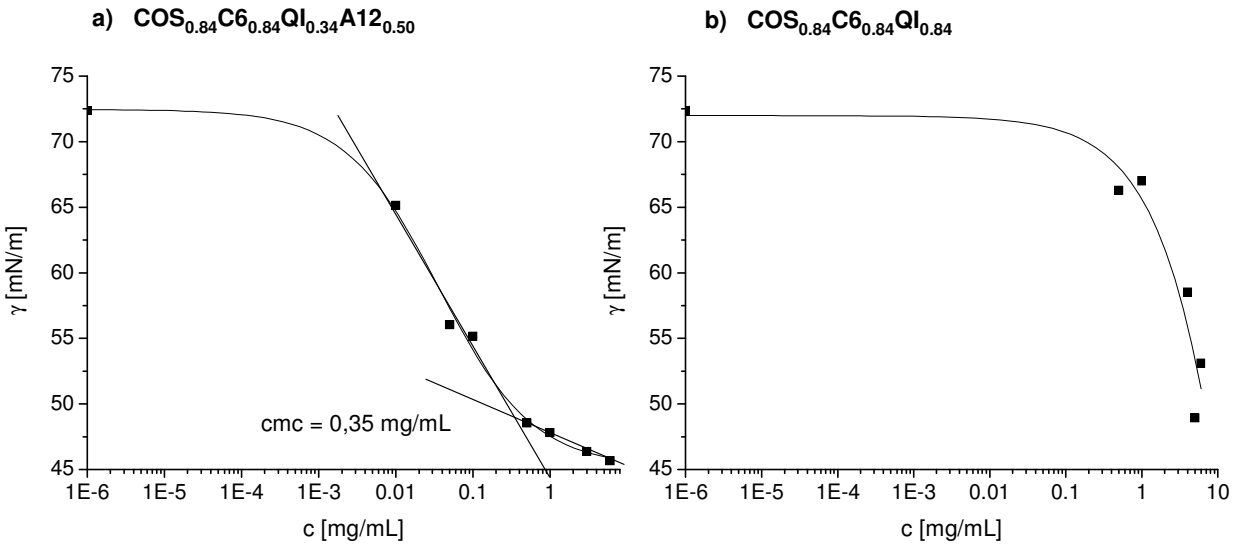
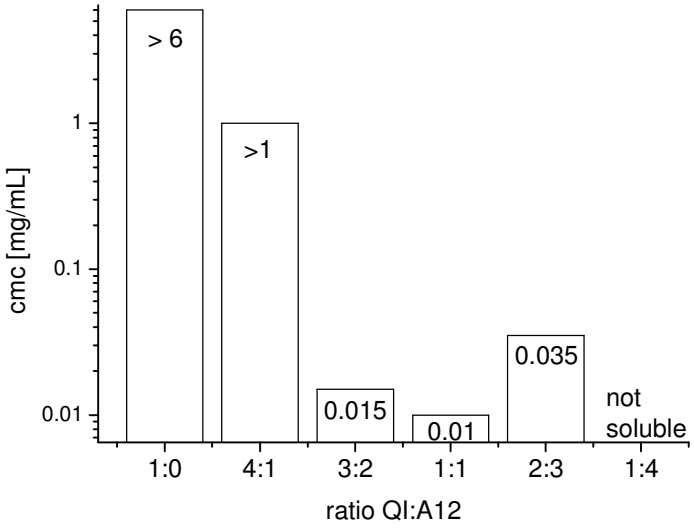


Figure 10 Surface tension vs. log concentration plot

The values for the other samples were determined in the same manner. The results obtained for the corresponding structures are summarized in Figure 11.



composition	QI:A12
$COS_{0.84}C6_{0.84}QI_{0.84}$	1:0
$COS_{0.84}C6_{0.84}QI_{0.67}A12_{0.17}$	4:1
$COS_{0.84}C6_{0.84}QI_{0.50}A12_{0.34}$	3:2
$COS_{0.84}C6_{0.84}QI_{0.42}A12_{0.42}$	1:1
$COS_{0.84}C6_{0.84}QI_{0.34}A12_{0.50}$	2:3
$COS_{0.84}C6_{0.84}QI_{0.17}A12_{0.67}$	1:4

Figure 11 Critical micelle concentration of $COS_{0.84}C6_{0.84}QI_yA12_z$

For the first sample (entry 1) with only ionic groups (no hydrophobic grafts) a distinct critical micelle concentration (cmc) could not be determined. Even at a concentration of 6 mg/mL no constant value of surface tension was observed and the measurement hence was stopped (see Figure 10 b). Only beginning with a ratio of the hydrophilic to the hydrophobic grafts of QI:A12 = 3:2 an influence on the aggregation behaviour of the samples was

observed. With decreasing QI:A12 ratio the surface tension decreases at lower sample concentrations. A four times higher molar amount of A12 than QI grafts results in a water-insoluble compound.

2.3 Conclusion

The carbonate coupler approach is a useful tool to prepare multifunctional polyamines.[26] This strategy was modified and successfully transferred to the natural polyamine chitosan. Two different starting materials which are soluble under alkaline conditions have been used for modification. In the case of glycol chitosan water-insoluble carbonate-functionalised products were obtained. IR spectroscopy clearly revealed the coupling of the cyclic carbonate moiety to the amino groups. It was possible to perform the nucleophilic addition of functional amines in a heterogeneous reaction; the same applies for the quaternisation reaction. Again via IR spectroscopy it was proven that the conversion of the tertiary amino groups into the quaternary ammonium groups was successful. At this point it should be mentioned that the resulting suspension of amphiphilic glycol chitosans fulfil prerequisites to be used in cosmetic formulations. Preliminary tests are on the way.

Starting with **COS,** mainly water-soluble functionalised products were obtained. These compounds vary in their degree of functionalisation and the ratio of hydrophilic to hydrophobic groups. These amphiphilic **COS** derivatives were examined, concerning their thermal stability and their solution properties. All products, independent of their microstructure – ratio of hydrophilic to hydrophobic grafts - the applied linker – C3 or C6 linker, showed similar thermal stability comparable to the starting **COS**. Lowest cmc values were observed for $\mathbf{COS_{0.84}C6_{0.84}QI_yA12_z}$ samples with a balanced ratio of the two grafts. The compound with a ratio of hydrophilic to hydrophobic grafts (QI:A = 1:4) was found to be insoluble.

As will be shown in a forthcoming paper, derivatives of soluble chitosan show remarkable antimicrobial activity against *E.coli* and *B.subtilis* and are hemocompatible.[33]

2.4 Experimental Part

2.4.1 Materials

Glycol Chitosan (**1**, *Aldrich*, DP ≥ 400), degree of deacetylation (DD) 88 % (determined by ^{1}H-NMR in D_2O),[28] chitosan oligosaccharide (**3**, DP~12, DD ~85 %, *Heppe medical chitosan*), (2-oxo-1,3-dioxolan-4-yl)methyl chloroformate (C3 linker, **2a**, 90%, *Aldrich*), $NaHCO_3$ (*KMF*), 3-dimethylamino-1-propylamine (DMAPA, *Aldrich*), methyl iodide (*Fluka*), N-methyl-pyrrolidone (NMP, *Fluka)* and 1-dodecylamine (*Acros Organics*) were used as received. In addition (2-oxo-1,3-dioxolan-4-yl)methyl chloroformate (C3 linker, **2a**) and 4-(2-oxo-1,3-dioxolan-4-yl)butyl chloroformate (C6 linker, **2b**) were prepared according to a general procedure of chloroformate synthesis.[34]

Dialysis was performed with Servapor dialysis tube (MWCO 12000-14000), obtained from SERVA Electrophoresis or in a SpectraPor dialysis membrane (MWCO 1000) from Spectrum Laboratories.

2.4.2 Instruments

FTIR spectra were recorded in KBr pellets on a Nicolet FT-IR spectrophotometer Nexus 470. NMR Spectra were recorded on a Varian VXR 300 or a Bruker DPX-300 FT-NMR spectrometer at 300 MHz and 75 MHz, respectively. Deuteriumoxide (D_2O) from Aldrich (99.9 %) was used as solvent. Thermogravimetric analyses (TGA) were performed on a TG 209 with a TA System Controller TASC 414/2 from Netzsch. The measurements were performed in nitrogen atmosphere with a heating rate of 10 K/min. Surface tensions were determined at room temperature according to the Wilhelmy-plate method on a Krüss

tensiometer and experiments were duplicated. All solutions were freshly prepared using seral water (resistivity=0.055 µS/cm). The critical micelle concentrations were estimated from the plot of the surface tension vs. log concentration.

2.4.3 Name of prepared products:

Products are named as described in the following: The first unit gives the applied chitosan derivative, which is either glycol chitosan (GC) or chitosan oligosaccharides (COS) and the index shows the DD. The next unit assigns the used linker (C3 or C6) and the index (x) the level of functionalisation based on the total amount of amino groups. The third and fourth indices (y,z) specify the ratio of the applied amines: QIp always presents the ionic precursor block, deriving from DMAPA, QI the ionic block with quaternary ammonium group and A12 presents the hydrophobic block with indication of the length of the carbon chain. QI* stands for a quaternary group at the chitosan backbone, deriving from the reaction of the primary amino group with methyl iodide. E.g.: $COS_{0.84}C3_{0.84}QI_{0.42}A12_{0.42}$ stands for the functionalisation of COS with C3 and nucleophilic addition with DMAPA and 1-dodecylamine in a ratio of 1:1 and subsequent quaternisation of the tertiary amino group.

2.4.4 Syntheses

The calculation of molar amount of chitosan derivatives is based on the molecular weight of the repeating unit.

N-(2-oxo-1,3-dioxolan-4-yl)methyl carbamate glycol chitosan ($GC_{0.88}C3_x$):

Chloroformate **2a** (276 mg, 1.38 mmol) was dissolved in 1,4-dioxane (1 mL). Glycol chitosan **1** (150 mg, 0.731 mmol) and $NaHCO_3$ (290.9 mg, 3.46 mmol) were dissolved in H_2O (4.2 mL) and 1,4-dioxane (1 mL) and drop wise added to the cooled chloroformate solution. The turbid mixture was stirred at room temperature for 16 h, extracted with $CHCl_3$ and either

used directly for further reactions or was purified via dialysis against water. The suspension was lyophilized to yield a cotton-like solid. Yield: not determined.

FTIR (KBr): 1797.2 (s, cycl. carbonate), 1697.5, 1551.8 (urethane), 1060.0 (glycoside) cm^{-1}.

N-(2-oxo-1,3-dioxolan-4-yl)butyl carbamate glycol chitosan ($GC_{0.88}C6_x$):

Chloroformate **2b** (339.1 mg, 1.37 mmol) was dissolved in 1,4-dioxane (1 mL). Glycol chitosan **1** (150 mg, 0.731 mmol) and $NaHCO_3$ (290.8 mg, 3.46 mmol) were dissolved in H_2O (4.2 mL) and 1,4-dioxane (1 mL) and drop wise added to the cooled chloroformate solution. The mixture became solid and was left at room temperature for 16 h, than diluted with H_2O and extracted with $CHCl_3$. The suspension was either used directly for further reactions or purified by dialysis against water and lyophilized to yield a cotton-like solid. Yield: not determined.

FTIR (KBr): 1791.8 (s, cycl. carbonate), 1696.7, 1552.8 (urethane), 1063.9 (glycoside) cm^{-1}.

Reaction of carbonate functionalized glycol chitosan with DMAPA in H_2O ($GC_{0.88}C3_xQIp_x$):

DMAPA (430 µL, 3.45 mmol) was added to a suspension of $GC_{0.88}C3_x$ (half the volume as obtained before). After 4.5 d stirring at 60 °C, the excess amine was extracted with $CHCl_3$ and the suspension was purified by dialysis against water and lyophilized to yield a cotton-like solid. The solid was analyzed by FTIR spectroscopy. Yield: not determined.

FTIR (KBr): 2829.1, 2790.5 (w, CH_2-$N(CH_3)_2$), 1699.0, 1556.3 (urethane), 1062.6 (glycoside) cm^{-1}.

The same procedure was applied for the synthesis of $GC_{0.88}C6_xQIp_x$.

FTIR (KBr): 2832.9, 2786.7 (w, CH_2-$N(CH_3)_2$), 1697.1, 1552.4 (urethane), 1062.6 (glycoside) cm^{-1}.

Reaction of $GC_{0.88}C3_x$ with DMAPA and 1-dodecylamine in NMP ($GC_{0.88}C6_xQIp_{0.7x}A12_{0.3x}$):

C6-functionalised glycol chitosan **$GC_{0.88}C6_x$** (161.3 mg, 0.431 mmol) was suspended in NMP (4 mL) and a mixture of 1-dodecylamine (170 µL, 0.734 mmol) and DMAPA (215 µL, 1.725 mmol) was added stirred at 70 °C for 2.5 d. The product was treated with Et_2O, filtrated, washed with acetone and dried in vacuo. Yield: 180.4 mg, white solid.

FTIR (KBr): 2827.1, 2782.7 (w, CH_2-$N(CH_3)_2$), 1698.0, 1551.7 (urethane), 1064.5 (glycoside) cm^{-1}.

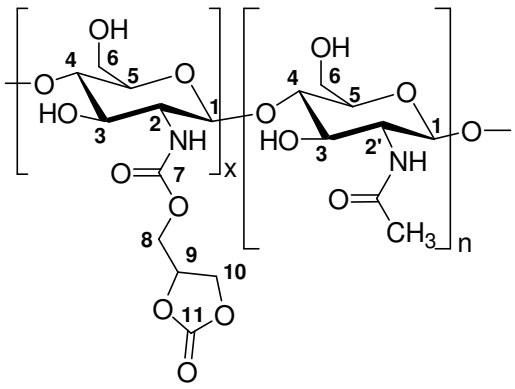
Quaternisation of functionalised glycol chitosan ($GC_{0.88}C6_xQI_{0.7x}A12_{0.3x}$):

Glycol chitosan **$GC_{0.88}C6_xQIp_{0.7x}A12_{0.3x}$** (171.5 mg, 0,353 mmol) was suspended in NMP (4 mL) and methyl iodide (115 µL, 1.85 mmol) was added and stirred at room temperature for 15 h. More methyl iodide (50 µL, 0.8 mmol) was added and after 17 h the product was treated with Et_2O, filtrated, washed with acetone and dried in vacuo. Yield: 185.4 mg, slightly yellow solid.

FTIR (KBr): 1698.5, 1542.7 (urethane), 1063.4 (glycoside) cm^{-1}.

N-(2-oxo-1,3-dioxolan-4-yl)methyl carbamate chitosan oligosaccharide (COS0.84C30.84, Method A):

Chitosan oligosaccharide **3** (1g, 5.97 mmol) was dissolved in H_2O (28 mL) and 1,4-dioxane (5 mL), $NaHCO_3$ (2.37 g, 28.3 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2a** (3.74 g, 18.6 mmol) in 1,4-dioxane (10 mL). The mixture was stirred at room temperature. After 16 h additional chloroformate **2a** (1.25 g, 6.22 mmol) was added. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. Inorganic salts were removed by dialysis in a cellulose tube with MWCO 1000 against water. The product was isolated by lyophilisation. Yield: 791 mg (46.0 %), white powder.



^{1}H-NMR (D_2O, 300 MHz):

δ = 1.97 (s, 3H, NAc), 3.40-3.85 (m, 7H, H-2, H-3, H-4, H-5, H-6), 4.14-4.47 (m, 6H, H-1, H-8, H-9, H-10), 4.64 (m, 1H, H-8'), 5.09 (m, 1H, H-9) ppm

FTIR (KBr): 1791.8 (cycl. Carbonate), 1726.1, 1549.0 (urethane), 1666.6 (N-acetyl), 1054.9 (glycoside) cm^{-1}.

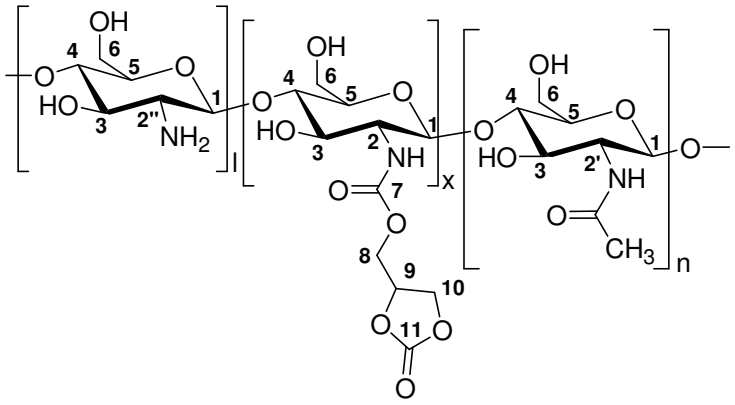
N-(2-oxo-1,3-dioxolan-4-yl)methyl carbamate chitosan oligosaccharide ($COS_{0.84}C3_{0.84}$, Method B):

Chitosan oligosaccharide **3** (3g, 17.9 mmol) was dissolved in H_2O (84 mL) and acetonitrile (15 mL), Et_3N (10.6 mL, 78.4 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2a** (11.22 g, 55.9 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature. After 16 h additional chloroformate **2a** (3.74 g, 18.6 mmol) was added. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. The product was isolated by precipitation in 2-propanol, filtrated and dried in vacuo. Yield: 3.07 g (59.2 %) white powder.

Synthesis of N-(2-oxo-1,3-dioxolan-4-yl)methyl carbamate chitosan oligosaccharide with degree of functionalisation < 100% ($COS_{0.84}C3_{0.46}$, Method C):

Chitosan oligosaccharide **3** (3g, 17.9 mmol) was dissolved in H_2O (84 mL) and Et_3N (2.7 mL, 19.5 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2a** (2.4 g, 12.0 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature. After 19 h the organic solvent was removed in vacuo and by-products by

extraction with chloroform. The product was isolated by precipitation in 2-propanol, filtration and dried in vacuo. Yield: 2.45 g



^{1}H-NMR (D_2O, 300 MHz):

δ = 1.93 (s, 3H, NAc), 2.93 (m, 1H, C*H*-NH_2) 3.38-3.85 (m, 7H, H-2, H-3, H-4, H-5, H-6), 4.14-4.47 (m, 6H, H-1, H-8, H-9, H-10), 4.64 (m, 1H, H-8'), 5.09 (m, 1H, H-9) ppm

The same procedure was applied to prepare **$COS_{0.84}C3_{0.50}$**. For details see Table 3.

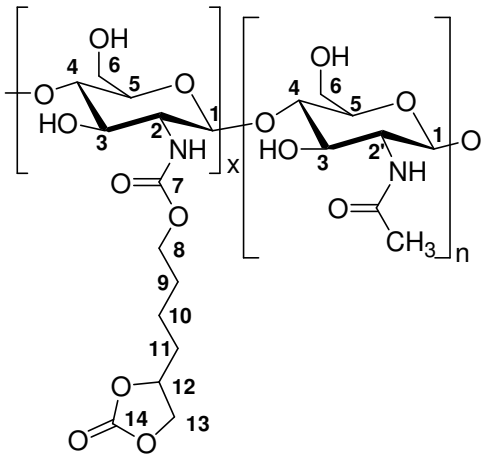
Table 3 N-(2-oxo-1,3-dioxolan-4-yl)methyl carbamate chitosan oligosaccharide with free amino groups

	Initial weight of C3 linker 3a	**Molar ratio C3:COS**	**Degree of functionalisation theoretical / achieved**	**Yield [%]**
$COS_{0.84}C3_{0.46}$	2.4 g, 12.0 mmol	0.64:1	67 % / 46 %	51.9
$COS_{0.84}C3_{0.50}$	2.05 g, 10.2 mmol	0.57:1	57 % / 50 %	53.2

N-(2-oxo-1,3-dioxolan-4-yl)butyl carbamate chitosan oligosaccharide ($COS_{0.84}C6_{0.84}$, Method A):

Chitosan oligosaccharide **3** (1g, 6 mmol) was dissolved in H_2O (28 mL) and 1,4 dioxane (5 mL), $NaHCO_3$ (1.85 g, 22 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2b** (3.07 g, 12.4 mmol) in 1,4 dioxane (10 mL). The mixture was stirred at room temperature. After 16 h additional chloroformate **2b** (1.5 g, 6.1 mmol) was

added. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. Inorganic salts were removed by dialysis in a cellulose tube with MWCO 1000 against water. The product was isolated by lyophilisation. Yield: 855 mg (44.3 %), white powder.



^{1}H-NMR (D_2O, 300 MHz):

δ = 1.38 (s, 2H, H-10), 1.59 (s, 2H, H-9), 1.70 (2H, H-11), 1.93 (s, 3H, NAc), 3.37-3.78 (m, 7H, H-2, H-3, H-4, H-5, H-6), 3.99 (s, 2H, H-8), 4.13 (m, 1H, H-13'), 4.45 (s, 1, H-1), 4.55 (m, 1H, H-13), 4.79 (m, 1H, H-12) ppm

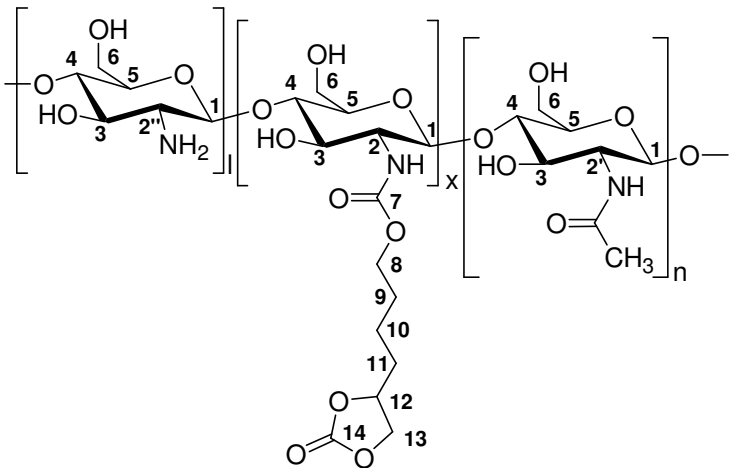
FTIR (KBr): 1785.8 (cycl. Carbonate), 1697.1, 1544.7 (urethane), 1066.5 (glycoside) cm^{-1}.

N-(2-oxo-1,3-dioxolan-4-yl)butyl carbamate chitosan oligosaccharide ($COS_{0.84}C6_{0.84}$, Method B):

Chitosan oligosaccharide **3** (3g, 17.9 mmol) was dissolved in H_2O (84 mL) and acetonitrile (15 mL), Et_3N (8 mL, 59.3 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2b** (9.24 g, 56 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature. After 16 h additional chloroformate **2b** (4.61 g, 18.6 mmol) was added. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. The product was isolated by precipitation in 2-propanol, filtrated and dried in vacuo. Yield: 2.25 g (38.6 %) white powder.

Synthesis of N-(2-oxo-1,3-dioxolan-4-yl)butyl carbamate chitosan oligosaccharide with degree of functionalisation < 100% ($COS_{0.84}C6_{0.70}$, Method C):

Chitosan oligosaccharide **3** (3g, 17.9 mmol) was dissolved in H_2O (84 mL) and Et_3N (2.7 mL, 19.5 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2b** (2.9 mg, 13.0 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. The product was isolated by precipitation in 2-propanol, filtrated and dried in vacuo. Yield: 2.40 g



^{1}H-NMR (D_2O, 300 MHz):

δ = 1.38 (s, 2H, H-10), 1.58 (s, 2H, H-9), 1.70 (2H, H-11), 1.93 (s, 3H, NAc), 2.98 (m, 1H, C*H*-NH_2), 3.35-3.76 (m, 7H, H-2, H-3, H-4, H-5, H-6), 3.99 (s, 2H, H-8), 4.13 (m, 1H, H-13'), 4.43 (s, 1, H-1), 4.55 (m, 1H, H-13), 4.80 (m, 1H, H-12) ppm

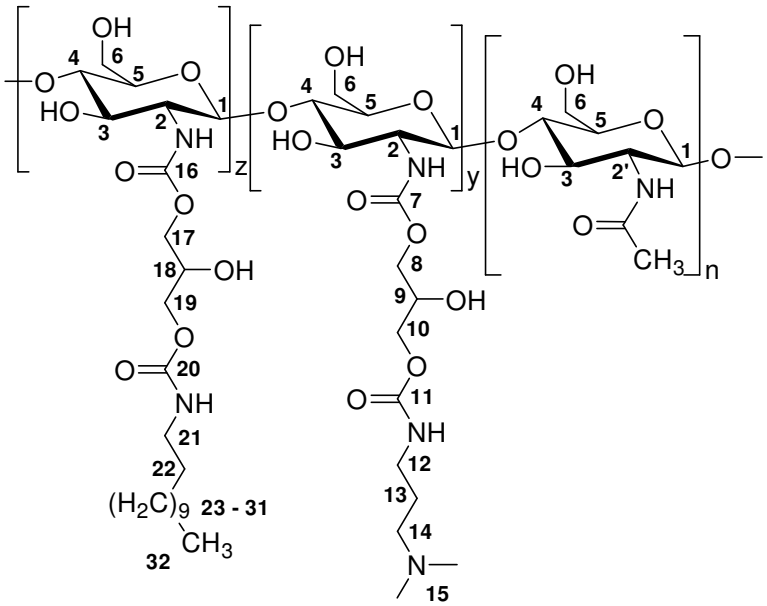
The same procedure was applied to prepare **$COS_{0.84}C6_{0.48}$**. For details see Table 4.

Table 4 N-(2-oxo-1,3-dioxolan-4-yl)butyl carbamate chitosan oligosaccharide with free amino groups

	Initial weight of C6 linker 2b	**Molar ratio C6:COS**	**Degree of functionalisation theoretical / achieved**	**Yield**
$COS_{0.84}C6_{0.70}$	2.9 g, 13.03 mmol	0.70:1	73 % / 70 %	44.2 %
$COS_{0.84}C6_{0.48}$	2.07 g, 9.30 mmol	0.52:1	52 % / 48 %	49.5 %

Reaction of carbonate-functionalised chitosan oligosaccharide with 1-dodecylamine and DMAPA ($COS_{0.84}C3_{0.84}QIp_{0.67}A12_{0.17}$):

The carbonate functionalised COS **$COS_{0.84}C3_{0.84}$** (400.0 mg, 1.38 mmol) was suspended in NMP (12 mL) and DMAPA (1.38 mL, 11.07 mmol) and 1-dodecylamine (642 µL, 2.77 mmol) were added. The reaction mixture was stirred at 70°C for 2.5 d. Upon heating all compounds dissolved. The product was isolated by precipitation in cooled Et_2O and used for the following step without further purification. Yield: 457.0 mg (85.0 %)



^{1}H-NMR (D_2O, 300 MHz):

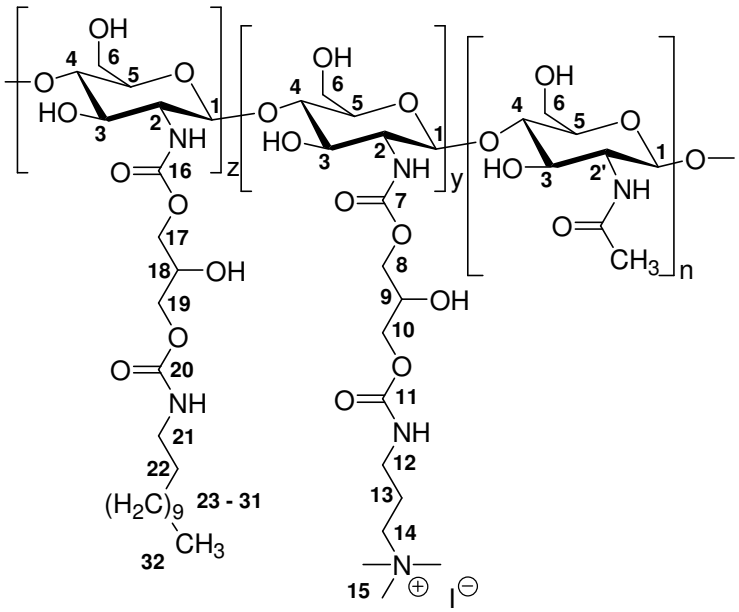
δ = 0.74 (m, 3H, H-32), 1.15 (m, 20H, H-22, H-23, H-24, H-25, H-26, H-27, H-28, H-29, H-30, H-31), 1.61 (m, 2H, H-13), 1.93 (s, 3H, NAc), 2.19 (s, 6H, H-15), 2.31-2.45 (m, 2H, H-14), 2.69 (m, 1H, H-2'), 3.06 (m, 4H, H-12, H-21), 3.36-4.00 (m, 11H, H-2, H-3, H-4, H-5, H-6, H-8, H-9, H-10), 4.43 (m, 1H, H-1) ppm.

The same procedure was applied to prepare other chitosan derivatives of the general composition **$COS_{0.84}C(3,6)_{0.84}QIp_yA12_z$** (see Table 5, step 1).

Quaternisation of multifunctional chitosan oligosaccharides ($COS_{0.84}C3_{0.84}QI_{0.67}A12_{0.17}$):

The multifunctional chitosan oligosaccharide **$COS_{0.84}C3_{0.84}QIp_{0.67}A12_{0.17}$** (429.2 mg, 1.1 mmol) was suspended in NMP (8.5 mL) and methyl iodide was added (430 µL, 6.9 mmol). Over night under protection of light a homogeneous solution was obtained at room

temperature. The product was isolated by precipitation in cooled Et_2O. The product was further purified by stirring in acetone p.a. at room temperature for 16 h, filtrated and dried in vacuo. Yield: 497.5 mg (93.0 %)



^{1}H-NMR (D_2O, 300 MHz):

δ = 0.79 (m, 3H, H-32), 1.20 (m, 20H, H-22, H-23, H-24, H-25, H-26, H-27, H-28, H-29, H-30, H-31), 1.98 (m, 5H, NHAc, H-13), 3.06 – 3.13 (m, 13H, H-12, H-15, H-21), 3.27-3.86 (m, 9H, H-2', H-2, H-3, H-4, H-5, H-6, H-14), 4.07 (m, 2H, H-8), 4.51 (m, 1H, H-1) ppm.

The same procedure was applied to prepare other chitosan surfactants (see Table 5, step 2).

Table 5 Overview of prepared COS surfactants: reaction of carbonate functionalized COS with amines (step 1) and quaternisation reaction (step 2)

	Product name	QI : A	Step 1				Step 2		
			Reactant mass [mg]			Yield (%)	Reactant mass [mg]		Yield (%)
			educt	QIp	A		educt	MeI	
1	$COS_{0.84}C3_{0.84}QI_{0.84}$	1:0	359[1)]	1270	--	100	444	433	75
2	$COS_{0.84}C6_{0.84}QI_{0.84}$	1:0	300[2)]	746	--	67	303	1254	57
3	$COS_{0.84}C3_{0.84}QI_{0.67}A12_{0.17}$	4:1	400[1)]	1132	513	85	429	978	93
4	$COS_{0.84}C3_{0.84}QI_{0.50}A12_{0.34}$	3:2	412[1)]	871	1040	79	380	992	59
5	$COS_{0.84}C3_{0.84}QI_{0.42}A12_{0.42}$	1:1	400[1)]	707	1283	65	355	182	70
6	$COS_{0.84}C3_{0.84}QI_{0.34}A12_{0.50}$	2:3	400[1)]	566	1540	74	380	866	67
7	$COS_{0.84}C3_{0.84}QI_{0.17}A12_{0.67}$	1:4	400[1)]	283	2053	67	362	825	80
8	$COS_{0.84}C6_{0.84}QI_{0.67}A12_{0.17}$	4:1	420[2)]	1059	480	100	445	1031	76
9	$COS_{0.84}C6_{0.84}QI_{0.50}A12_{0.34}$	3:2	314[2)]	458	563	76	240	513	54
10	$COS_{0.84}C6_{0.84}QI_{0.42}A12_{0.42}$	1:1	450[2)]	709	1286	66	355	809	80
11	$COS_{0.84}C6_{0.84}QI_{0.34}A12_{0.50}$	2:3	420[2)]	529	1440	78	426	971	67
12	$COS_{0.84}C6_{0.84}QI_{0.17}A12_{0.67}$	1:4	420[2)]	265	1921	51	303	68	43
13	$COS_{0.84}C3_{0.46}QI_{0.23}A14_{0.23}\ QI^*_{0.39}$	1:1	600[1)]	318	665	74	444	182	96
14	$COS_{0.84}C3_{0.46}QI_{0.23}A12_{0.23}\ QI^*_{0.39}$	1:1	600[1)]	318	577	61	345	137	86
15	$COS_{0.84}C3_{0.46}QI_{0.23}A8_{0.23}\ QI^*_{0.39}$	1:1	600[1)]	318	403	64	351	137	95
16	$COS_{0.84}C6_{0.70}QI_{0.35}A14_{0.35}\ QI^*_{0.15}$	1:1	600[2)]	378	790	41	203	68	79
17	$COS_{0.84}C6_{0.70}QI_{0.35}A12_{0.35}\ QI^*_{0.15}$	1:1	600[2)]	378	686	70	345	125	92
18	$COS_{0.84}C6_{0.70}QI_{0.35}A8_{0.35}\ QI^*_{0.15}$	1:1	600[2)]	378	478	84	436	137	96

[1)] $COS_{0.84}C3_{0.84}$ [2)] $COS_{0.84}C6_{0.84}$

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Chapter 3 Biocompatible chitosan biocides

3.1 Introduction

Infectious diseases become more and more an important issue. The development of bacterial resistance is increasing, whereas the development of new antibiotics is stagnating and not comparable with the gold rush years after the development of penicillin. Today, two resistant bacterial strains, methicillin-resistant *Staphyloccus aureus* (MRSA) and vancomycin-resitant *Enterococci* (VRE) are present in hospital and are a problem in the care of immunologically weak patients. Moreover, the occurrence of these pathogens has already been described occasionally in schools, restaurants and other public places.[1]

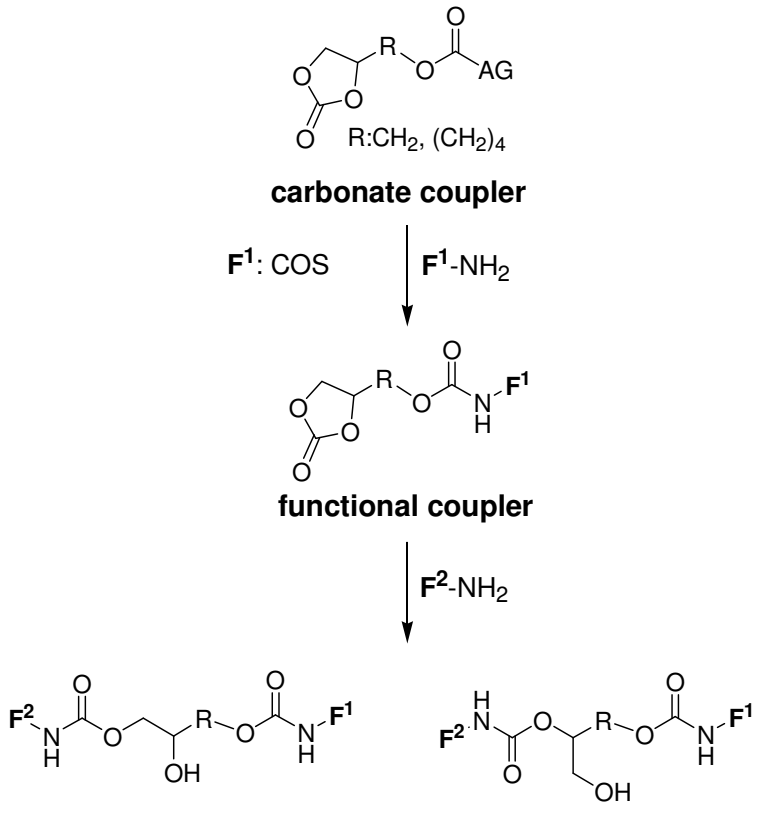
The targeted destruction of bacteria is one approach, the other is the prevention of bacterial growth, which is achieved by standard disinfection. Materials having an intrinsic ability to prevent biofilm formation are of special interest since they do not target a certain molecule, but exhibit a broad mode of action, thus preventing resistance development of bacteria.[2] Especially biocidal polymers, which mimic so-called “antimicrobial peptides” (AMP) or better said “host defense peptides” (HDP) are among such active compounds. Among HDPs a broad variety of primary and secondary structures were observed. However, their key similarity is their facially amphipathic structure with a cationic and a non-polar side, which effectively interacts with the negatively charged cell membrane of bacteria.[3] Scientist are trying to mimic such structures by inserting polar groups like quaternary ammonium, quaternary pyridinium, guanidinium, biguanide or phosphonium groups and hydrophobic groups such as aryl or alkyl segments.[4-9] Some of these polymeric biocides are impressively effective with minimal inhibitor concentrations (MIC) equal to 0.025 mg/mL, but they exhibit a lack of selectivity. This means they already act at low concentrations as biocide, but also

interact with mammalian cells, like red blood cells (RBCs), fibroblasts or keratinocytes. The selectivity is normally defined as the factor of the haemolytic concentration HC_{50} (haemolytic concentration at which 50 % of RBCs lyse) and the MIC.[1]

Other compounds are already commonly used as disinfectant like polyhexamethylene biguanide (PHMB), a polymeric biocide, which is used in contact lens solutions at concentrations around 0.0001 wt%.

Beside synthetically produced polymeric biocides, chitosan is a natural polyamine with moderate antimicrobial effect and good biocompatibility.[10] The action against microorganisms, bacteria as well as fungi, was investigated intensively. Nevertheless results are difficult to summarise since the activity is influenced by different parameters, which influence the structure of chitosan. There is the molecular weight, the molecular weight distribution, the degree of deacetylation and the resource for this natural material. The latter influencing impurities like lipids, proteins and salt, which are sometimes still present in the tested product. A broad study about chitosan from different suppliers and with different molecular weights ranging from 28 kDa to 1671 kDa for chitosan and with 1 kDa to 22 kDa for chitosan oligosaccharides (COS) was published in 2002. The activity against four gram-negative and seven gram-positive bacteria was investigated, but neglecting the degree of deacetylation (DD). MIC values were found in the range of 0.5 mg/mL and 1 mg/mL or higher in the case of chitosan with molecular weights higher than 28 kDa. The general observation was that chitosan is more effective than chitosan oligosaccharides and more effective against gram-positive than gram-negative bacteria, which was also shown by other studies. However, they could not draw a linear correlation between MIC and molecular weight.[11] Beside the effect of molecular weight on the antimicrobial activity it was observed that chitosan is more active against bacteria at low pH because of better solubility and because of the protonation of the amino groups under acidic conditions.

Since positive charges favour the antibacterial activity, permanent covalent modifications at the amino group by positively charged groups was followed by different groups. Therefore asparagine, was coupled to chitosan oligosaccharide (MW<10 kDa) and the obtained derivative showed enhanced antimicrobial activity with MIC against *E. coli* of 0.02 mg/mL.[12] Another modification strategy widely found is the derivatisation of chitosan and its oligomers using glycidyltrimethylammonium chloride (GTMAC). In the case of COS (degree of polymerisation DP=3) compounds with different degrees of substitution showed MICs between 0.05 mg/mL and 0.3 mg/mL against *Staphylococcus aureus*.[13] Quaternary ammonium groups are a potent group in antimicrobial agents. Therefore our attempt was to use cationic groups to increase the inherent biocidal activity of chitosan oligosaccharide by the carbonate coupler approach (Scheme 9).[14] This strategy has also been followed for the preparation of antimicrobial multifunctional poly(ethylene imine) derivatives with MIC values between 0.3 mg/mL and 0.5 mg/mL against *E. coli*.[15]



Scheme 9 Carbonate coupler approach, (AG: activating group, e.g.: Cl, OPh, OC_6H_4-pNO_2)

The carbonate coupler is a bifunctional reagent bearing an activating group like a chlorine or a phenoxy group and another amino-reactive five-membered cyclic carbonate moiety. The activating group reacts easily with primary amines via substitution already at room temperature while the ethylene carbonate unit can be reacted under nucleophilic addition (ring-opening) with primary amines at higher temperatures (> 50°C). Following this procedure, we report here on the preparation of a broad variety of multifunctional chitosan biocides.

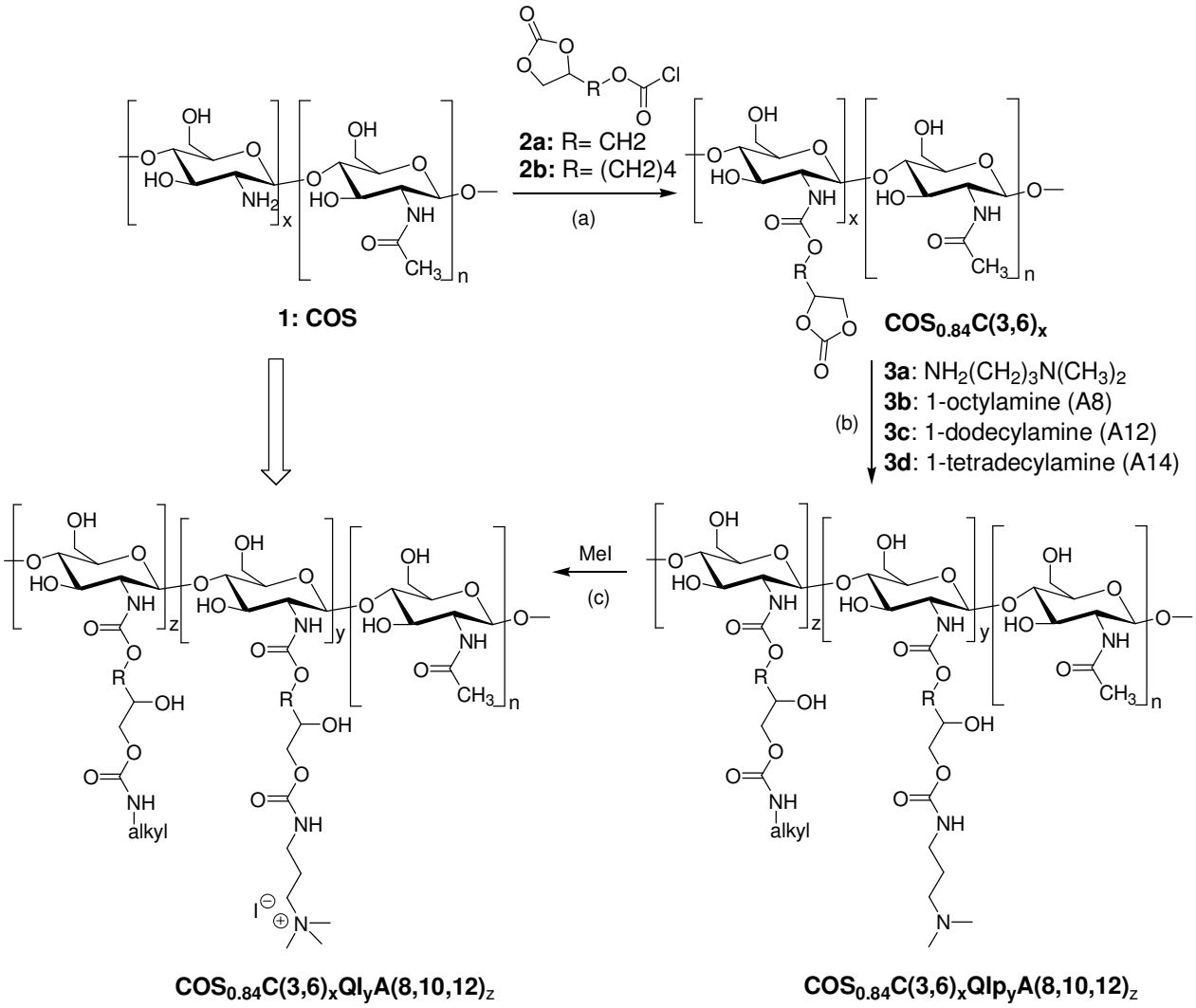
3.2 Results and Discussion

The aim of the investigation, carried out in this work was the preparation of a new class of chitosan biocides, which are decorated with quaternary ammonium groups and alkyl side chains. By this means amphiphilic molecules were obtained with ionic and hydrophobic branches, mimicking host defence peptides like defensins, found in the innate immune system. The synthesis of these biocides is presented and the antimicrobial activity is demonstrated in terms of evaluation of the minimal inhibitor concentration. The toxic potential was determined for samples with high antimicrobial activity by investigating the haemolysis of red blood cells and the behaviour in solution was determined by light scattering experiments:

3.2.1 Preparation of multifunctional chitosan oligosaccharides (COS)

The chitosan biocides were synthesised according to the "carbonate coupler approach" (Scheme 9), which was established at our institute.[14] The adaption of this approach to COS is shown in Scheme 10: In the first step the chitosan starting material **1** was modified with (2-oxo-1,3-dioxolan-4-yl)methyl chloroformate, hence named C3 linker **2a** or with 4-(2-oxo-1,3-dioxolan-4-yl)butyl chloroformate, hence named C6 linker **2b**. Secondly, a mixture of two amines **3**; (i) alkyl amines with a chain length of A8, A12 or A14, and (ii) 3-dimethylamino-

1-propylamine (DMAPA) were reacted with the carbonate-functionalised chitosan **$COS_{0.84}C3_{0.84}$** and **$COS_{0.84}C6_{0.84}$** under ring-opening to yield products **$COS_{0.84}C3_{0.84}QIp_yA(8,12,14)_z$** and **$COS_{0.84}C6_{0.84}QIp_yA(8,12,14)_z$** with different ratios of applied amines. In the last step of the synthesis the tertiary amino group of DMAPA was quaternised with methyl iodide (see Scheme 10, step c) to give an ionic moiety.



Scheme 10 Synthetic pathway for the preparation of chitosan biocides $COS_{0.84}C3_{0.84}QI_yA(8,12,14)_z$ and $COS_{0.84}C6_{0.84}QI_yA(8,12,14)_z$

Initially the carbonate functionalisation with the C3 linker **2a** and the C6 linker **2b** was performed under Schotten-Baumann conditions according to a published procedure for the protection of the amino group of monosaccharides.[16] Following this procedure, the **COS** and

$NaHCO_3$ were dissolved in water and added drop wise to the excess of cooled chloroformate solution in 1,4-dioxane. The excess of chloroformate was removed by extraction and the product was purified by dialysis against water. In the future work this procedure was modified: First of all acetonitrile was used instead of 1,4-dioxane to assure effective cooling (Melting point of 1,4-dioxane is 11.8 °C, of acetonitrile is -45 °C). Moreover acetonitrile is still easy to remove by evaporation. Furthermore triethylamine was used as acid scavenger instead of the inorganic $NaHCO_3$. The advantage is that the product now could be precipitated in isopropanol. Thus, time-consuming dialysis and subsequent lyophilisation was avoided and furthermore a long-time treatment of functionalised **COS** with water, which bears the risk of hydrolytic degradation. In this way a higher yield of functionalisation (up to 59 %) in shorter time was obtained. Nevertheless the product still contained traces of triethylamine hydrochloride and similarly to the work-up by dialysis low-molecular weight fractions were removed.

The products were analysed by means of ^{1}H-NMR spectroscopy (see Figure 12) to confirm successful acylation of the amino groups.

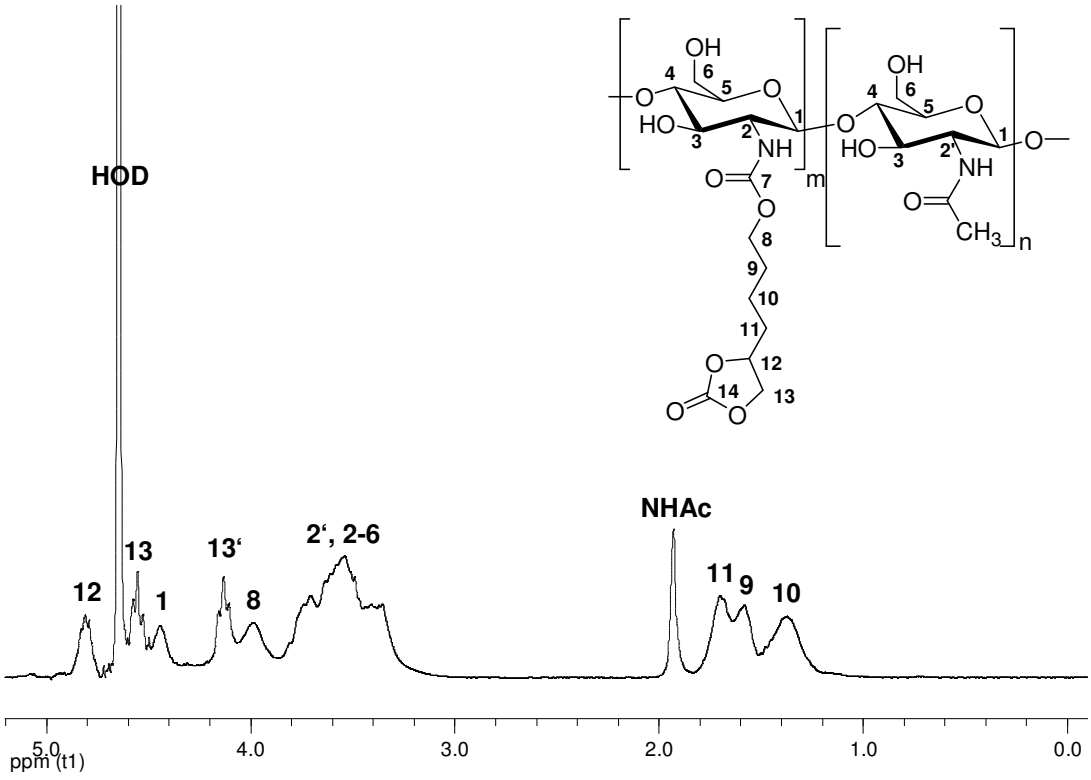


Figure 12 ^{1}H-NMR spectrum of C6-functionalised chitosan oligosaccharide $\mathbf{COS_{0.84}C6_{0.84}}$ in D_2O

The characteristic signals of the cyclic carbonate appear at a chemical shift of $\delta = 4.13$ to 4.79 ppm (H-13, H-13' and H-12) and the signals from three methylene groups of the linker (H-9, H-10, H-11) at lower field from $\delta = 1.38$-1.93 ppm.

In the second approach not all amino groups of **COS** were modified with the carbonate linker. Such a partial functionalisation can be achieved with both linkers by applying less than 1 eq of chloroformate per amino group. Thereby carbonate-functionalised **COS** were prepared having a degree of free amino groups between 15 % and 50 %. In the case of a partial functionalisation a signal at $\delta = 2.93$ ppm of the proton H-2 adjacent to the free amino group remains, beside the signal of H-2 adjacent to the carbamate group (multiplett region from $\delta = 2.93$ - 3.8ppm.

Further functionalisation was performed via nucleophilic addition of amines **4** to the cyclic carbonate moiety (see step (b) in Scheme 10). Fully functionalised $\mathbf{COS_{0.84}C3_{0.84}}$ and

$COS_{0.84}C6_{0.84}$, as well as partially functionalised **$COS_{0.84}C6_{0.46}$** with 38 % free amino groups and **$COS_{0.84}C6_{0.70}$** with 14 % free amino groups, served as starting materials. The reaction was performed in NMP at 70 °C for 2.5 d and applying a mixture of DMAP and hydrophobic amine, such as octyl- (A8), dodecyl- (A12) or tetradecylamine (A14). By varying the ratio of the two amines different chitosan derivatives with compositions described in Table 2 were obtained.

Table 6 Ratio of ionic to hydrophobic grafts in chitosan biocides **$COS_{0.84}C3_{0.84}QI_yA(8,10,12)_z$** and **$COS_{0.84}C6_{0.84}QI_yA(8,10,12)_z$**

	y : z							
A	1:0	4:1	3:2	1:1	1:1	1:1	2:3	1:4
8		+	+	+	+[a)]	+[b)]	+	
12	+	+	+	+	+[a)]	+[b)]	+	+
14			+	+	+[a)]	+[b)]	+	

starting material was also [a)]**$COS_{0.84}C3_{0.46}$** and [b)]**$COS_{0.84}C6_{0.70}$**

Quaternisation of the inserted tertiary amino group with methyl iodide was completed in NMP at room temperature. The products were isolated by precipitation in cooled Et_2O and washed with acetone to remove NMP impurities. The brown solids became cream-white powders by this treatment.

3.2.2 Antimicrobial assessment

The activity of the prepared compounds was screened against the gram-negative bacteria *E. coli* and the gram-positive bacteria *B. subtilis* in solution to evaluate the minimal inhibitor concentration (MIC). This concentration is evaluated by measuring the bacterial growth by turbidity as optical density (OD) at λ=612 nm and corresponds to the concentration at which the number of colony forming units (CFU) is reduced by log 4. The dependence of the MIC on the composition of the chitosan derivatives will be presented in dependence on (i) the ratio

of ionic to hydrophobic branches (ratio of QI:A), (ii) on the length of the hydrophobic branch (A(8,12,14)), (iii) the applied linker (C3, **2a** or C6, **2b**) and (iv) in the case of the ratio of 1:1 on the fully or partial functionalisation of amino groups of **COS**. The MIC value against *B. subtilis* was tested for all samples; the value against *E. coli* (which is the less sensitive microorganism) was tested for 10 samples less. Additionally the values of the starting material (**COS**) and of a chitosan derivative (**4**), which was directly quaternised with methyl iodide , were evaluated.

Antimicrobial assessment against B. subtilis

The MIC of the original **COS** was determined as equal to 1 mg/mL for *B. subtilis*. All prepared derivatives of **COS** showed higher activity against the investigated microorganisms than the original chitosan oligosaccharide. The lowest MIC values against the gram-positive bacterium *B. subtilis* were obtained for derivatives decorated with the A12 branch (0.01 mg/mL), while the highest MIC (>0.2 mg/mL) was evaluated for COS functionalised with an A8 branch. An overview of the activity against *B. subtilis* of all samples divided into C3 and C6 functionalisation is shown in Figure 13.

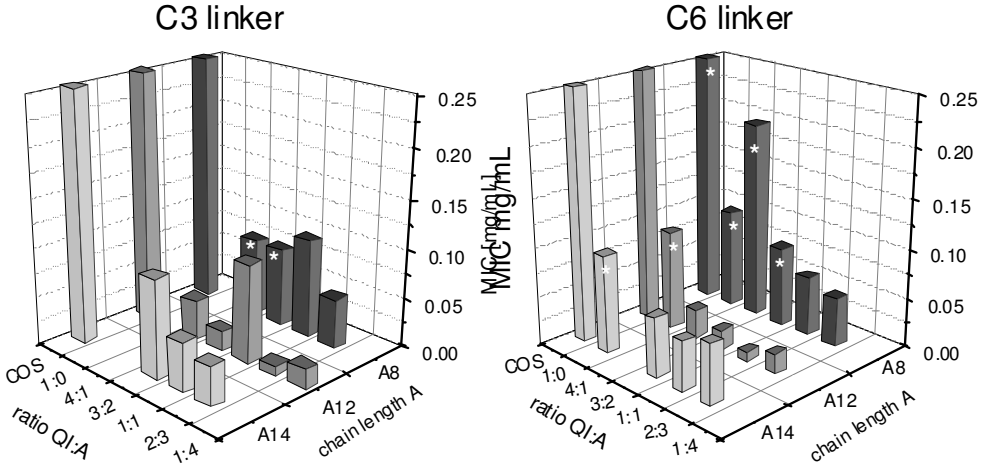


Figure 13 MIC values of $COS_{0.84}C3_{0.84}QI_yA(8.12,14)_z$ and $COS_{0.84}C6_{0.84}QI_yA(8.12,14)_z$ against *B. subtilis* (MIC of COS: 1 mg/mL); * Determination of MIC was stopped at shown concentrations because reduction was lower than log4

The MIC values for the derivatives **$COSC3_{0.84}QI_yA8_z$** and **$COSC6_{0.84}QI_yA8_z$** decrease with an increasing content of the A8 chain, this trend is observed for both linkers. The lowest MIC in the case of decoration with A8 was determined as 0.05 mg/mL in the case of the segment ratio of 2:3 (**$COS_{0.84}C3_{0.84}QI_{0.34}A8_{0.50}$** and **$COS_{0.84}C6_{0.84}QI_{0.34}A8_{0.50}$**).

For chitosan biocides with an A12 graft the best MIC value found was 0.01 mg/mL for **$COS_{0.84}C3_{0.84}QI_{0.34}A12_{0.50}$** and **$COS_{0.84}C6_{0.84}QI_{0.42}A12_{0.42}$**. For C6-functionalisation the MIC values decrease with increasing fraction of hydrophobic A12 grafts until a ratio of 2:3. The derivative with the highest fraction of hydrophobic grafts $COS_{0.84}C6_{0.84}QI_{0.17}A12_{0.67}$ (1:4) could not be determined due to only partial solubility in water, probably because of too high concentration of hydrophobic groups. The same trend was observed for the **$COS_{0.84}C3_{0.84}QI_yA12_z$** derivatives, except that the sample **$COS_{0.84}C3_{0.84}QI_{0.42}A12_{0.42}$** showed an exceptional high MIC value. This sample was found to be only partial soluble in water, even though ^{1}H-NMR spectroscopy revealed functionalisation. The sample **$COS_{0.84}C3_{0.84}QI_{0.17}A12_{0.67}$** (1:4) was not soluble in PBS buffer – similar to the corresponding C6 derivative - and was therefore measured in aqueous solution (MIC = 0.02 mg/mL).

A similar tendency was observed for the samples with the A14 branch, which was the longest hydrophobic chain inserted. The influence of the chain length follows the range A12>A14>A8. The observation that also COS-A14 was less efficient than COS-A12 was surprising, since a longer hydrophobic “sword” should be more capable to penetrate/permeate the membrane of *B. subtilis*. Presumably the poor membrane interaction derives from differences in solubility. Modification with the C6 linker resulted in a slightly better activity than the modification with the C3 linker.

Beside the investigation of fully functionalized samples, COS derivatives with an amount of branches less than the DD and an equal ratio of QI:A were also examined for their activity against *B. subtilis* (**$COS_{0.84}C3_{0.46}QI_{0.23}A(8,12,14)_{0.23}QI^*_{0.38}$** and

$COS_{0.84}C6_{0.69}QI_{0.35}A8,12,14_{0.35}QI^*_{0.14}$). The values of all samples, fully and partially functionalized, having a ratio of QI:A equal to 1:1 and different length of A, are shown in Figure 14.

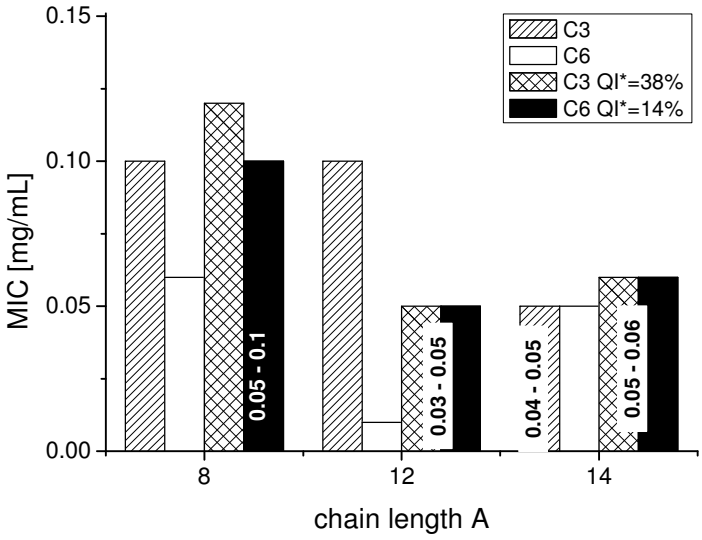


Figure 14 MIC values (*B. subtilis*) of COS derivatives with QI:A=1:1 with full and partial functionalisation (MIC value of marked samples is between the denoted values)

Again the tendency A12>A14>A8 was observed. The activity was the highest for the full functionalisation of COS amino groups, however, especially in the case of A14 the difference between full and partial conversion is only marginal. Interestingly the partial functionalisation with the C3 linker, where 39 % of the amino groups remained unreacted, showed an activity similar to the partial C6-functionalised samples, although the amount of side branches here was lower for the partially functionalized COS-C3 derivatives. One has to bear in mind, that the free amino groups do not remain as primary amino groups, but are also quaternised by the treatment with methyl iodide. Thereby leading to a structure, in which the positive charge is more close to the backbone though, but is also sterically more separated from the hydrophobic branch. This steric discrimination might enhance the amphipathic character of the molecules.

Antimicrobial assessment against E. coli

The growth of the gram-negative *E.coli* is more difficult to influence, because of its multiple membrane system and the lower content of negatively charged phospholipids in the cell membrane compared to gram-positive bacteria (see later Table 7). Therefore the evaluated MIC values of the investigated samples are a factor of ten higher than against *B. subtilis*. The samples $\mathbf{COS_{0.84}C3_{0.84}QI_yA12_z}$ and $\mathbf{COS_{0.84}C6_{0.84}QI_yA12_z}$ showed MIC values between 0.1 mg/mL and 1 mg/mL (see Figure 15). Again, samples, which were modified with the C6 linker showed a higher activity than the samples functionalized with the C3 linker. For the inhibition of *E. coli* an increasing fraction of hydrophobic grafts resulted in a decrease in MIC samples. The lowest value (0.1 mg/mL) was observed for the samples $\mathbf{COS_{0.84}C6_{0.84}QI_{0.50}A12_{0.34}}$ (QI:A=3:2) and $\mathbf{COS_{0.84}C6_{0.84}QI_{0.42}A12_{0.42}}$ (QI:A=1:1). The original **COS** instead exhibited an activity, which was higher than 1 mg/mL.

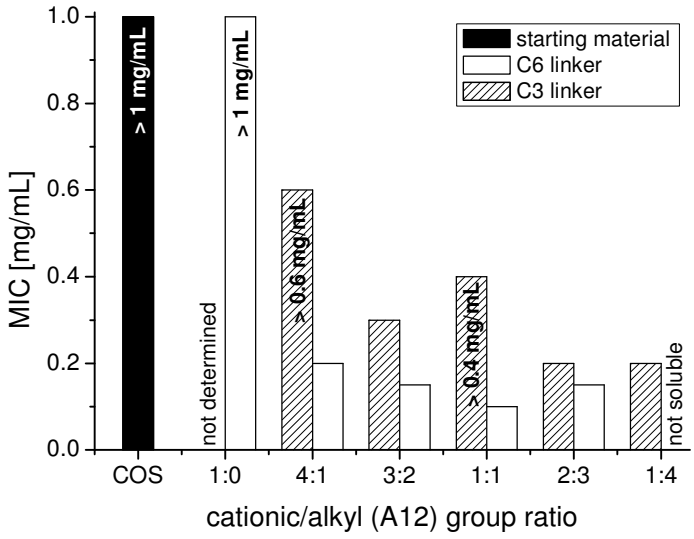


Figure 15 MIC values $COSC3_{0.84}QI_xA12_y$ and $COSC6_{0.84}QI_xA12_y$ against *E. coli*

Analysis of randomly chosen samples of chitosan biocides with A8 and A14 grafts against *E. coli* revealed a lower activity than derivatives with A 12 branches. Thus, the trend A12>A14>A8 was confirmed for *E. coli*.

3.2.3 Haemolytic activity of chitosan biocides

A key factor in the development of new compounds with antimicrobial activity, whether antibiotics or polymer biocides, is their non-toxicity. This means high selectivity in the targeting of prokaryotic cells and low interaction with eukaryotic cells. Due to the different membrane structures, with respect to membrane proteins and phospholipids composition, antimicrobial agents should be capable to act selectively. Especially the low content of anionic lipids in mammalian cells (see Table 7) makes polycationic biocides highly interesting as biocompatible antimicrobial macromolecule (AMM).

Table 7 Lipid distribution in RBC compared to *E. coli* and *B. subtilis*. Anionic lipids are marked

Cell type	**PC (%)**	**PE (%)**	**PG (%) ⊖**	**PS (%) ⊖**	**SM (%)**	**CL (%) ⊖**	**CH (%)**
E. coli	-	80	20	-	-	5	-
B. subtilis	-	12	70	-	-	4	-
RBC (outer leaflet)	33	9	-	-	18	-	25
RBC (inner leaflet)	10	25	-	10	5	-	-

PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PS: phosphatidylserine; SM: spingomyelin; CL: cardiolipin; CH: cholesterol

Therefore samples with low MIC values were tested regarding their haemolytic activity. The lysis of human RBCs was evaluated at three different concentrations: 2, 20 and 100 µg/mL (see Figure 16). All samples exhibited good hemocompatibility (rate of hemolysis < 5 %). Since not even at the highest applied sample concentration lysis was observed, it can be concluded that the HC_{50} value (haemolytic concentration at which 50% of RBCs lyse) is much higher than 100 µg/mL. Samples which were prepared using the C6 linker **2b** showed a slightly higher haemolytic activity than the samples, which were synthesized with the C3 linker **2a**. The haemolysis is also correlated to the ratio of ionic to hydrophobic branches and

increases with increasing content of A12-side chains. This is reasonable, because these blocks have the ability to interact with the hydrophobic tails of the phospholipids in the cell membrane.

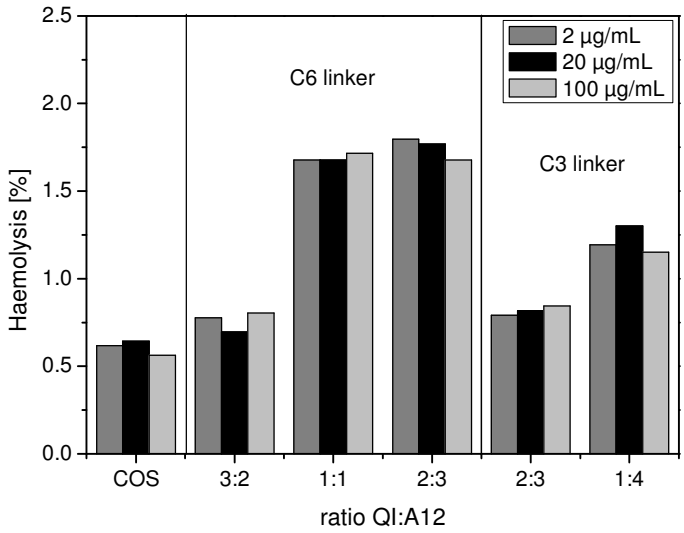


Figure 16 Rate of haemolysis induced by the chitosan biocides at three concentrations: rate of haemolyis is < 5 %, thus investigated compounds are non-haemolytic

Generally, the selectivity of the samples is estimated from the MIC and the HC_{50} value. However, since the HC_{50} was not yet reached at a concentration of 100 µg/mL, the selectivity is probably much higher as it can be concluded from the shown results.

Table 8 Selectivity of chitosan biocides

entry	sample	ratio QI:A12	MIC [µg/mL] B. subtlis	Cytotoxicity (HC_{50}) [µg/mL]	Selectivity (HC_{50}/MIC)
1	**COS**		1000	> 100	> 0.1
2	**$COS_{0.84}C6_{0.84}QI_{0.50}A12_{0.34}$**	3:2	20	> 100	> 5
3	**$COS_{0.84}C6_{0.84}QI_{0.42}A12_{0.42}$**	1:1	10	> 100	> 10
4	**$COS_{0.84}C6_{0.84}QI_{0.34}A12_{0.50}$**	2:3	20	> 100	> 5
5	**$COS_{0.84}C3_{0.84}QI_{0.34}A12_{0.50}$**	2:3	10	> 100	> 10
6	**$COS_{0.84}C3_{0.84}QI_{0.17}A12_{0.67}$**	1:4	20	> 100	> 5

3.2.4 Dynamic light scattering (DLS) investigations

During MIC and haemolysis estimation it was observed that some samples were not soluble in PBS buffer, e.g. **$COS_{0.84}C3_{0.84}QI_{0.17}A12_{0.67}$** (QI:A12=1:4) or in pure water like **$COS_{0.84}C6_{0.84}QI_{0.17}A12_{0.67}$** (QI:A12=1:4) due to the high content of hydrophobic branches. Furthermore, the haemolytic activity was not dose dependent (see Figure 16), suggesting a limit of solubility of these compounds in aqueous media. Therefore their behavior in aqueous solution was investigated by dynamic light scattering, which can provide useful information on solution properties of polymers.

At first, light scattering measurement of aqueous solutions of the chitosan biocides, which were tested for their haemolytic activity was performed without any filtration of the solutions to have an entire view on the dissolution process. These measurements were done at three different concentrations, namely 100 µg/mL, 500 µg/mL and 2000 µg/mL, while data were taken at 60 min, 165 min and 24 h (see Figure 17) after preparation of the solutions.

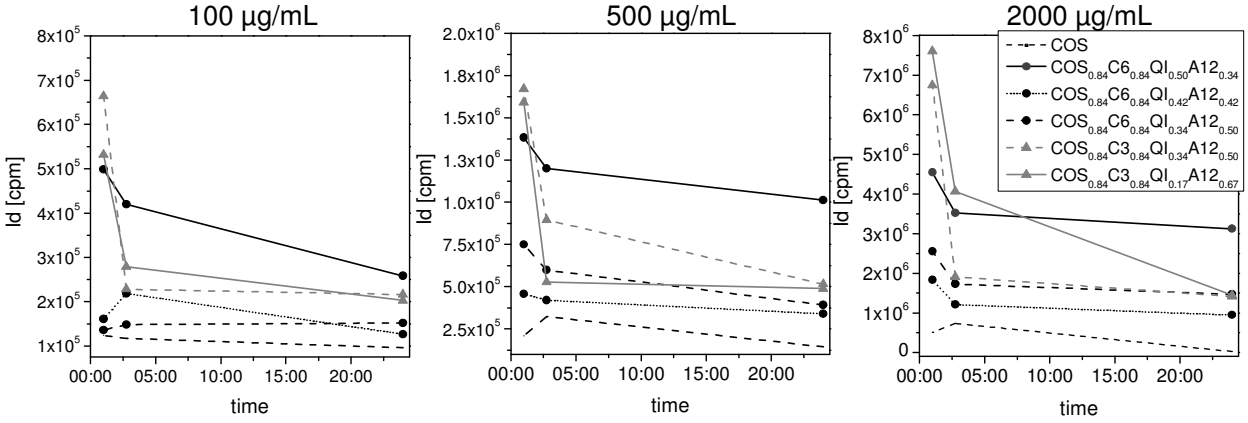


Figure 17 Mean intensity of scattered light (Id) as a function of time, measured at three concentrations, namely 100 µg/mL, 500 µg/mL and 2000 µg/mL

In Figure 17 averaged intensity of scattered light is plotted against time for unmodified chitosan and modified oligomers. It was observed that within the first two hours of experiments there was a decrease of Id, probably due to sedimentation of particles containing

modified chitosan. In the case of pure chitosan oligosaccharide **COS** the intensity was constant within investigated range of time. Moreover, only at the highest investigated concentration and in the case of the samples with the highest content of hydrophobic segments the intensity was further decreasing.

From the autocorrelation functions obtained by dynamic light scattering for unfiltered solutions it was not possible to determine the hydrodynamic diameters D_h. Therefore measurements were repeated for four samples of the biocides (**$COS_{0.84}C3_{0.84}QI_{0.67}A12_{0.17}$**, **$COS_{0.84}C3_{0.84}QI_{0.34}A12_{0.50}$**, **$COS_{0.84}C6_{0.84}QI_{0.67}A12_{0.17}$** and **$COS_{0.84}C6_{0.84}QI_{0.34}A12_{0.50}$**) in aqueous solution after filtration through 0.45 μm Nylon filters. The ratio of QI:A12 was equal to 4:1 and 2:3, respectively. During these measurements the intensity of the filtrated sample solutions did not decrease over time, confirming sample stability. From the autocorrelation function it was possible to determine the distributions of hydrodynamic diameter of the samples (see Figure 18).

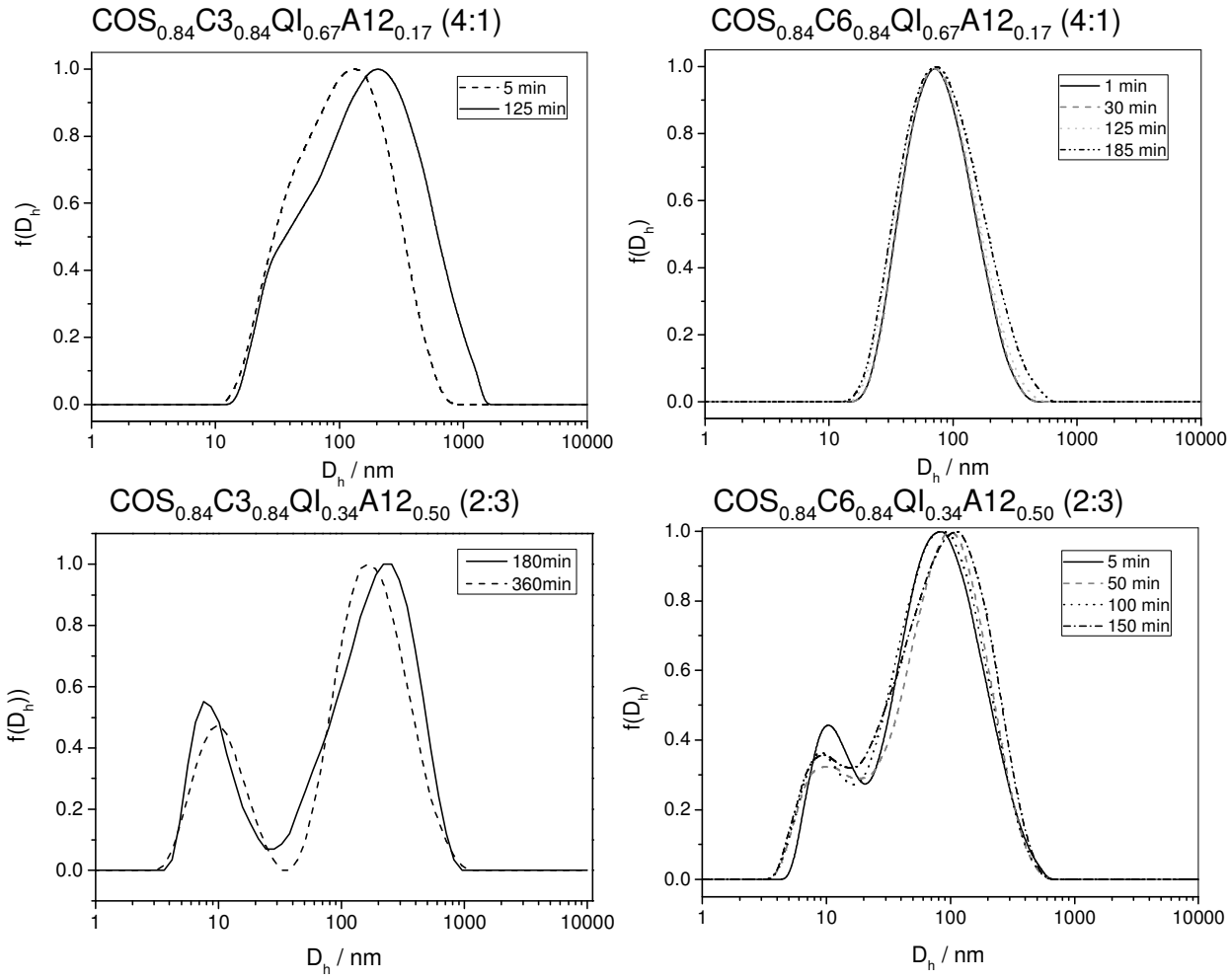


Figure 18 Distributions of hydrodynamic diameters for selected samples containing ratios of QI:A12 equal to 4:1 (top) and 2:3 (bottom) and either the C3 linker (left) or the C6 linker (right)

Both samples with a ratio of 4:1 (see Figure 18, top) are characterized by monomodal and relatively broad distributions of hydrodynamic diameters with aggregates having a D_h around 100 nm, but the distribution of sample **$COS_{0.84}C3_{0.84}QI_{0.67}A12_{0.17}$** changed in time, which was also the case for the other C3 functionalized sample **$COS_{0.84}C3_{0.84}QI_{0.34}A12_{0.50}$**. This sample showed a bimodal distribution. The solutions of the samples that were prepared using the more hydrophobic C6 linker were stable and the sample with a ratio of 2:3 showed a bimodal distribution, with smaller aggregates with D_h equal to 10 nm and bigger aggregates with a D_h higher than 100 nm. This fact can be connected with the difference in the ratio of hydrophobic

to ionic pendant groups in the oligomer chain. For surfactants containing dominant amount of ionic linkers, the aggregation process was weak, however when the ratio of QI:A12 was changed to 2:3 (see Figure 18, bottom), the derivatives showed lower stability and aggregated to bigger particles. Thus, it can be assumed that the observed increase of size in comparison with pure chitosan is a consequence of introduction of hydrophobic grafts into the oligosaccharide structure. Moreover, one can conclude that grafts containing C6 linker led to more stable aggregates in comparison with derivatives, which were prepared using the C3 linker.

3.3 Conclusion

Different chitosan biocides were prepared via a three-step-synthesis according to the carbonate coupler approach. The products varied in the applied carbonate linker, which resulted in shorter or longer spacers between main chain and functionality. The degree of functionalisation of the glucosamine amino groups was either 100 % or partial with 61 % for C3 linker or 85 % for the C6 linker. The octyl- (A8), dodecyl- (A12), or tetradecylamine (A14) branches were attached as hydrophobic units and DMAPA as precursor of the cationic side-chain, obtained by quaternisation with methyl iodide.

Products with different ratios of hydrophobic and ionic branches were tested for their activity against the gram-positive *B. subtilis* and the gram-negative *E. coli* bacteria. Highest activity was determined for derivatives prepared with the C6 linker, A12 branches and moderate (1:1, 2:3) ratios of QI:A12. The hemocompatibility of samples with low MICs was established and the haemolysis rate was lower than 5 % in all cases. A slight decrease in haemolytic activity was observed for increasing content of the alkyl chain.

The light scattering experiments revealed the formation of aggregates with a size of 10 nm and 100 nm, respectively, which were stable for $\mathbf{COS_{0.84}C6_{0.84}QI_yA12_z}$ derivatives in the

filtrated solutions. In the case of unfiltrated biocide solutions, especially with high contents of A12 branches, bigger unstable particles were formed.

The activity of chitosan biocides with amphipathic character can be influenced by the degree of functionalisation, as well as the length and the content of the hydrophobic block.

3.4 Experimental Part

3.4.1 Materials

Chitosan oligosaccharide (**1**, DP~12, DD ~85 %, *Heppe medical chitosan*), (2-oxo-1,3-dioxolan-4-yl)methyl chloroformate (C3 linker, **2a**, 90%, *Aldrich*), $NaHCO_3$ (*KMF*), 3-dimethylamino-1-propylamine (**3a**, DMAPA, *Aldrich*), methyl iodide (*Fluka*), N-methyl-pyrrolidone (NMP, *Fluka)*, 1-dodecylamine (**3b**, *Acros Organics*), 1-octylamine (**3c**, *Aldrich*) and 1-tetradecylamine (**3d**, *Fluka*) were used as received. In addition (2-oxo-1,3-dioxolan-4-yl)methyl chloroformate (C3 linker, **2a**) and 4-(2-oxo-1,3-dioxolan-4-yl)butyl chloroformate (C6 linker, **2b**) were prepared according to the general procedure of chloroformate synthesis.

Dialysis was performed with Servapor dialysis tube (MWCO 12000-14000), obtained from SERVA Electrophoresis or in a SpectraPor dialysis membrane (MWCO 1000) from Spectrum Laboratories.

3.4.2 Name of prepared products:

Products are named as described in the following: The first unit gives the applied chitosan derivative, which is chitosan oligosaccharides (COS) and the index shows the degree of deacetylation (DD). The next unit assigns the linker used (C3 or C6) and the index (x) the level of functionalisation based on the total amount of amino groups in the chitosan substrate. The third and fourth indices (y,z) specify the ratio of the applied amines: QIp always presents the ionic precursor block, deriving from DMAPA, QI the ionic block with quaternary

ammonium group and A(8,12,14) presents the hydrophobic block with indication of the length of the carbon chain. In addition, QI* stands for a quaternary ammonium group at the chitosan backbone, deriving from the reaction of the primary amino group with methyl iodide. E.g.: $COS_{0.84}C3_{0.84}QI_{0.42}A12_{0.42}$ stands for the functionalisation of COS with C3 and nucleophilic addition with DMAPA and 1-dodecylamine in a ratio of 1:1 and subsequent quaternisation of the tertiary amino group; no quaternary ammonium groups at the chitosan backbone are present (no QI*).

3.4.3 Instruments

FTIR spectra were recorded with KBr pellets on a Nicolet FT-IR spectrophotometer Nexus 470. NMR Spectra were recorded on a Varian VXR 300 or a Bruker DPX-300 FT-NMR spectrometer at 300 MHz and 75 MHz, respectively. Deuteriumoxide (D_2O) from Aldrich (99.9 %) was used as solvent. Dynamic light scattering was performed on a Photocor Goniometer - Brookhaven BI9000 correlator at an angle of 90° without filtration or on a Zetasizer Nano ZS Malvern Instruments at an angle of 173° after filtration of the COS solutions in pure water with 0.45 µm nylon syringe filter. Centrifugation was performed with a MiniSpin Plus centrifuge.

3.4.4 Microorganisms

The strains employed in this work were the gram-negative bacterium *Escherichia coli* (DSMZ 498) and the gram-positive bacterium *Bacillus subtilis* (DSMZ 347).

3.4.5 Solutions

Nutrient solution pH 7 contained 5.0 g/L of peptone, 3.0 g/L meat extract in bidistilled water. Phosphate-buffered saline (PBS) contained 9.0 g/L NaCl in 0.1 M disodium hydrogenphosphate/sodium dihydrogenphosphate buffer solution, adjusted to pH 6.5. Soft

agar was prepared from 10.0 g/L of peptone, 3.0 g/L of meat extract, 6.0 g/L of NaCl and 7.0 g/L of agar-agar in bidistilled water. All solutions were autoclaved for 15 min at 120 °C prior to use.

3.4.6 Antimicrobial assessment

A suspension of strains with known colony forming units (CFU; *E. coli*: 6 x 10^8CFU/mL; *B. subtilis*: 8 x 10^7 CFU/mL) was incubated at 37 °C in nutrient solutions with different concentrations of the test substances. The growth of the bacteria was followed during the incubation over 20 h by measuring the optical density at 612 nm every 30 min by using a microplate reader/incubator. The minimal inhibitor concentration (MIC) corresponds to the concentration of the test substance at which a log 4 reduction (corresponds to 99.99 % inhibition) of the growth of the inoculated bacteria was observed by comparison with control samples without test substance. This test does not clarify whether the sample is bacteriostatic or bactericidal. Experiments were triplicated. The standard deviations are not significant in comparison with the growth curves. The growth curves obtained from samples with different oligomer concentrations are significantly different.

3.4.7 Haemolytic activity test

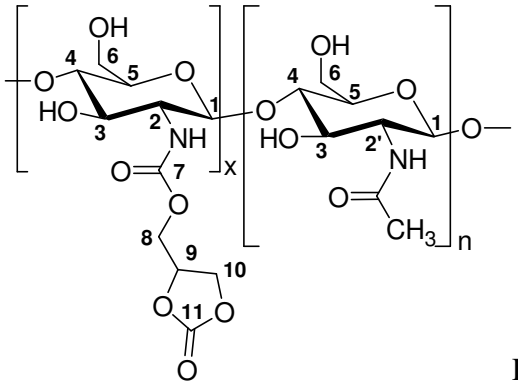
The haemolytic activity of test samples was performed according to a standard procedure.[16] Stock solutions of the test substance were prepared in phosphate-buffered saline with a concentration of 2 mg/mL. These solutions were further diluted to a concentration of 20, 200 and 1000 µg/ml and incubated (15 µL) in contact with normal human whole blood (135 µL) for 15 min at 37 °C. The plasma was isolated by centrifugation at 600g for 5 min at room temperature. Plasma (50 µL) was treated with Drabkins reagent (950 µL) and the haemoglobin concentration in 250 µL of the sample was colorimetrically determined at 540 nm. The measurements were duplicated.

3.4.8 Syntheses

The calculation of molar amount of chitosan derivatives is based on the molecular weight of the repeating unit.

N-(2-oxo-1,3-dioxolan-4-yl)methyl carbamate chitosan oligosaccharide ($COS_{0.84}C3_{0.84}$, Method A):

Chitosan oligosaccharide **1**(1g, 5.97 mmol) was dissolved in H_2O (28 mL) and 1,4-dioxane (5 mL), $NaHCO_3$ (2.37 g, 28.3 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2a** (3.74 g, 18.6 mmol) in 1,4-dioxane (10 mL). The mixture was stirred at room temperature. After 16 h additional chloroformate **2a** (1.25 g, 6.22 mmol) was added. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. Inorganic salts were removed by dialysis in a cellulose tube with MWCO 1000 against water. The product was isolated by lyophilisation. Yield: 791 mg (46.0 %), white powder.



Formula 1 $COS_{0.84}C3_{0.84}$

^{1}H-NMR (D_2O, 300 MHz):

δ = 1.97 (s, 3H, NAc), 3.40-3.85 (m, 7H, H-2, H-3, H-4, H-5, H-6), 4.14-4.47 (m, 6H, H-1, H-8, H-9, H-10), 4.64 (m, 1H, H-8'), 5.09 (m, 1H, H-9) ppm

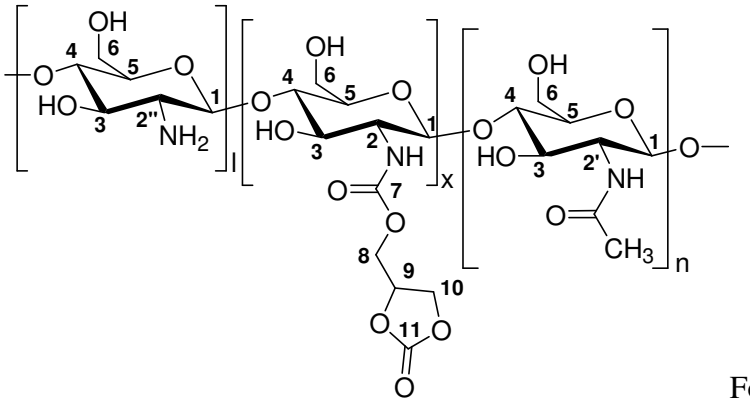
FTIR (KBr): 1791.8 (cycl. Carbonate), 1726.1, 1549.0 (urethane), 1666.6 (N-acetyl), 1054.9 (glycoside) cm^{-1}.

N-(2-oxo-1,3-dioxolan-4-yl)methyl carbamate chitosan oligosaccharide ($COS_{0.84}C3_{0.84}$, Method B):

Chitosan oligosaccharide **1** (3g, 17.9 mmol) was dissolved in H_2O (84 mL) and acetonitrile (15 mL), Et_3N (10.6 mL, 78.4 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2a** (11.22 g, 55.9 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature. After 16 h additional chloroformate **2a** (3.74 g, 18.6 mmol) was added. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. The product was isolated by precipitation in 2-propanol, filtrated and dried in vacuo. Yield: 3.07 g (59.2 %) white powder.

Synthesis of N-(2-oxo-1,3-dioxolan-4-yl)methyl carbamate chitosan oligosaccharide with degree of functionalisation < 100% ($COS_{0.84}C3_{0.46}$, Method C):

Chitosan oligosaccharide **1** (3g, 17.9 mmol) was dissolved in H_2O (84 mL) and Et_3N (2.7 mL, 19.5 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2a** (2.4 g, 12.0 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. The product was isolated by precipitation in 2-propanol, filtration and dried in vacuo. Yield: 2.45 g (51.9 %).



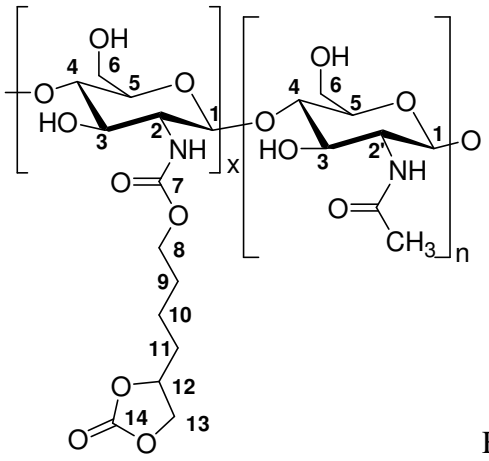
Formula 2 $COS_{0.84}C3_{0.46}$

^{1}H-NMR (D_2O, 300 MHz):

δ = 1.93 (s, 3H, NAc), 2.93 (m, 1H, C*H*-NH_2) 3.38-3.85 (m, 7H, H-2, H-3, H-4, H-5, H-6), 4.14-4.47 (m, 6H, H-1, H-8, H-9, H-10), 4.64 (m, 1H, H-8'), 5.09 (m, 1H, H-9) ppm.

N-(2-oxo-1,3-dioxolan-4-yl)butyl carbamate chitosan oligosaccharide ($COS_{0.84}C6_{0.84}$, Method A):

Chitosan oligosaccharide **1** (1g, 6 mmol) was dissolved in H_2O (28 mL) and 1,4 dioxane (5 mL), $NaHCO_3$ (1.85 g, 22 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2b** (3.07 g, 12.4 mmol) in 1,4 dioxane (10 mL). The mixture was stirred at room temperature. After 16 h additional chloroformate **2b** (1.5 g, 6.1 mmol) was added. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. Inorganic salts were removed by dialysis in a cellulose tube with MWCO 1000 against water. The product was isolated by lyophilisation. Yield: 855 mg (44.3 %), white powder.



Formula 3 $COS_{0.84}C6_{0.84}$

^{1}H-NMR (D_2O, 300 MHz):

δ = 1.38 (s, 2H, H-10), 1.59 (s, 2H, H-9), 1.70 (2H, H-11), 1.93 (s, 3H, NAc), 3.37-3.78 (m, 7H, H-2, H-3, H-4, H-5, H-6), 3.99 (s, 2H, H-8), 4.13 (m, 1H, H-13'), 4.45 (s, 1, H-1), 4.55 (m, 1H, H-13), 4.79 (m, 1H, H-12) ppm.

FTIR (KBr): 1785.8 (cycl. Carbonate), 1697.1, 1544.7 (urethane), 1066.5 (glycoside) cm^{-1}.

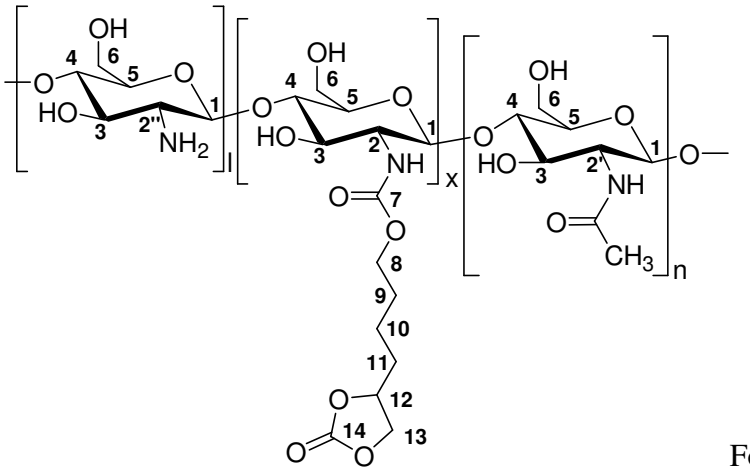
N-(2-oxo-1,3-dioxolan-4-yl)butyl carbamate chitosan oligosaccharide ($COS_{0.84}C6_{0.84}$, Method B):

Chitosan oligosaccharide **1** (3g, 17.9 mmol) was dissolved in H_2O (84 mL) and acetonitrile (15 mL), Et_3N (8 mL, 59.3 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2b** (9.24 g, 56 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature. After 16 h additional chloroformate **2b** (4.61 g, 18.6 mmol) was added. After 19 h the organic solvent was removed in vacuo and by-products by extraction with

chloroform. The product was isolated by precipitation in 2-propanol, filtrated and dried in vacuo. Yield: 2.25 g (38.6 %) white powder.

Synthesis of N-(2-oxo-1,3-dioxolan-4-yl)butyl carbamate chitosan oligosaccharide with degree of functionalisation < 100% ($COS_{0.84}C6_{0.70}$, Method C):

Chitosan oligosaccharide **1** (3g, 17.9 mmol) was dissolved in H_2O (84 mL) and Et_3N (2.7 mL, 19.5 mmol) was added. This solution was dropped to a cooled solution of chloroformate **2b** (2.9 mg, 13.0 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature. After 19 h the organic solvent was removed in vacuo and by-products by extraction with chloroform. The product was isolated by precipitation in 2-propanol, filtrated and dried in vacuo. Yield: 2.40 g (44.2 %)



Formula 4 $COS_{0.84}C6_{0.70}$

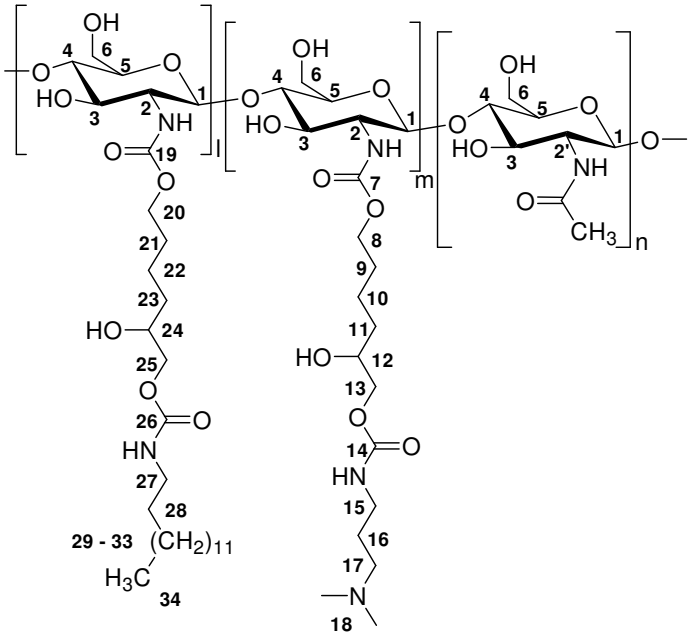
^{1}H-NMR (D_2O, 300 MHz):

δ = 1.38 (s, 2H, H-10), 1.58 (s, 2H, H-9), 1.70 (2H, H-11), 1.93 (s, 3H, NAc), 2.98 (m, 1H, C*H*-NH_2), 3.35-3.76 (m, 7H, H-2, H-3, H-4, H-5, H-6), 3.99 (s, 2H, H-8), 4.13 (m, 1H, H-13'), 4.43 (s, 1, H-1), 4.55 (m, 1H, H-13), 4.80 (m, 1H, H-12) ppm.

Reaction of carbonate-functionalised chitosan oligosaccharide with 1-octylamine and DMAPA ($COS_{0.84}C6_{0.84}QIp_{0.42}A8_{0.42}$):

The carbonate functionalised COS **$COS_{0.84}C6_{0.84}$** (400.0 mg, 1.23 mmol) was suspended in NMP (12 mL) and DMAPA **3a** (796 µL, 6.17 mmol) and 1-octylamine **3b** (1,028 mL, 6.17 mmol) were added. The reaction mixture was stirred at 70°C for 2.5 d. Upon heating all

compounds dissolved. The product was isolated by precipitation in cooled Et_2O and used for the following step without further purification. Yield: 344.31.0 mg (70.0 %)



Formula 5 $COS_{0.84}C6_{0.84}QIp_{0.42}A8_{0.42.84}$

^{1}H-NMR (D_2O, 300 MHz):

δ = 0.73 (m, 3H, H-34), 1.14 (m, 10H, H-29, H-30, H-31, H-32, H-33), 1.26-1.66 (m, 8H, H-9, H-10, H-11, H-16, H-28), 1.92 (s, 3H, NAc), 2.25 (s, 6H, H-18), 2.44, (m, 2H, H-17), 3.06 (m, 4H, H-15, H-27), 3.33-3.95 (m, 9H, H-2, H-2', H-3, H-4, H-5, H-6, H-8), 4.43 (m, 1H, H-1) ppm.

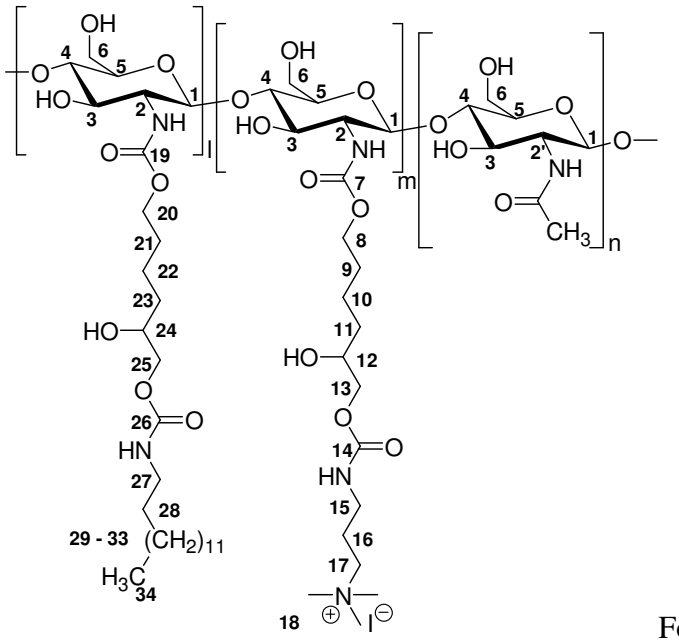
FTIR (KBr): 2825.9, 2782.4 ($CH_2N(CH_3)_2$), 1699.3, 1548.2 (urethane), 1666.4 (acetamide) 1070.4 (glycoside) cm^{-1}.

The same procedure was applied to prepare other chitosan derivatives of the general composition $\mathbf{COS_{0.84}C(3,6)_{0.84}QIp_yA(8,12,14)_z}$ (see Table 9, step 1).

Quaternisation of multifunctional chitosan oligosaccharides ($COS_{0.84}C6_{0.84}QI_{0.42}A8_{0.42}$):

The multifunctional chitosan oligosaccharide $\mathbf{COS_{0.84}C6_{0.84}QIp_{0.42}A8_{0.42}}$ (309.8 mg, 0.78 mmol) was suspended in NMP (7 mL) and methyl iodide was added (60 µL, 1.1 mmol). Over night under protection of light a homogeneous solution was obtained at room temperature. The product was isolated by precipitation in cooled Et_2O. The product was

further purified by stirring in acetone p.a. at room temperature for 16 h, filtrated and dried in vacuo. Yield: 283.7 mg (75.9 %)



Formula 6 $COS_{0.84}C6_{0.84}QI_{0.42}A8_{0.42}$

^{1}H-NMR (D_2O, 300 MHz):

δ = 0.75 (m, 3H, H-34), 1.17 (m, 10H, H-29, H-30, H-31, H-32, H-33), 1.28-1.54 (m, 8H, H-9, H-10, H-11, H-28), 1.92 (s, 5H, Nac, H-16), 3.01-3.08 (m, 13H, H-15, H-18, H-27), 3.26-3.82 (m, 9H, H-2, H-2', H-3, H-4, H-5, H-6, H-17), 3.97 (m, 2H, H-8), 4.45 (m, 1H, H-1) ppm.

FTIR (KBr): 1697.5, 1538.9 (urethane), 1068.2 (glycoside) cm^{-1}.

The same procedure was applied to prepare other chitosan surfactants of the general composition **$COS_{0.84}C(3,6)_xQI_yA(8, 12, 14)_z$** (see Table 9, step 2).

Table 9 Overview of prepared **COS** surfactants: reaction of carbonate functionalized **COS** with amines (step 1) and quaternisation reaction (step 2); s.m.:starting material

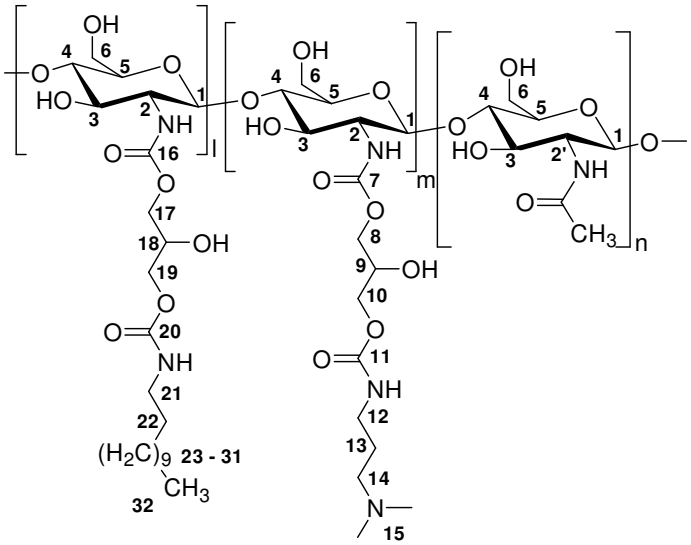
entry	Product name	QI : A	Step 1				Step 2		
			Reactant mass [mg]			Yield (%)	Reactant mass [mg]		Yield (%)
			s.m.	QIp	A		s.m.	MeI	
1	$COS_{0.84}C3_{0.84}QI_{0.84}$	1:0	359[1)]	1270	--	100	444	433	75
2	$COS_{0.84}C6_{0.84}QI_{0.84}$	1:0	300[2)]	746	--	67	303	1254	57
3	$COS_{0.84}C3_{0.84}QI_{0.67}A12_{0.17}$	4:1	400[1)]	1132	513	85	429	978	93
4	$COS_{0.84}C3_{0.84}QI_{0.50}A12_{0.34}$	3:2	412[1)]	871	1040	79	380	992	59
5	$COS_{0.84}C3_{0.84}QI_{0.42}A12_{0.42}$	1:1	400[1)]	707	1283	65	355	182	70
6	$COS_{0.84}C3_{0.84}QI_{0.34}A12_{0.50}$	2:3	400[1)]	566	1540	74	380	866	67
7	$COS_{0.84}C3_{0.84}QI_{0.17}A12_{0.67}$	1:4	400[1)]	283	2053	67	362	825	80
8	$COS_{0.84}C6_{0.84}QI_{0.67}A12_{0.17}$	4:1	420[2)]	1059	480	10	445	1031	76
9	$COS_{0.84}C6_{0.84}QI_{0.50}A12_{0.34}$	3:2	314[2)]	458	563	76	240	513	54
10	$COS_{0.84}C6_{0.84}QI_{0.42}A12_{0.42}$	1:1	450[2)]	709	1286	66	355	809	80
11	$COS_{0.84}C6_{0.84}QI_{0.34}A12_{0.50}$	2:3	420[2)]	529	1440	78	426	971	67
12	$COS_{0.84}C6_{0.84}QI_{0.17}A12_{0.67}$	1:4	420[2)]	265	1921	51	303	68	43
13	$COS_{0.84}C3_{0.84}QI_{0.67}A8_{0.17}$	4:1	370[1)]	1047	331	35	144	114	87
14	$COS_{0.84}C3_{0.84}QI_{0.50}A8_{0.34}$	3:2	370[1)]	785	662	66	329	194	97
15	$COS_{0.84}C3_{0.84}QI_{0.42}A8_{0.42}$	1:1	370[1)]	654	828	67	337	160	74
16	$COS_{0.84}C3_{0.84}QI_{0.34}A8_{0.50}$	2:3	370[1)]	524	993	69	325	137	60
17	$COS_{0.84}C6_{0.84}QI_{0.67}A8_{0.17}$	4:1	400[2)]	1009	319	48	581	137	38
18	$COS_{0.84}C6_{0.84}QI_{0.50}A8_{0.34}$	3:2	400[2)]	756	638	63	330	148	99
19	$COS_{0.84}C6_{0.84}QI_{0.42}A8_{0.42}$	1:1	400[2)]	630	797	59	310	137	93
20	$COS_{0.84}C6_{0.84}QI_{0.34}A8_{0.50}$	2:3	400[2)]	504	957	37	467	68	37
21	$COS_{0.84}C3_{0.84}QI_{0.50}A14_{0.34}$	3:2	500[1)]	531	739	63	351	205	72
22	$COS_{0.84}C3_{0.84}QI_{0.42}A14_{0.42}$	1:1	500[1)]	442	923	23	311	137	88
23	$COS_{0.84}C3_{0.84}QI_{0.34}A14_{0.50}$	2:3	500[1)]	354	1108	27	177	68	87
24	$COS_{0.84}C6_{0.84}QI_{0.50}A14_{0.3}$	3:2	442[2)]	418	582	34	450	342	84
25	$COS_{0.84}C6_{0.84}QI_{0.42}A14_{0.42}$	1:1	450[2)]	355	740	42	238	114	87
26	$COS_{0.84}C6_{0.84}QI_{0.34}A14_{0.50}$	2:3	422[2)]	525	832	78	280	171	90
27	$COS_{0.84}C3_{0.46}QI_{0.23}A14_{0.23}QI^{*}_{0.38}$	1:1	600[1)]	318	665	74	444	182	96
28	$COS_{0.84}C3_{0.46}QI_{0.23}A12_{0.23}QI^{*}_{0.38}$	1:1	600[1)]	318	577	61	345	137	86
29	$COS_{0.84}C3_{0.46}QI_{0.23}A8_{0.23}QI^{*}_{0.38}$	1:1	600[1)]	318	403	64	351	137	95

30	**$COS_{0.84}C6_{0.70}QI_{0.35}A14_{0.35}QI^*_{0.14}$**	1:1	600[2)]	378	790	41	203	68	79
31	**$COS_{0.84}C6_{0.70}QI_{0.35}A12_{0.35}QI^*_{0.14}$**	1:1	600[2)]	378	686	70	345	125	92
32	**$COS_{0.84}C6_{0.70}QI_{0.35}A8_{0.35}QI^*_{0.14}$**	1:1	600[2)]	378	478	84	436	137	96

[1)] $COS_{0.84}C3_{0.84}$ [2)] $COS_{0.84}C6_{0.84}$

Exemplary analytical data of COS derivatives:

$COS_{0.84}C3_{0.46}QIp_{0.23}A12_{0.23}$

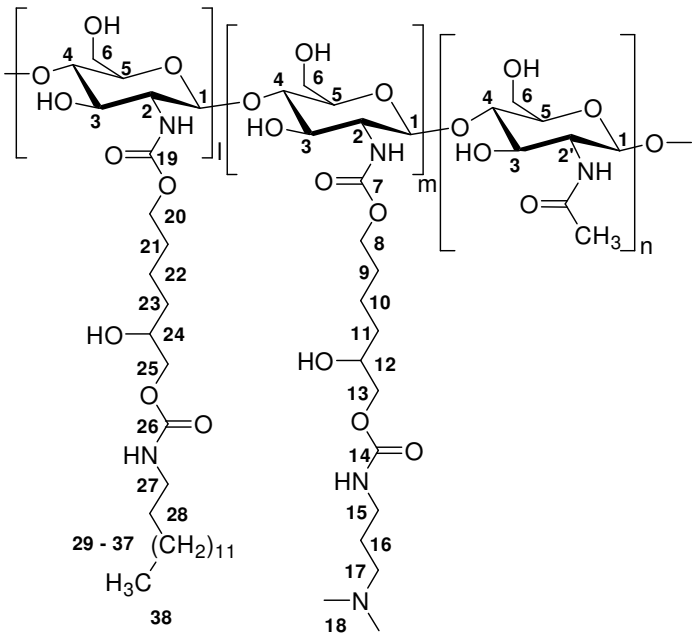


^{1}H-NMR (D_2O, 300 MHz):

δ = 0.73 (m, 3H, H-32), 1.13 (m, 18H, H-23, H-24, H-25, H-26, H-27, H-28, H-29, H-30, H-31), 1.69 (m, 2H, H-13, H-22), 1.92 (s, 3H, NAc), 2.27 (s, 6H, H-15), 2.45-2.51 (m, 2H, H-14), 2.79 (m, 1H, H-2'), 3.05 (m, 4H, H-12, H-21), 3.40-4.00 (m, 11H, H-2, H-3, H-4, H-5, H-6, H-8, H-9, H-10), 4.43 (m, 1H, H-1) ppm.

FTIR (KBr): 2783.2 ($CH_2N(CH_3)_2$), 1705.1, 1548.2 (urethane), 1662.9 (acetamide) 1070.9 (glycoside) cm^{-1}.

$COS_{0.84}C6_{0.84}QIp_{0.50}A12_{0.34}$

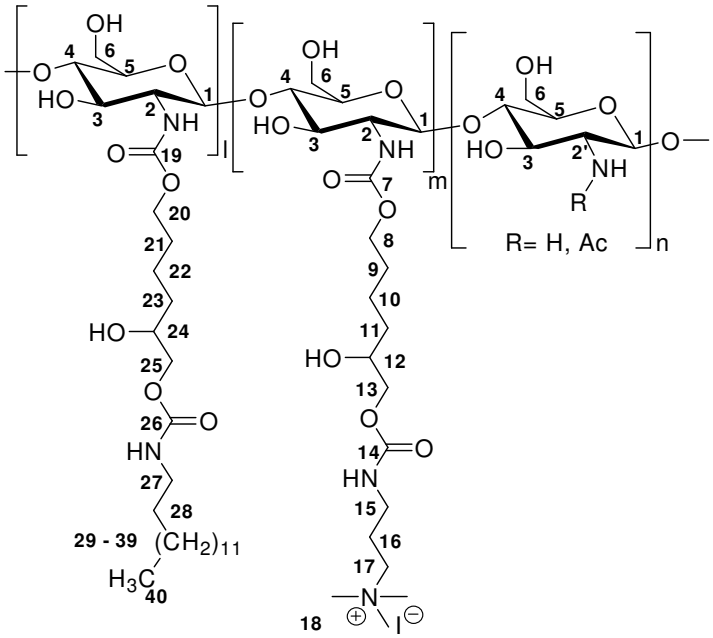


^{1}H-NMR (D_2O, 300 MHz):

δ = 0.76 (m, 3H, H-38), 1.17 (m, 12H, H-29, H-30, H-31, H-32, H-33, H-34, H-35, H-36, H-37), 1.26-1.61 (m, 10H, H-9, H-10, H-11, H-16, H-28), 1.92 (s, 3H, NAc), 2.17 (s, 6H, H-18), 2.38 (m, 2H, H-17), 3.05 (m, 4H, H-15, H-27), 3.45-3.96 (m, 9H, H-2, H-2', H-3, H-4, H-5, H-6, H-8), 4.45 (m, 1H, H-1) ppm.

FTIR (KBr): 2782.7 ($CH_2N(CH_3)_2$), 1769.1, 1552.7 (urethane), 1670.5 (acetamide), 1069.9 (glycoside) cm^{-1}.

$COS_{0.84}C6_{0.7}QI_{0.35}A14_{0.35}$

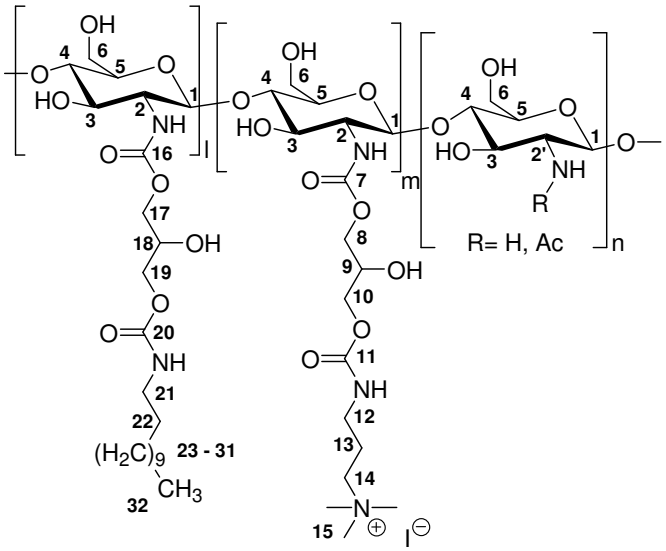


^{1}H-NMR (D_2O, 300 MHz):

δ = 0.75 (m, 3H, H-40), 1.15 (m, 10H, H-29, H-30, H-31, H-32, H-33, H-34, h-35, h-36, H-37, H-38, H-39), 1.30-1.52 (m, 8H, H-9, H-10, H-11, H-28), 1.93 (s, 5H, Nac, H-16), 3.00-3.07 (m, 13H, H-15, H-18, H-27), 3.12 (m, 2H, H-17), 3.26-3.97 (m, 7H, H-2, H-2', H-3, H-4, H-5, H-6), 3.97 (m, 2H, H-8), 4.44 (m, 1H, H-1) ppm.

FTIR (KBr): 1696.3, 1538.0 (urethane), 1069.1 (glycoside) cm^{-1}.

$COS_{0.84}C3_{0.46}QI_{0.23}A12_{0.23}$



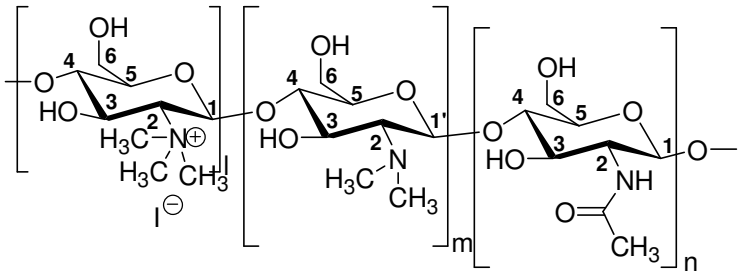
^{1}H-NMR (D_2O, 300 MHz):

δ = 0.73 (m, 3H, H-32), 1.13 (m, 18H, H-23, H-24, H-25, H-26, H-27, H-28, H-29, H-30, H-31), 1.69 (m, 2H, H-13, H-22), 1.92 (s, 3H, NAc), 2.27 (s, 6H, H-15), 2.45-2.51 (m, 2H, H-14), 2.79 (m, 1H, H-2'), 3.05 (m, 4H, H-12, H-21), 3.40-4.00 (m, 11H, H-2, H-3, H-4, H-5, H-6, H-8, H-9, H-10), 4.43 (m, 1H, H-1) ppm.

FTIR (KBr): 1701.6, 1541.1 (urethane), 1666.0 (acetamide), 1069.1 (glycoside) cm^{-1}.

Quaternisation of chitosan oligosaccharide 4 :

Chitosan oligosaccharide (**1**, 500 mg, 2.98 mmol) was suspended in NMP (12 mL) and methyl iodide was added (780 µL, 12.53 mmol) and stirred at room temperature for 16 h under protection of light. The product was isolated by precipitation in cooled 2-propanol, filtrated and dried in vacuo.



Yield: 401.9 mg (43.4 %)

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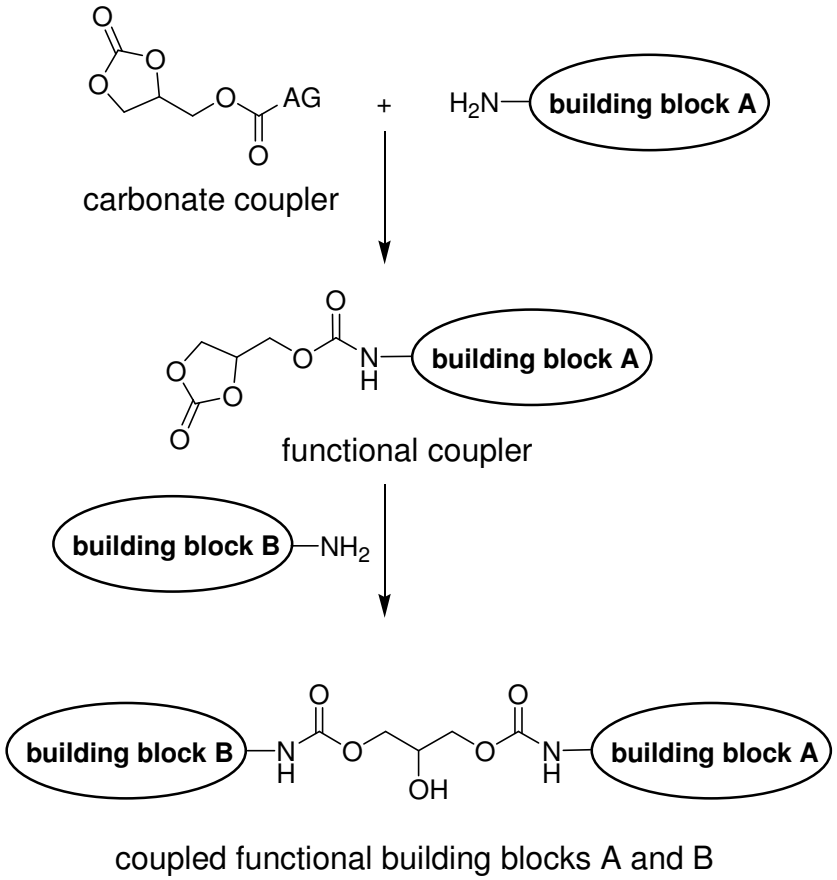
Chapter 4 Carbonate couplers and functional cyclic carbonates from amino acids and glucosamine

4.1 Introduction

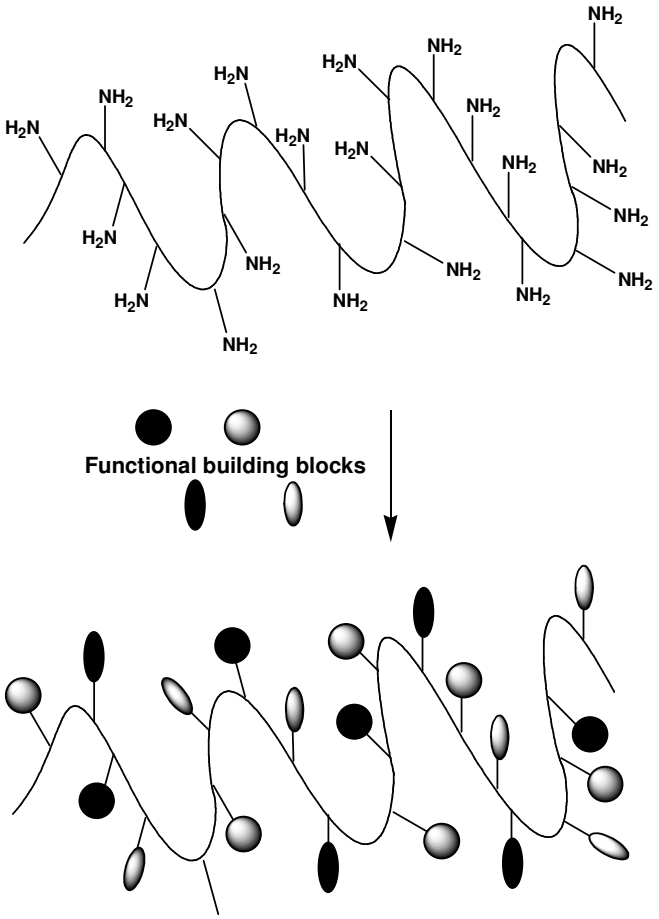
Post-synthetic modification of polymers and functionalisation of surfaces is an important scientific topic and important for industrial applications, too. Via plasma activation and plasma etching the properties of surfaces are modified uniformly by the introduction of additional functionalities: hydroxyl and carboxyl groups are introduced in oxygen plasma and amino groups are introduced in ammoniac plasma. This leads to a modification of properties of hard surfaces and fibre surfaces, e.g. biocompatibility, soil-resistance, hydrophilic and adhesive finishing can be improved.

Functionalisation of polymers is often used for compatibilization of immiscible polymers, for alteration of solubility properties, for the preparation of polymers for release systems and biomedical applications in general. One of the procedures applied to prepare multifunctional polymers is based on couplers. Recently, we developed a carbonate coupler, (2-oxo-1,3-dioxolan-4-yl)methyl phenyl carbonate (**2a**), to combine monodisperse or polymeric, functional building blocks within a single molecule (Scheme 11).[1-3]



Scheme 11 Coupling of two monodisperse or polymeric functional building blocks, using the carbonate coupler strategy. (AG = activating group)

Within this scheme, amine functionalised oligomeric or polymeric substrates are successfully linked within one molecule leading for example to amphiphilic molecules (coupled functional building block A and B, where A is a hydrophilic and B a hydrophobic building block). With such new polymers, functionalised and nano-patterned surfaces are prepared based on the self organization of the groups linked together. In case different functional couplers are linked to a polyamine a multifunctional polyamine is obtained (see Scheme 12).



Scheme 12 Synthesis of multifunctional polyamines, using the carbonate coupler strategy

Carbonylbiscaprolactam (CBC) is another coupling agent or chain extender which reacts with amines and alcohols either by ring elimination (RE) or by ring opening (RO). The quantitative CBC conversion with functional building blocks containing an amine end group occurs in bulk at 100°C in the absence of catalysts, yielding N-carbamoyl caprolactam terminated oligomers and caprolactam (RE pathway). The reaction with building blocks containing hydroxy end groups occurs at 100 to 150°C in the presence of catalysts such as zirconium alcoholates, magnesium bromide or dibutyltindilaurate (DBTDL) yielding N-carbamoyl caprolactam end groups via nucleophilic attack of the hydroxy group at one of the CBC caprolactam rings and subsequent ring opening (RO pathway).[4, 5]

A bifunctional coupling agent, which enables the coupling of two compounds with different functionalities, was realized by the combination of an oxazoline and an oxazinone

group.[6] The oxazoline group reacts with carboxylic acid groups, the oxazinone group reacts either with amino or hydroxy groups. Thereby different polymers, but in each case carboxy- or amino-terminated, such as carboxy-terminated polypropylene and amino terminated polyamide have been coupled. In the case of bifunctional starting materials the coupler was utilized as a chain extender, e.g. for polyamides.[7]

Another approach of post-synthetic modification to prepare multifunctional polymers deals with the insert of reactive groups into polymers, which do not bear any reactive groups after their synthesis. But due to their intrinsic properties they are interesting for special applications, where further modification is needed. The procedure is a rather individual process and has to be adapted for every purpose. In such a way amino groups were inserted in a poly(D,L)-lactic acid in a two-step procedure; the amino groups being used as anchoring group for the peptide sequence RGD.[8] Especially for the preparation of polymer-protein or protein-protein hybrids a variety of cross-linking agents are described and several are commercially available.[9, 10] However, they are designed for special applications and have to be selected carefully in every case.

Cyclic carbonate units are often used as reactive units at the chain ends or in the side chains for further modifications with amines or even hydroxy functionalised building blocks.[11] In the first step of the modification the reactive group is introduced in the molecule by an addition reaction resulting in a urethane/carbonate group and a free hydroxy group. This hydroxy group can be used as initiator for grafting with lactones. Next some examples are given: The end-groups of 3-aminopropyl terminated poly(dimethylsiloxane) (PDMS) were transferred into mono- or dihydroxy groups by using ethylene carbonate or glycerol carbonate, respectively.[12] The resulting hydroxy groups were then further reacted with ε-caprolactone (CL) to yield PCL-PDMS-PCL triblock copolymers. The same starting polymer was used to prepare quaternary ammonium functionalised PDMS.[13] Therefore, the

amino-terminated polymer was either transferred into a cyclic carbonate-terminated polymer, which then was reacted with 3-(dimethylamino)-1-propylamine and subsequently quaternised. These ammonium functionalised PDMS polymers show the formation of self-organised structures in the melt, in thin film and in water as a consequence of inter- and/or intramolecular interactions in the melt and of hydrophobic/hydrophilic domain formation in thin film and aqueous solution. In addition these polymers are antimicrobial active.

Optically active polyurethanes bearing carboxy and hydroxy side groups were prepared starting from a bifunctional five-membered cyclic carbonate monomer and lysine.[14] The hydroxy groups are formed via ring-opening of the ethylene carbonate with the amino groups of lysine, while the carboxylic acid groups remain unconverted.

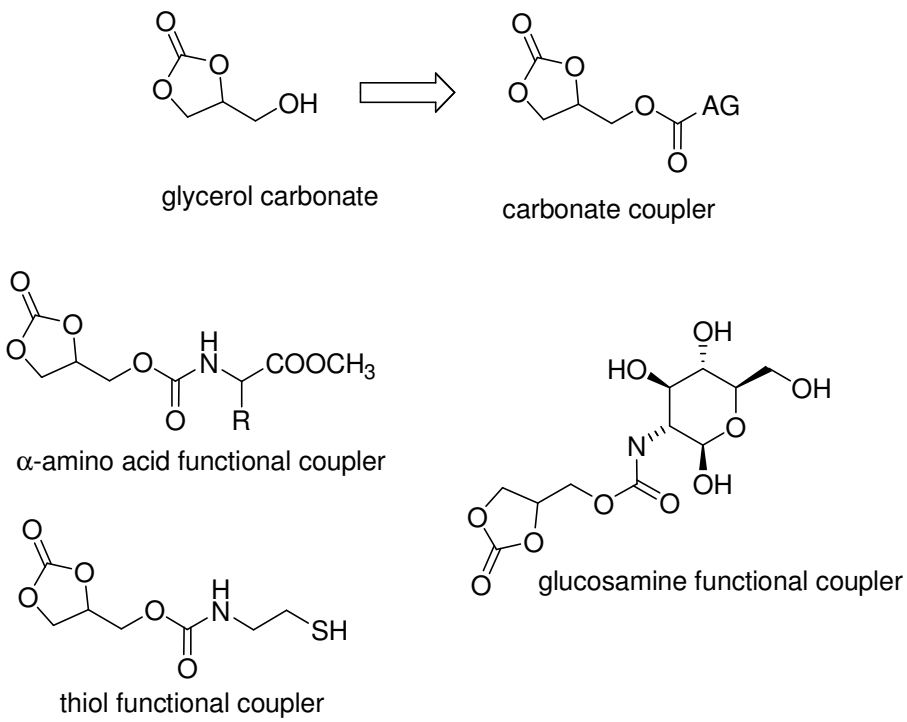
Our work presented here, is based in the conversion of amino acids into the corresponding carbamate, which is an important tool in peptide chemistry since 1932, when Bergmann and Cervas introduced the benzyloxycarbonyl (Cbz) protection group.[15] Instead of using this conversion only temporarily as protection for the amine group we used this strategy to permanently modify amino acid derivatives and glucosamine to introduce a cyclic carbonate moiety by applying carbonate couplers. Due to the cyclic carbonate moiety these compounds are flexibly deployable building blocks for post-synthetic modifications of polymeric materials.

4.2 Results and Discussion

The preparation of multifunctional polymers starts either from functional monomers which are copolymerized via an adequate polymerization procedure or from reactive polymers which are functionalized in a second step. It was our aim to prepare bifunctional coupling agents with high, but controllable reactivity toward amines which could be used for functionalisation of polyamines. These couplers were designed to have an ethylene carbonate moiety and an

activated carbonate group showing different reactivity towards primary amines (Scheme 11). The scope of such couplers is to combine two functional moieties in one molecule or to graft a polymer with functional carbonate couplers (Scheme 12).

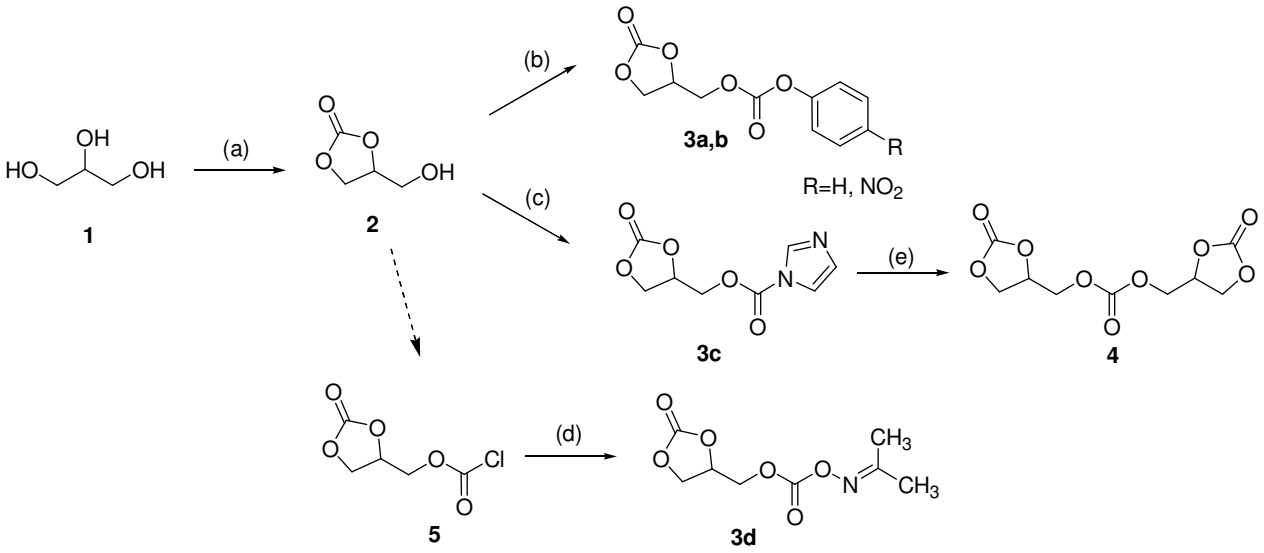
At low temperatures functional building blocks bearing an amine group react first by releasing the activating group in a substitution reaction, and by increasing the temperature a second building block bearing an amine function or a polyamine will react via ring-opening with the cyclic carbonate unit. In this work the first reaction is studied: the dependence of the coupler activity on the type of activating group. So-called "functional couplers" (see Scheme 13): α-amino acid functional couplers, thiol functional couplers and glucosamine functional couplers were synthesised to achieve building blocks of natural origin for the functionalisation of polyamines.



Scheme 13 Synthetic approach for the preparation of carbonate couplers and functional couplers. (AG: activating group)

Synthesis of bifunctional couplers

The bifunctional couplers were synthesized in a two step procedure (see Scheme 14): in the first step glycerol **1** is reacted with dimethyl carbonate to yield glycerol carbonate **2**. Two different routes were developed to prepare four different bifunctional couplers, starting either with glycerol carbonate **2** or with corresponding chloroformate **5**. Glycerol carbonate chloroformate (**5**) can be obtained from glycerol carbonate (**2**) and phosgene and is the most reactive carbonate coupler. All products obtained are white or slightly colored powders, which were synthesized in a one- or two-step reaction, respectively, in moderate to good yields.



Scheme 14: Synthesis of different bifunctional carbonate couplers: (a) dimethylcarbonate, DABCO, 75 °C; (b) phenyl chloroformate, pyridine, 0 °C, and r.t., THF; (c) 1,1'-carbonyldiimidazole (CDI), 0 °C, and r.t., CH_2Cl_2; (d) acetone oxime, Et_3N, –5 °C, and r.t., THF; (e) undesired side reaction of **3c** with **2**

Conversion of glycerol carbonate **2** with phenyl chloroformate leads to the already known (2-oxo-1,3-dioxolan-4-yl)methyl phenyl carbonate **3a**.[1, 16] Aliphatic amines easily react with this coupler, however, a higher reactivity is expected by replacing the phenyl group with the 4-nitrophenyl group. Therefore carbonate coupler **3b** was synthesized under similar reaction conditions with a good yield and was obtained as a slightly colored solid.

The ^{1}H NMR spectrum of the coupler **3b** in DMSO-d_6 shows the typical signals of the cyclic carbonate ring between δ = 4.40 and 5.20 ppm (see Figure 19). The signals of the glycerol moiety appear in the same region as for coupler **3a**; while for the 4-nitrophenyl group the expected AA'-BB' signal pattern appears at lower field.

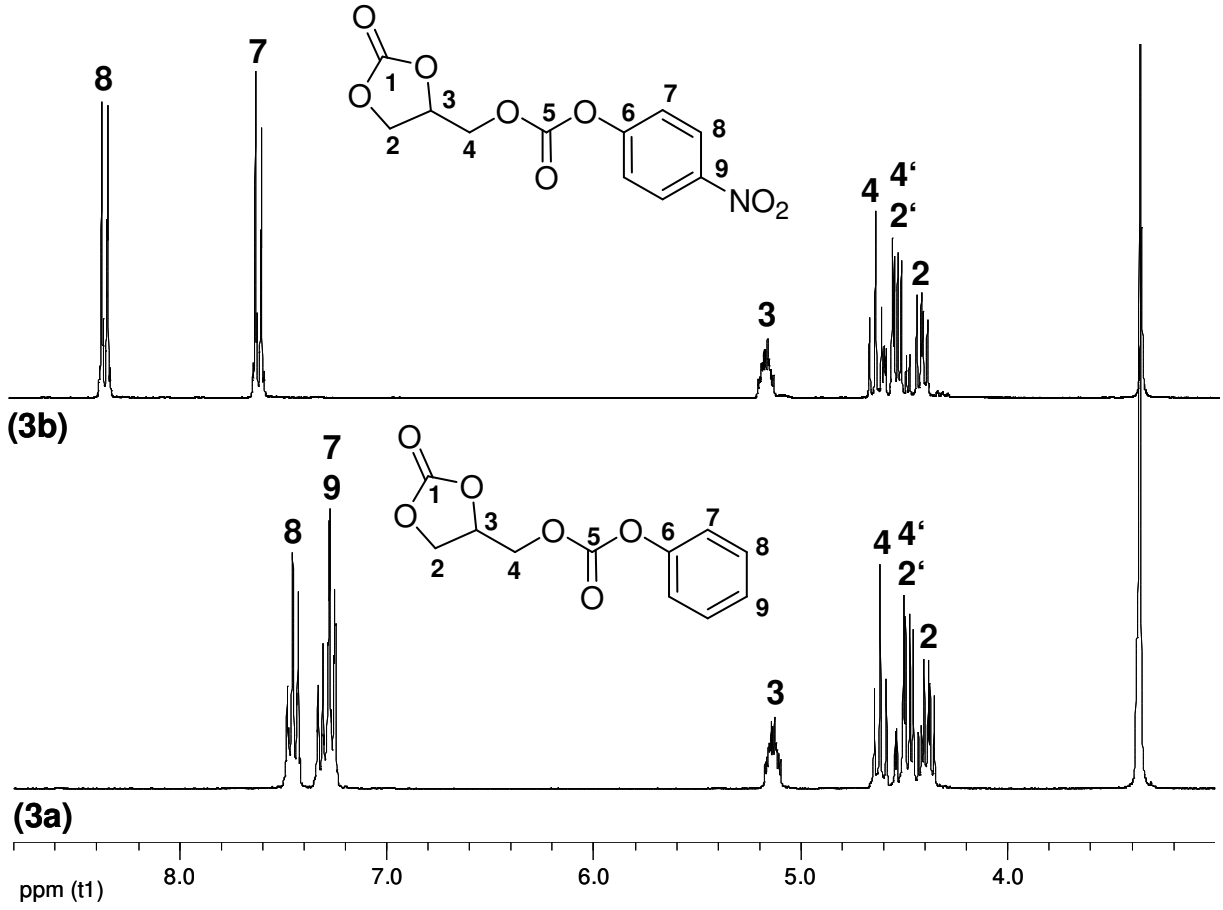


Figure 19: ^{1}H-NMR spectra of **3a** and **3b** in DMSO-d_6

Furthermore, glycerol carbonate **2** also served as starting material for the preparation of coupler **3c** (Scheme 2). The synthesis was performed in dichloromethane with 1,1'-carbonyl-diimidazole instead of using a imidazole chloroformate. 1,1'-Carbonyldiimidazole (CDI), which might be considered as an activated carbonate, is easy to handle, since it is a solid. Only very pure CDI (>97 %) has been used for the synthesis, because otherwise the product is contaminated with di(glycerol carbonate)carbonate **4**. This side-product is either formed during the synthesis or during the work-up. Nevertheless, the obtained product still contained

traces of the tricarbonate **4** and of imidazole. This side reaction is an evidence for a high reactivity of the imidazole carbonate coupler towards hydroxyl groups, which has already been described in literature.[17]

The fourth coupler **3d**, bearing acetone oxime as activating group, was prepared in a one step reaction starting with glycerol carbonate chloroformate **5** and acetone oxime. Acetone oxime phenyl carbonates (*O*-[phenyloxy)carbonyl]oxime) has been used for the regioselective modification of nucleotides using Lipase B from *Candida Antarctica* (CALB); hereby the primary hydroxyl group of the saccharide unit was selectively converted to the corresponding phenyl carbonate.[18] The synthetic procedure for **3d** is comparable to that of **3a** and **3b**; THF served as the solvent and dry triethylamine as acid scavenger. In the reaction beside the desired product **3d** the tricarbonate **4** was formed and as a consequence relatively low yield (39 %) of **3d** was obtained. However, the product did not contain side products after work-up and hence the preparation procedure was not further optimized for higher yields.

Comparable to the NMR spectra of **3a** and **3b**, the signals of the cyclic carbonate moiety of **3c** and **3d** appear at a chemical shift between δ = 4.27 and 5.20 ppm (Figure 20).

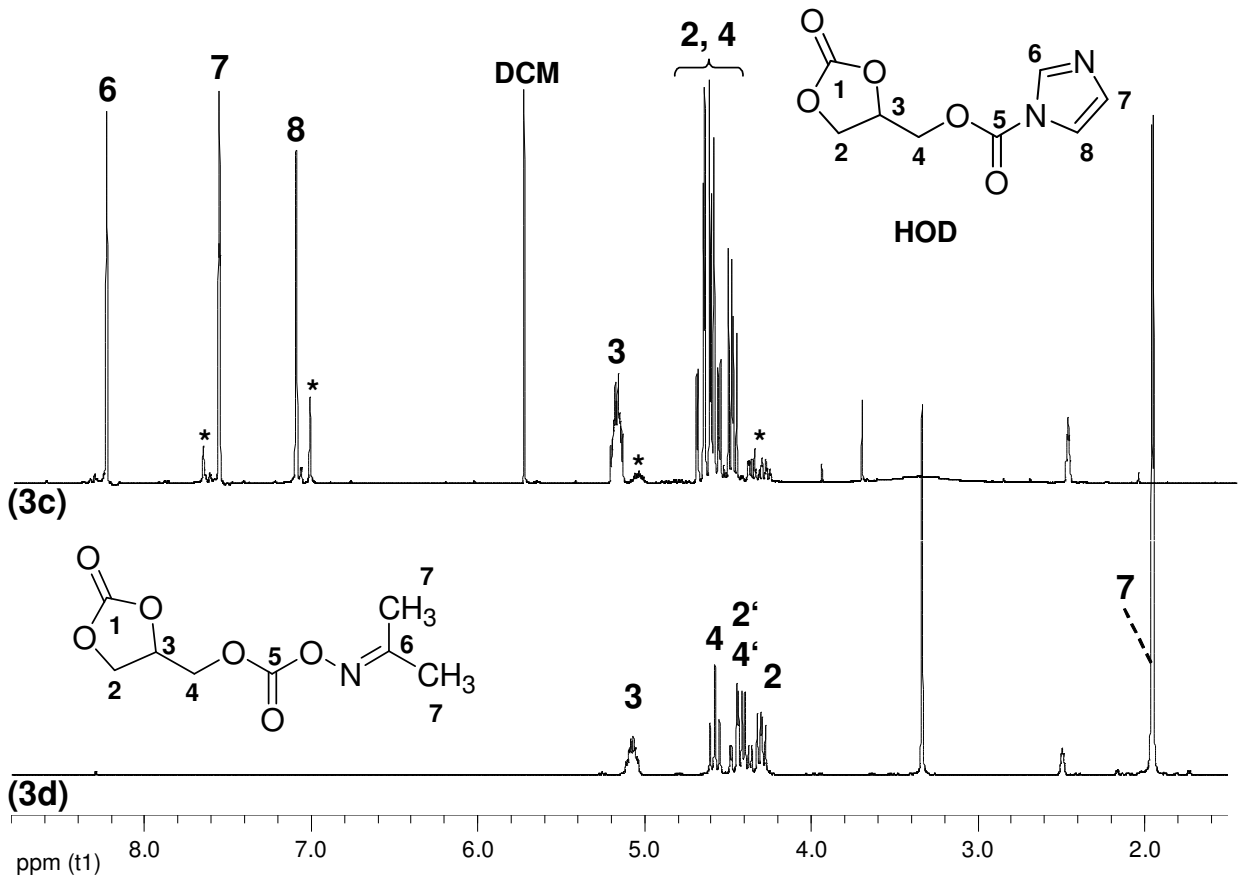


Figure 20: ^{1}H-NMR spectra of coupler **3c** and **3d** in DMSO-d_6 (DCM: dichloromethane; HOD: water signal; *: impurities)

In the case of the coupler with acetone oxime as leaving group, the corresponding signals are shifted to lower field, as is found for the tricarbonate **4**, too. The signal of the methyl groups appear at a higher chemical shift (Δ = 0.22 ppm) than in the starting material acetone oxime (^{1}H-spectrum not shown).

The successful synthesis of the coupler becomes more evident from the ^{13}C-spectrum (Figure 21). Especially the signals of the methyl group 7_Z and the carbon 6 are shifted to lower field, whereas the signals of the cyclic carbonate appear at the usual values.

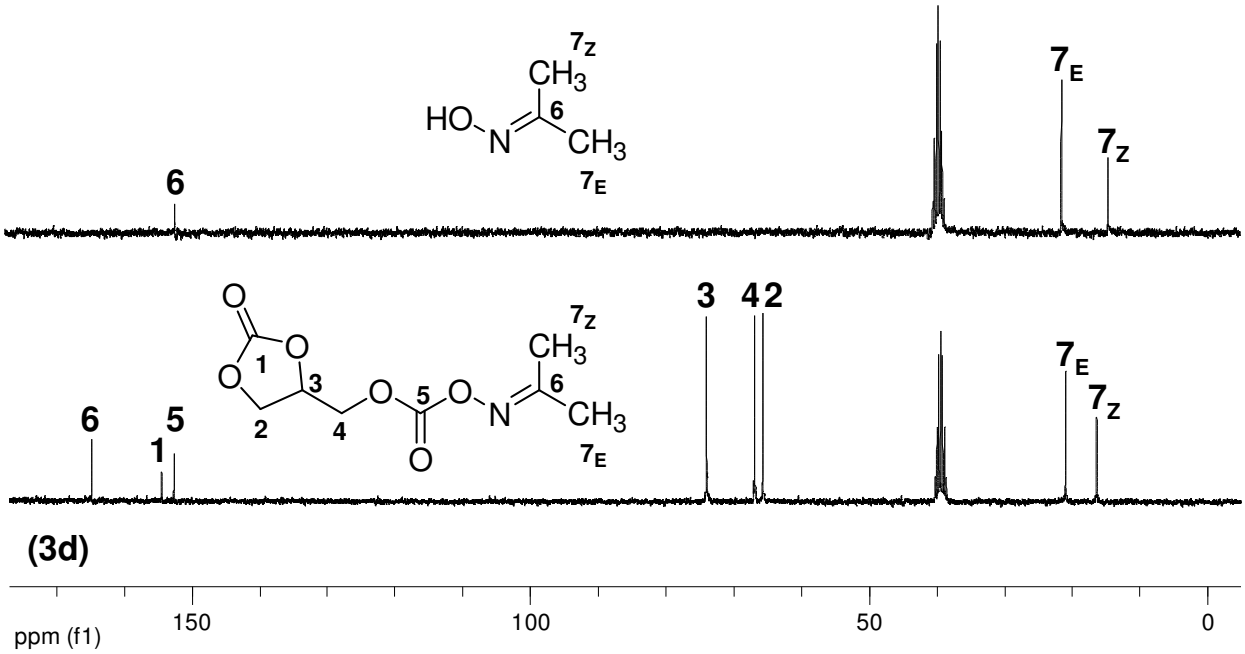
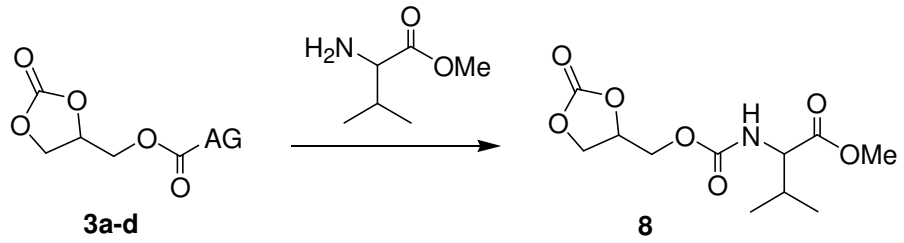


Figure 21: ^{13}C-spectra of coupler 3d compared with the starting material acetone oxime

4.2.1 Reactivity of the carbonate couplers

As the synthesised bifunctional coupling agents had different activating groups, different reactivities were expected. The investigation of the reactivity was determined by reaction of the coupling agent with valine methyl ester hydrochloride (see Scheme 15). This amino acid derivative was chosen because of its solubility in the selected solvents, the absence of other functional groups and a relative low nucleophilicity of the amine group due to the neighbourhood of the the iso-propyl group.



Scheme 15 Evaluation of the reactivity of bifunctional carbonate couplers: (a) AG = -OC_6H_5, (b) AG = -OC_6H_4-pNO_2, (c) AG = $C_3H_3N_2$ (imidazol), (d) AG = -N=$C(CH_3)_2$

Reactions were performed at room temperature in $CDCl_3$ and DMSO respectively, to study the solvent effect; the conversion was followed via NMR spectroscopy. In order to rule out participation of the ethylene carbonate ring in the reaction with the amine a model experiment was performed in which ethylene carbonate was reacted with valine methyl ester. No conversion was detected at room temperature, neither in $CDCl_3$, nor in DMSO-d_6; only at 70°C in DMSO-d_6 was a conversion detected. The results for the reaction of the four couplers with valine methyl ester hydrochloride in $CDCl_3$ at r.t. are shown in Figure 22.

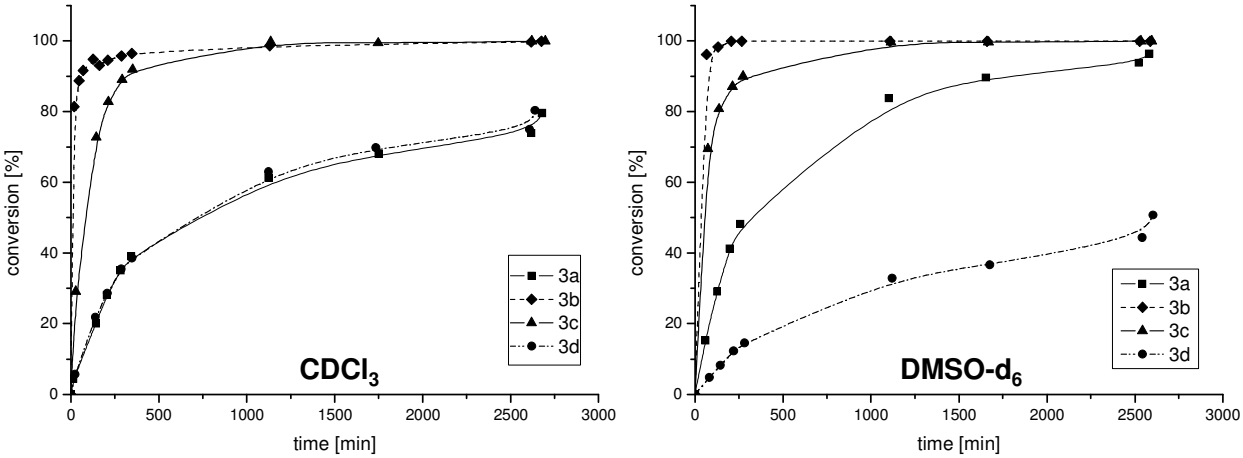


Figure 22: Conversion of valine methyl ester hydrochloride with the couplers **3a-d** in $CDCl_3$ (left) and DMSO-d_6 (right)

In chloroform solution (Figure 4, left) the fastest conversion was achieved for coupler **3b**, bearing the 4-nitrophenyl group as activating group. 4-Nitrophenyl carbonate or ester groups are often used in PEGylation chemistry for the pharmacokinetic protection of peptides and proteins.[19] Further, full conversion in a reasonable time was also reached with the imidazole coupler **3c**. Like 4-nitrophenyl carbonate groups, imidazole activating groups are also widely used for the inhibition of a fast degradation of proteins via PEGylation.[19] The reactivity of couplers **3a** and **3d** was much lower in comparison to previously mentioned couplers. With these two compounds a full conversion could not be obtained during ca. 44 h in $CDCl_3$. Almost the same curve was achieved with coupler **3a**, having the phenyl carbonate group and

with coupler **3d**, which contains acetone oxime as leaving group. Higher reaction temperatures are recommended for the modification of amino acid derivatives in a solvent like $CDCl_3$.

The results for the corresponding reactions of valine methyl ester with the carbonate couplers in DMSO-d_6 (Figure 22, right) show larger differences as in chloroform especially for **3a** and **3d**. The fastest conversion was achieved again for coupler **3b** and **3c** and the rate difference in these two solvents is marginal. The use of DMSO-d_6 influenced, however, significantly the reactivity for the couplers **3a** and **3d**. The carbonate coupler **3a** reacted faster in DMSO-d_6 than in $CDCl_3$ and the coupler with the acetone oxime group **3d** reacted faster in $CDCl_3$ than in DMSO-d_6. The latter effect is surprising, since a higher reaction rate is expected for polar solvents.[11]

The dependence of the reactivity on the nature of the leaving group can be explained by the impact of the different groups on the reaction center at the carbonyl atom. The reaction rate is higher the more electrophilic the carbonyl group is. The 4-nitrophenyl- and imidazole-groups are more electron withdrawing than the unsubstituted phenyl group or the acetone oxime, which is an electron donor, thus reducing the reaction rate at room temperature.

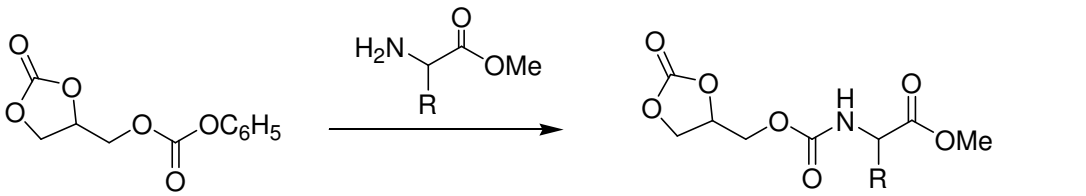
In conclusion a range of carbonate couplers was obtained suitable for the preparation of building blocks with amine groups of different reactivity.

4.2.2 Synthesis of α-amino acid functional, thiol functional and glucosamine functional couplers

The reaction of coupler **3a** with aliphatic amines has already been studied for different applications.[20, 21] However, preparation of functional couplers with naturally occurring amine building blocks such as α-amino acids, thiol amines and glucosamine has not been studied so far. Such compounds are of special interest for biofunctionalisation of polymer surfaces and

preparation of biocompatible materials. Simple amino acids, like glycine, alanine and valine are of interest to study the influence of the side group on the reaction and to prepare optically active functional couplers with only one reactive group. With functional amino acids, like serine, cysteine and lysine carbonate building blocks can be prepared that bear another reactive group beside the cyclic carbonate. The same situation occurs with thiol amines, which offer reversible coupling by the formation of disulfide bonds.[22]

By using the phenyl coupler **3a**, different amino acid methyl esters were converted into the corresponding amino acid functional couplers (Scheme 16). All syntheses were successfully performed in acetonitrile to avoid high-boiling dipolar aprotic solvents like dimethylformamide or dimethylsulfoxide.



Scheme 16 Synthesis of α-amino acid functional couplers

In the next paragraphs the preparation and characterization of these α-amino acid functional couplers starting from the coupler **3a** and the corresponding α-amino acid esters will be described in detail. Further investigations of this reaction will be performed by comparing the reactivity of the α-amino acid esters with alkylamines, the influence of solvent and base on the conversion will be discussed in each case with the used amino acid methyl esters.

- Glycine methyl ester

Glycine methyl ester, the most simple α-amino acid and due to the secondary carbon atom between the amino and carboxy group also the most reactive α-amino acid, was converted into (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-glycine methyl ester (**6**) at 40 °C in 4 h with

78.4 % yield. No reaction of the ethylene carbonate ring was observed under these conditions. This is consistent with the previously mentioned model reaction with ethylene carbonate which showed no conversion, too. In order to evaluate the reactivity of glycine in comparison with aliphatic amines, hexylamine was chosen. Both amines were dissolved in $CDCl_3$ and triethylamine was added as acid scavenger, either under competitive conditions (1 eq coupler and 2eq amines in the ratio 1:1) or under non-competitive reaction conditions (1 eq coupler and 1 eq amines in the ratio 1:1). The conversion at room temperature was followed by NMR spectroscopy by comparing the signal intensity of certain peaks of each starting material and the corresponding functional coupler. The comparison of the conversion of both amines – glycine methyl ester and hexylamine – shows, that the aliphatic amine reacts faster with the carbonate coupler (**3a)** than the natural amine (see Figure 23).

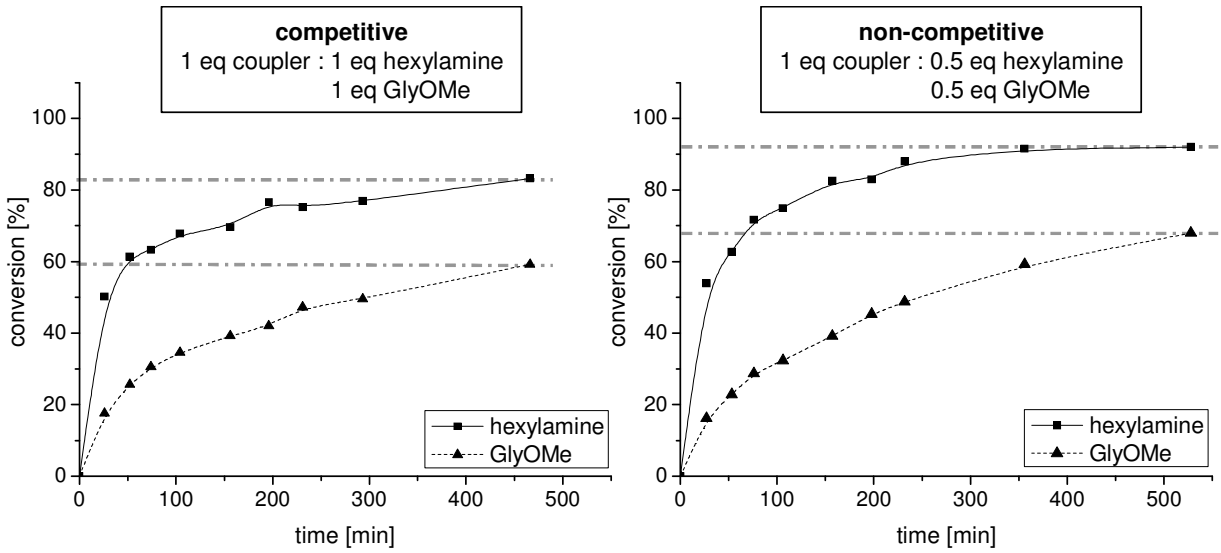


Figure 23: Comparison of hexylamine and glycine methyl ester in the reaction with coupler **3a** at room temperature. The conversion is based on the amines, not on the coupler

Both amines were dissolved in $CDCl_3$ and triethylamine was added as acid scavenger, either under competitive conditions (1 eq coupler and 2 eq amines in the ratio 1:1) or under non-competitive reaction conditions (1 eq coupler and 1 eq amines in the ratio 1:1). The

conversion at room temperature was followed by NMR spectroscopy by comparing the signal intensity of certain peaks of each starting material and the corresponding functional coupler. The comparison of the conversion of both amines – glycine methyl ester and hexylamine – shows, that the aliphatic amine reacts faster with the carbonate coupler 3a than the natural amine. Since under competitive conditions (Figure 23, left graph) not enough coupler is provided to fully convert both amines into their respective functional couplers, the high conversion of amines can only be explained by both reactions: The substitution of the phenyl carbonate and the ring-opening reaction of the ethylene carbonate ring. In the non-competitive reaction again the higher reactivity of the alkylamine is observed; within 8 hours over 90% of hexylamine and ca. 70 % of glycine methyl ester is converted.

- Alanine methyl ester

Alanine methyl ester hydrochloride, the simplest enantiomeric α-amino acid derivative, was reacted with coupler **3a** in acetonitrile to yield (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-alanine methyl ester (**7**). The ^{1}H-NMR spectrum (Figure 24) shows the characteristic signals of the components and the linking urethane group: The signals of the cyclic carbonate appear at $\delta = 4.97$, 4.58 and 4.25-4.41 ppm, the urethane group at $\delta = 5.61$ and 5.87 ppm (transoid and cisoid constitution),[23] and the signal at $\delta = 1.43$ ppm and at $\delta = 3.75$ ppm are attributed to the α-methyl group of alanine and the one of the methoxy group.

However, it would be desirable to convert free amino acids (not with esters) with the coupler in order to further link amino acids and peptides via known carbodiimide chemistry to this unit. Nevertheless, a reaction of the amino acid would be possible only in water due to their insolubility in organic solvents. In order to check the stability of the coupler in aqueous media the successful reaction of alanine methyl ester and the coupler in acetonitrile was performed in a mixture of water and tetrahydrofuran (THF): Dissolving the coupler and the

base in THF and AlaOMe hydrochloride in H_2O and mixing the solutions resulted in a two-phase system instead of the expected one-phase system. NMR-analyses after 10 h at 50 °C revealed that 50 % of the carbonate coupler 3a was still present in the reaction mixture. The mixture contained the desired product; however unidentified side products derived from the hydrolysis of the cyclic carbonate ring or of the phenyl carbonate group were also present. As a consequence, the mixture of water with an organic solvent is not acceptable for the preparation of α-amino acid functional couplers.

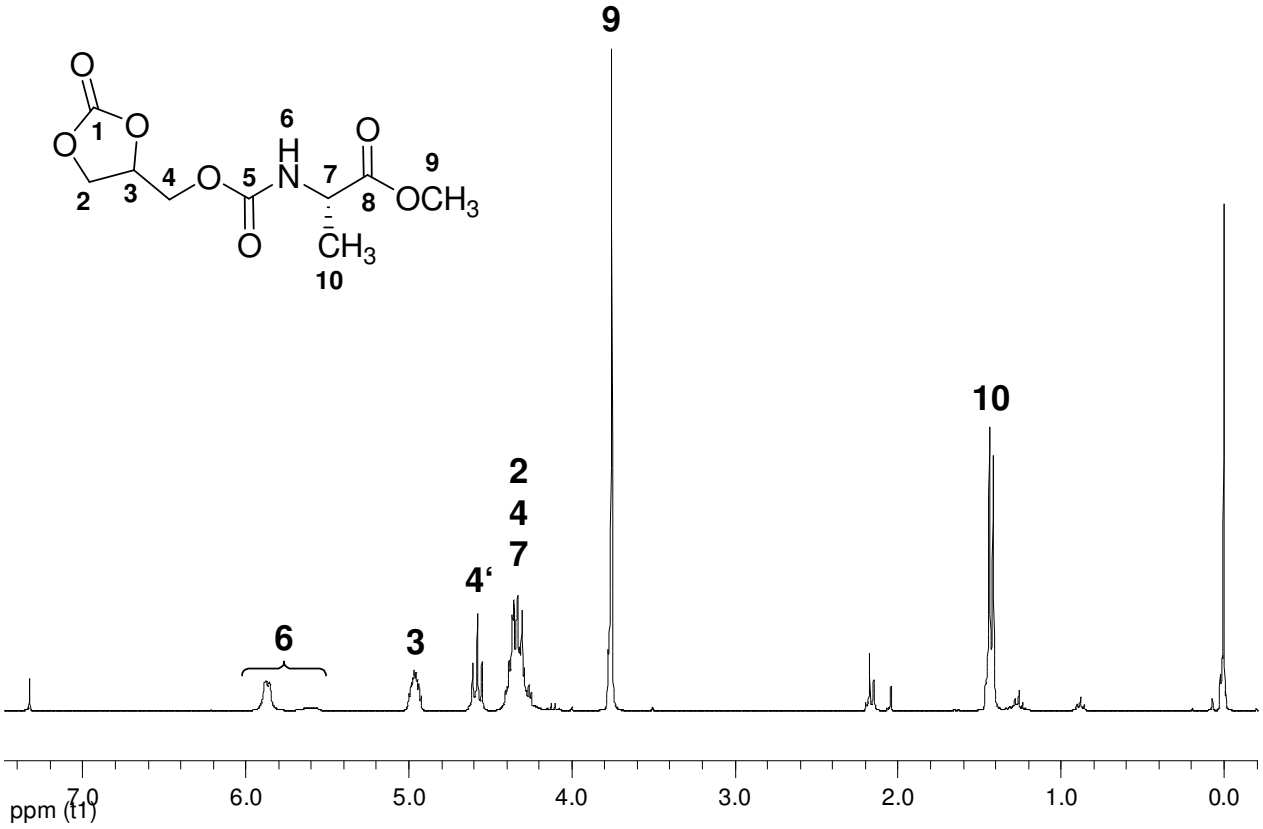


Figure 24: ^{1}H-NMR spectrum of (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-alanine methyl ester (**7**) in $CDCl_3$

- Valine methyl ester

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-valine methyl ester (**8**) was synthesized also in acetonitrile at 40 °C. After 23 h and chromatographic purification a slightly yellow oil was

obtained in 55.3 % yield. Due to the reaction of an enantiomeric pure L-amino acid ester with a racemic glycerol carbonate two diastereomers are formed. They show a slightly different chemical shift in the ^{13}C NMR spectrum, e.g. the three peaks of the glycerol carbonate unit 2, 3 and 4 (Figure 25).

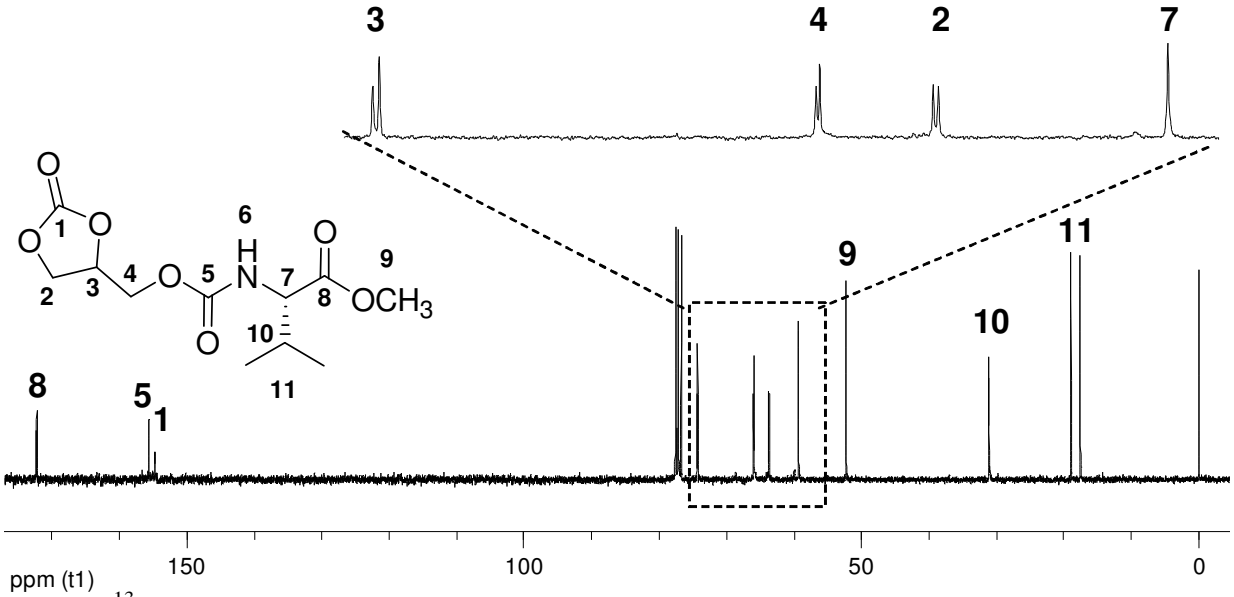


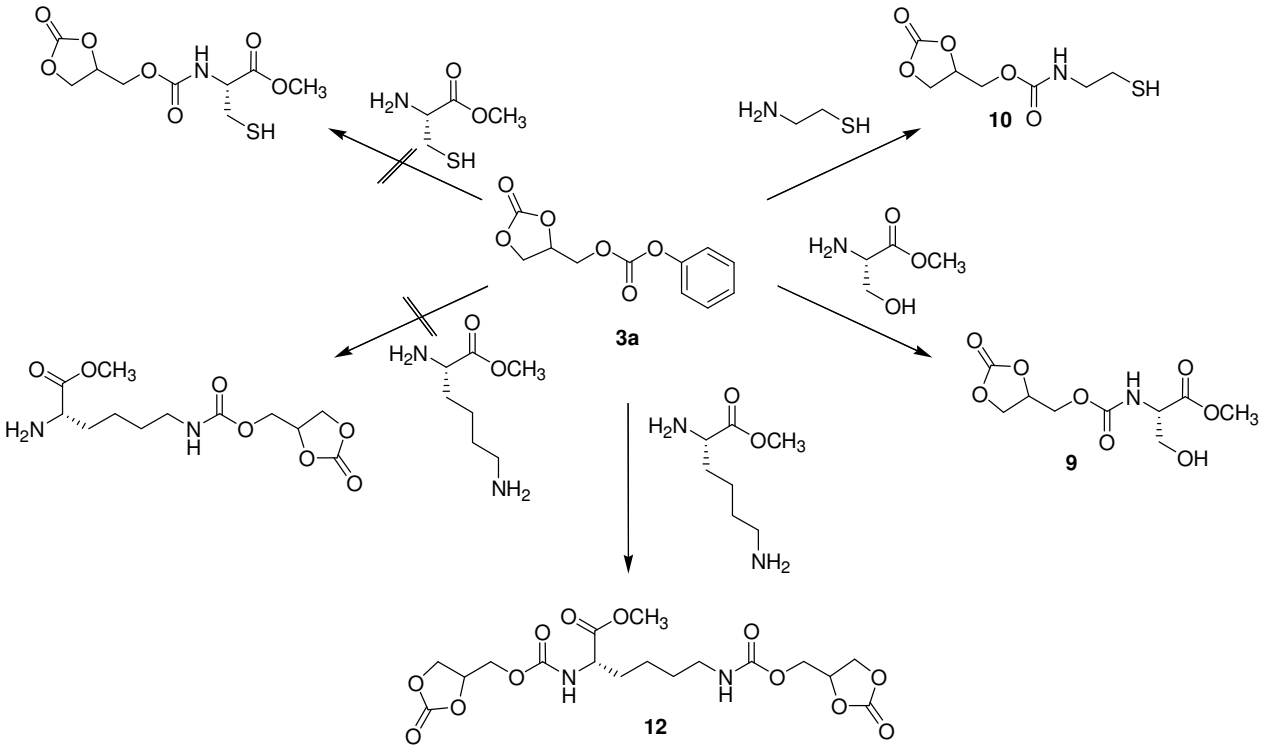
Figure 25: ^{13}C-NMR spectrum of (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-valine methyl ester (**8**) in $CDCl_3$

The signal of the tertiary carbon C-3 at δ = 74.4 ppm and the peaks for the secondary carbons C-2 and C-4 at δ = 63.7 and 66.0 ppm, respectively occur as doublets.

For this reaction the influence of the base on the conversion was investigated: triethylamine, 1,8-diazabicyclo[5.4.0]undec-7-en (DBU) and 4-dimethylyamino pyridine (DMAP) were tested. ValOMe hydrochloride reacted either with coupler **3a** at 70 °C in the presence of 1.2 eq Et_3N, at 50 °C with 1.2 eq DBU and at 50 °C with 1.2 eq DMAP and the conversion was followed by NMR spectroscopy. Only with DBU and DMAP a high conversion was attained, but the use of DBU resulted also in a complex product mixture, so that DMAP is the preferred base for the preparation of carbonate building blocks from amino acid methyl esters.

- Serine methyl ester

In the case of serine methyl ester hydrochloride, diisopropylethylamine (DIPEA) was used together with a catalytic amount of DMAP to prepare (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-serine methyl ester (**9**, Scheme 17). However, only after addition of an equivalent amount of DMAP the reaction progressed and the product **9** was obtained after 8 h at 40 °C in 57.3 % yield. No reaction at the hydroxy group occurred, which was also observed in the model reaction of 2-aminoethanol with coupler **3a** in the presence of DMAP.



Scheme 17 Desired and prepared functional couplers from α-amino acid esters with additional functionality and cysteamine

- Cysteine methyl ester, cysteamine

The reaction of the cysteine methyl ester hydrochloride with coupler **3a** resulted in a complex product mixture, so that the desired product could not be isolated (Scheme 17). An explanation for this result is the comparable nucleophilicity of the amine and thiol group and the sensibility of the thiol group to oxidation. However, we were interested in a carbonate building block with a thiol group and therefore we choose cysteamine (2-aminoethanethiol) as a substrate. The reaction product (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-cysteamine **10**

was obtained after 5 h at 40 °C in THF and chromatographic purification in 47.6 % yield. The successful selective conversion of only the amine group and not the thiol group of cysteamine is due to the higher nucleophilicity of the amino group in cysteamine compared to the amino group of an α-amino acid (which is deactivated by the ester group in α-position). For cysteamine the difference in reactivity is sufficient for the selective reaction.

Attempts to modify the corresponding disulfide cystamine failed due to the low solubility of cystamine.

- Lysine methyl ester

First model reaction was performed where lysine was converted with diphenyl carbonate (DPC) to test the selectivity (difference in reactivity of the α- and ε-amino groups) of the two amino groups towards DPC. However, under the typical reaction conditions with DMAP as base in acetonitrile at 40°C both the α- and the ε-amino group were converted to the corresponding carbamate. The NMR spectrum with all assignments is shown in Figure 26.

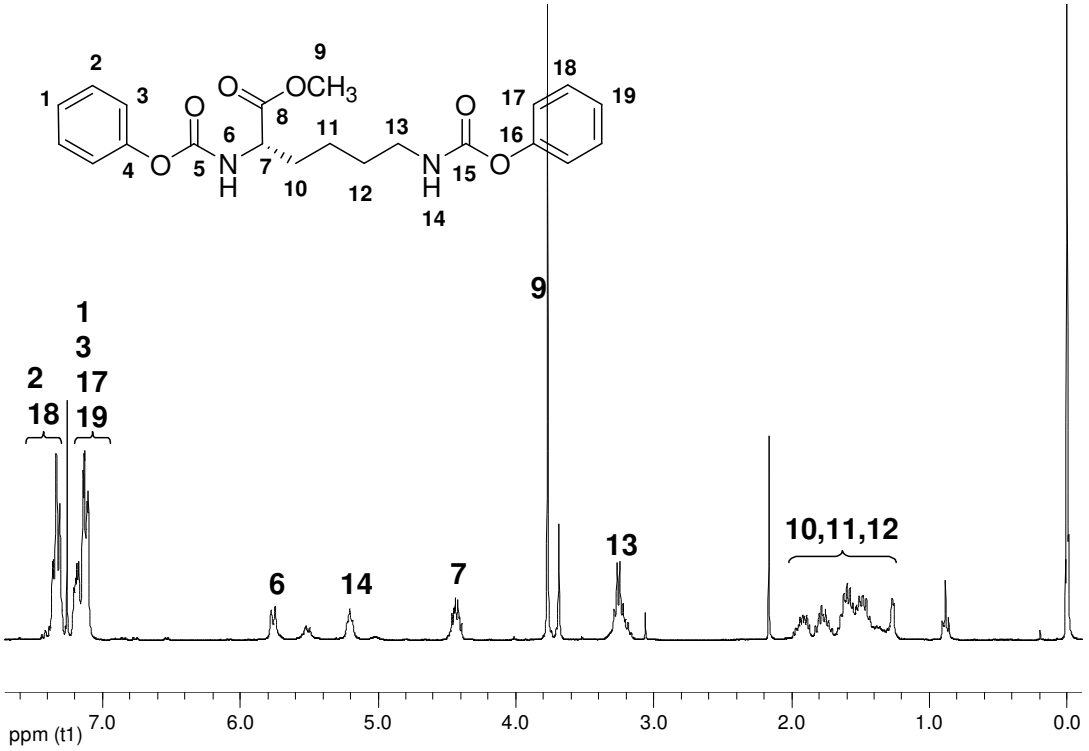
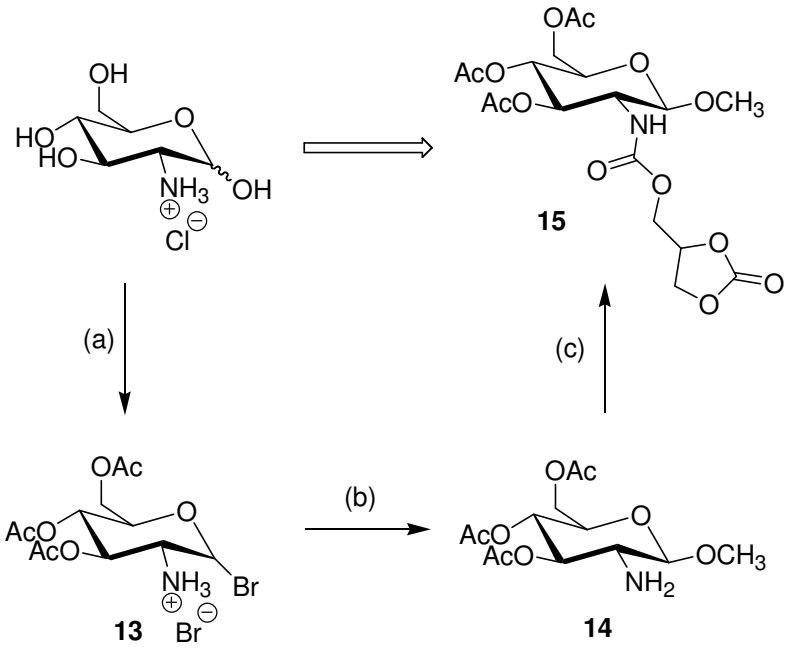


Figure 26 ^{1}H-NMR spectrum of N,N'-bis-(phenoxycarbonyl)-lysine methyl ester (**11**) in $CDCl_3$

The reaction of lysine methyl ester with the carbonate coupler **3a** was even more complex: Again, both amine groups were converted. The derivatisation was followed in DMSO-d_6 by the shift of the α-methine proton (H-7) from δ = 3.81 ppm to δ =4.2 ppm and of the ε-methyline protons (H-13) from δ =2.72 ppm to δ =2.95 ppm. In conclusion it must be stated that selective conversion of only the ε-amino group in lysine with coupler was not possible.

- Glucosamine

Our interest in the synthesis of a functional coupler made from glucosamine derives from the aim to prepare immobilized oligosaccharides as biomimetic structures. Glucosamine represents the starting material for the enzymatic synthesis to oligosaccharides.[24] The amino sugar, however, was not directly transferred to the cyclic carbonate functionalized derivative, because of its insolubility in organic solvents and to avoid any side reaction at the hydroxy groups. First the hydroxy groups were protected by acetylation of hydroxy groups at C-2, C-3 and C-6 with acetyl bromide, followed by methoxylation at C-1 (see Scheme 18).[25]



Scheme 18 Synthesis of functional coupler (**15**) from glucosamine: (a) AcBr, 3d; (b) MeOH, pyridine, 75 min, r.t.; (c) **3b**, CH_2Cl_2, HOBt, 65 h, 50 °C; or glycerol carbonate chloroformate, THF, pyridine, -5 °C and r.t., 16 h

Reaction of methyl tri-O-acetyl-2-amino-2-deoxy-β-D-glucopyranoside **14** with coupler **3a** was not possible, therefore coupler **3b** was chosen. The synthetic conditions using 1-hydroxybenzotriazol (HOBt) in dichloromethane followed the synthesis of ureido sugars from amino sugar **14** and di(4-nitro-phenly carbonate).[26] The desired product **15** was obtained after a reaction time of 65°h and chromatographic purification in 41.6 % yield, which is not comparable to the preparation described for ureido sugars.

However, our results are consistent with the results of Vatèle, who described a similar synthesis using glucosamine and a 4-nitrophenyl carbonate bearing compound in dichloromethane at room temperature and observed low conversion, too.[27] Our results show, that glucosamine is even less nucleophilic than the previously described amino acid methyl esters.

The glucosamine functional coupler could be more easily prepared from methyl tri-O-acetyl-2-amino-2-deoxy-β-D-glucopyranoside (**14**) and glycerol carbonate chloroformate (**5**) with pyridine in THF at r.t. in 16 h and 93.6 % yield (^{1}H NMR spectrum see Figure 27).

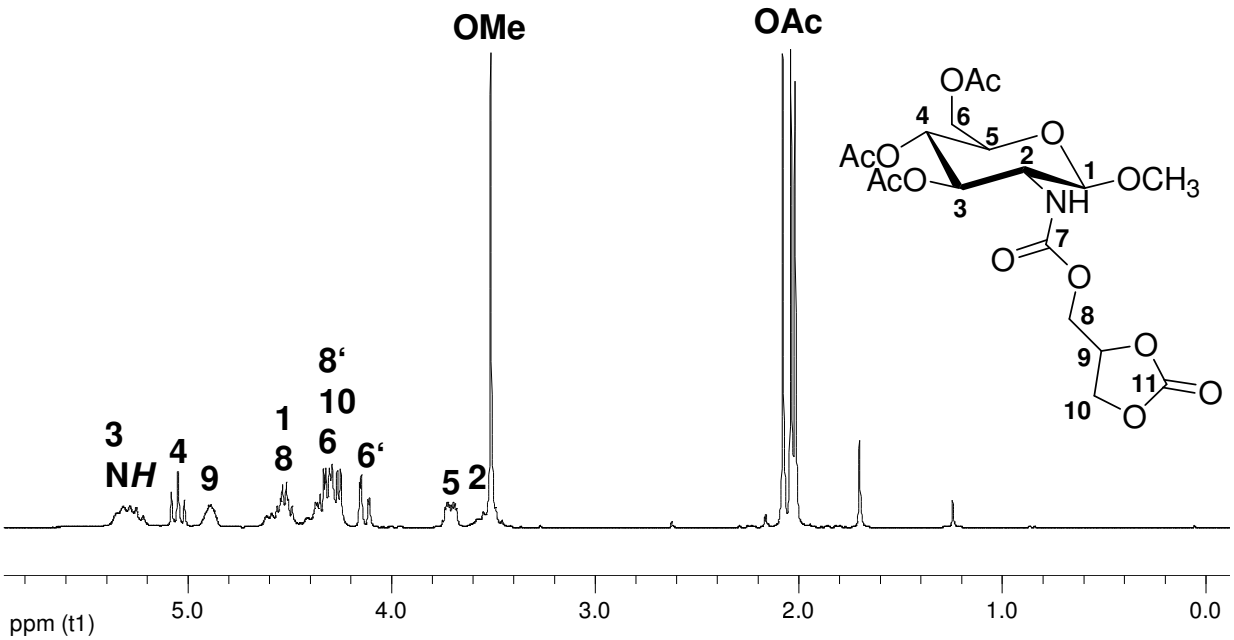


Figure 27 ^{1}H-NMR spectrum of glucosamine functional coupler **15** in $CDCl_3$

The 1H NMR spectrum shows three singuletts for the protective acetyl groups at $\delta = 1.96$, 1.98 and 2.02 ppm, respectively. The singulett at $\delta = 3.46$ ppm is attributed to the methoxy group at C-1. The signals of the protons at the pyranose ring, the cyclic carbonate moiety and the urethane group appear in the region from $\delta = 3.49$ ppm to $\delta = 5.29$ ppm.

4.3 Conclusion

Three new carbonate couplers with different reactivity have been synthesized, which complement the already known (2-oxo-1,3-dioxolan-4-yl)methyl phenyl carbonate (**3a**). These compounds can be easily prepared in two- or three-step reactions. The evaluation of reactivity was performed in the reaction with valine methyl ester hydrochloride and revealed differences as a function of the activating group and used solvent. (2-Oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate (**3b**) was the most reactive coupler in $CDCl_3$ as well as in DMSO-d_6. The more reactive couplers **3a** and **3b** were necessary for the preparation of functional couplers from amines with low nucleophilicity. The amino acid methyl esters with one reactive group (glycine, alanine and valine), with two reactive groups (serine, cysteine and lysine), cysteamine and glucosamine exhibited a much lower reactivity as aliphatic amines. The amino group of serine methyl ester and cysteamine could be modified selectively. A selective functionalization of only the amine group was not possible with cysteine methyl ester, where both reactive groups – amino and thiol – reacted with the carbonate coupler **3a**. In the case of lysine methyl ester again only both amino groups – α- and ε-group - could be converted to the corresponding carbamate. The amino sugar glucosamine was successfully transferred into the glucosamine functional coupler **15** with (2-oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate (**3b**), but more easily with glycerol carbonate chloroformate (**5**).

The obtained functional couplers are suitable for the modification of amino functional surfaces and polyamines to result in multifunctional polymers.[3]

4.4 Experimental Part

4.4.1 Materials

Starting materials and reagents were used as received unless specified. Acetone oxime (*Acros*), glycerol (*Acros*), 1,1'-carbonyldiimidazole (CDI, *Fluka*) 1,4-diazabicyclo-[2.2.2]octane (DABCO, *ABCR*), glycerol carbonate chloroformate (90%, *Aldrich*), phenyl chloroformate (*Aldrich*), dimethyl carbonate (*Acros*), 4-nitrophenyl chloroformate (*ABCR*), 4-dimethylaminopyridine (DMAP, *Aldrich*), 1-hydroxybenzotriazol (Hobt, *Fluka*) and L-amino acid methyl ester hydrochlorides (*Iris*) were used as received. Glycine methyl ester hydrochloride was synthesized according to standard procedures.[28] Tetrahydrofuran (THF, *KMF*) was dried by refluxing over potassium, methanol by refluxing over sodium. Pyridine and triethylamine were purchased from *Fluka* in anhydrous form and used without further purification. Dichloromethane (CH_2Cl_2, *Fluka*) was dried by refluxing over P_2O_5 and distillation. Thin-layer chromatography was performed on precoated plates (TLC aluminium sheets with fluorescence indicator, (Macherey-Nagel, Düren) with detection by UV light or developing with common reagents and subsequent heating: 5% molybdato phosphoric acid in ethanol was used for all cyclic carbonate containing compounds, 10 % H_2SO_4 in ethanol for glucosamine derivatives. Column chromatography was carried out on silica gel from Acros Organics (particle size 35-70 μm for flash chromatography and 60-200 μm for chromatography at ambient conditions).

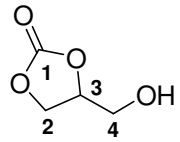
4.4.2 Instruments

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR 300 or Bruker DPX 300 FT-NMR spectrometer at 300 MHz and 75 MHz, respectively. Chloroform-*d* ($CDCl_3$) and dimethyl sulfoxide-d_6 (DMSO-d_6) were used as solvents, and tetramethylsilane served as internal standard.

4.4.3 Syntheses

4-Hydroxymethyl-[1,3]dioxolan-2-one (glycerol carbonate, 2):

Glycerol (**1**) (54,54 g, 592 mol), dimethyl carbonate (149,84 g, 1663 mmol) and DABCO (667,5 g, 5,96 mmol) were heated at 75 °C for 16 h. Then methanol and excess dimethyl carbonate were removed by distillation. Glycerol carbonate **2** was obtained in quantitative yield and was used without further purification for the next step.

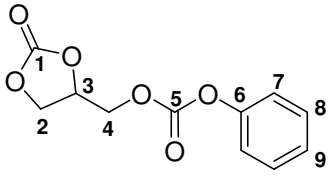


^{1}H-NMR (300 MHz, DMSO-d_6):
δ = 3.49 – 3.55 (m, 1 H, C*H*$_2$OH), 3.66 – 3.73 (m, 1 H, C*H*$_2$OH), 4.28 – 4.32 (m, 1 H, C*H*$_2$OCO$_2$), 4.46 – 4.52 (m, 1 H, C*H*$_2$OCO), 4.76 – 4.83 (m, 1 H, C*H*), 5.29 (s, br, O*H*) ppm.
^{13}C-NMR (75 MHz, DMSO-d_6):
δ = 60.5 (*C*H$_2$OH), 65.7 (*C*H$_2$OCO$_2$), 77.0 (*C*H), 155.2 (*C*=O) ppm.

(2-Oxo-1,3-dioxolan-4-yl)methyl phenyl carbonate (3a):

Glycerol carbonate **2** (49.8 g, 421.5 mmol) was dissolved in dry THF (500 mL) and pyridine (37.8 mL, 463.6 mmol). The solution was cooled to -5 °C and phenyl chloroformate (72.6 g, 463.7 mmol), dissolved in dry THF (70 mL), was slowly added during 1 h. The reaction was stirred at room temperature for 16 h. Pyridinium hydrochloride was removed by filtration and washed with THF. The solvent was removed under reduced pressure and the

crude product crystallized from $CHCl_3$. A colorless powder was obtained. Yield: 60.7 g, 60.4 %.



^{1}H-NMR (300 MHz, $CDCl_3$):

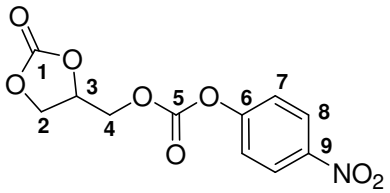
δ = 4.37 – 4.44 (m, 4 H, H-2, H-4), 4.52 – 4.64 (m, 2 H, H-2´, H-4´), 4.97 – 5.01 (m, 1 H, H-3), 7.17 – 7.30 (m, 3 H, H-7, H-7´, H-9), 7.38 – 7.43 (m, 2 H, H-8, H-8´) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$):

δ = 65.6 (C-2), 66.5 (C-4), 73.4 (C-3), 120.7 (C-7, C-7´), 126.4 (C-9), 129.5 (C-8, C-8´), 150.7 (C-6), 153.1 (C-5), 154.2 (C-1) ppm.

(2-oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate (3b):

Glycerol carbonate **2**, (15 g, 127 mol) was dissolved in dry THF (150 mL) and pyridine (11.5 mL, 141 mmol). The solution was cooled to -5 °C and 4-nitrophenyl chloroformate (28.2 g, 140 mmol), dissolved in dry THF (50 mL), was added slowly. The reaction was stirred at room temperature for 16 h. Pyridinium hydrochloride was removed by filtration and the solvent was removed by distillation at reduced pressure. The product was crystallized from $CHCl_3/Et_2O$ (7:1). Yield: 21.4 g, 59.6 %.



^{1}H-NMR (DMSO-d_6, 300 MHz):

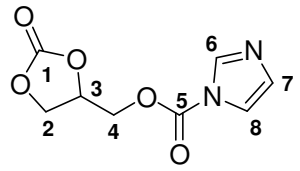
δ = 4.40-4.68 (kB, 4H, H-2, H-4), 5.13-5.20 (m, 1H, H-3), 7.59-7.62 (m, 2H, H-7), 8.32-8.36 (m, 2H, H-8) ppm.

^{13}C-NMR (DMSO-d_6, 75 MHz):

δ = 65.7 (C-2), 68.0 (C-4), 73.8 (C-3), 122.5 (C-7), 125.4 (C-8), 145.2 (C-9), 151.6 (C-5), 154.5 (C-1), 155.1 (C-6) ppm.

(2-oxo-1,3-dioxolan-4-yl)methyl 1-imidazole carbonate (3c):

1,1'-Carbonyldiimidazole (4.7 g, 29.0 mmol) was suspended in dry CH_2Cl_2 (50 mL) under N_2 and cooled to -5 °C and glycerol carbonate (3.11 g, 26.3 mmol) was added drop wise. The reaction mixture was stirred at room temperature for 4 h and then washed three times with H_2O. The organic phase was dried over $MgSO_4$, filtrated, the solvent was removed by distillation and the residual oil was dried under vacuum. Upon standing the oil crystallized. Yield: 3.79 g, 67.9 %



^{1}H-NMR (DMSO-d_6, 300 MHz):

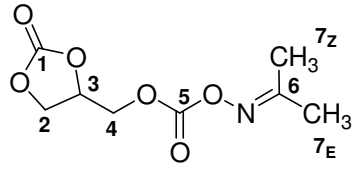
δ = 4.49 – 4.68 (m, 4H, H-2, H-4), 5.17 – 5.24 (m, 1H, H-3), 7.12 (s, 1H, H-8), 7.57 (s, 1H, H-7), 8.25 (s, 1H, H-6) ppm.

^{13}C-NMR (DMSO-d_6, 75 MHz):

δ = 66.42 (C-4), 67.37 (C-2), 74.34 (C-3), 117.89 (C-7), 130.93 (C-8), 137.67 (C-6), 148.33 (C-5), 155.09 (C-1) ppm.

(2-oxo-1,3-dioxolan-4-yl)methyl 4-(propan-2-ylideneaminooxy) carbonate (3d):

Glycerol carbonate chloroformate (14.95 g, 83 mmol) dissolved in 10 mL dry THF was added slowly to a solution of acetone oxime (5 g, 68 mmol) in dry THF (40 mL) and triethylamine (11 mL, 79 mmol), cooled to -5 °C. The reaction was stirred at room temperature for 19 h. After removing triethylamine hydrochloride by filtration and evaporation of THF a brown liquid was obtained. The crude product was crystallized from $CHCl_3$ / Et_2O. Yield: 5.74 g, 38.6 %.



^{1}H-NMR (DMSO-d_6, 300 MHz):

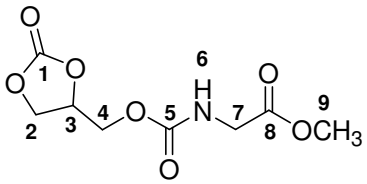
δ = 1.94 (s, 3H, H-7_E), 1.95 (s, 3H, H-7_Z), 4.27-4.48 (m, 3 H, H-2, H-4), 4.54-4.56 (t, 1H, H-2'), 5.03-5.10 (m, 1H, H-3) ppm.

^{13}C-NMR (DMSO-d_6, 75 MHz):

δ = 16.4 (C-7_Z), 21.0 (C-7_E), 65.7 (C-2), 66.9 (C-4), 74.0 (C-3), 152.7 (C-5), 154.5 (C-1), 164.8 (C-6) ppm.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-glycine methyl ester (6):

Glycine methyl ester hydrochloride (5 g, 39.9 mmol), carbonate coupler **3a** (9.5 g, 39.8 mmol) and DMAP (5.85 g, 47.8 mmol) were dissolved in acetonitrile (80 mL) and stirred at 40 °C for 4 h. The product was isolated as colorless oil by column chromatography (eluent: pentane/ethyl acetate 3:1 to 1:1). Yield: 7.3 g, 78.4 %



^{1}H-NMR (300 MHz, $CDCl_3$):

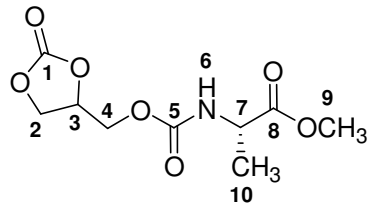
δ = 3.74 (s, 3 H, H-9), 3.93 (d, 2 H, H-7), 4.26 – 4.41 (kB, 3 H, H-2, H-4), 4.60 (t, 1 H, H-4'), 4.96 – 5.03 (m, 1 H, H-3), 5.87 (t, 1 H, H-6_Z), 6.20 (t, 1 H, H-6_E) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$):

δ = 42.6 (C-7), 52.3 (C-9), 63.9 (C-2), 66.1 (C-4), 74.7 (C-3), 155.1 (C-1), 156.1 (C-5), 170.6 (C-8) ppm.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-alanine methyl ester (7):

Alanine methyl ester hydrochloride (850 mg, 6.09 mmol), carbonate coupler **3a** (1.52 g, 6.38 mmol) and DMAP (893 mg, 7.31 mmol) were dissolved in acetonitrile (15 mL) and stirred at 40 °C for 6 h. The solvent was removed by distillation under reduced pressure and the product was isolated as colorless oil by column chromatography (eluent: pentane/ethyl acetate). Yield: 842 mg, 56.1 %, colorless oil.



^{1}H-NMR (300 MHz, $CDCl_3$):

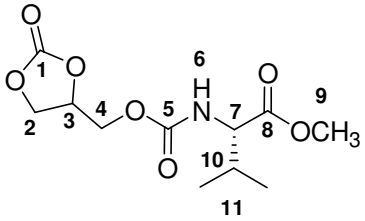
δ = 1.43 (d, 3H, H-10), 3.75 (s, 3H, H-9), 4.25 – 4.41 (m, 4H, H-2, H-4, H-7), 4.58 (t, 1H, H-4´), 4.97 (m, 1H, H-3), 5.61 (d, 1H, H-6_Z), 5.87 (d, 1H, H-6_E) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$):

δ = 17.9 (C-10), 49.6 (C-7), 52.3 (C-9), 63.4 (C-2), 65.7 (C-4), 74.2 (C-3), 154.6 (C-1), 154.9 (C-5), 173.1 (C-8) ppm.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-valine methyl ester (8):

Valine methyl ester hydrochloride (1.52 g, 9.07 mmol), carbonate coupler **3a** (2.16 g, 9.07 mmol) and DMAP (1.33 g,10.9 mmol) were dissolved in acetonitrile (25 mL) and stirred at 40 °C for 23 h. Acetonitrile was removed by distillation under reduced pressure and the crude product was dissolved in $CHCl_3$ (10 mL). After washing the organic phase with diluted sodium hydroxide and diluted hydrochloric acid, the product was isolated as colorless oil by column chromatography (eluent: pentane/ethyl acetate 3:1 to 1:1). Yield: 1.38 g, 55.3 %, yellowish oil.



^{1}H-NMR (300 MHz, $CDCl_3$):

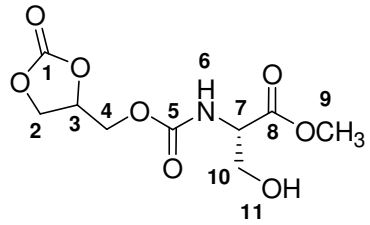
δ = 0.87 (d, 3 H, H-11), 0.93 (d, 3 H, H-11´), 2.13 (m, 1 H, H-10), 3.71 (s, 3 H, H-9), 4.18 – 4.37 (kB, 4 H, H-2, H-4, H-7), 4.53 (t, 1 H, H-4´), 4.91 – 4.97 (m, 1 H, H-3), 5.38 (d, 1H, H-6_Z), 5.61 (d, 1H, H-6_E) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$):

δ = 17.6 (C-11), 18.9 (C-11´), 31.1 (C-10), 52.3 (C-9), 59.3 (C-7), 63.7 (C-2), 66.0 (C-4), 74.3 (C-3), 154.7 (C-1), 155.6 (C-5), 172.2 (C-8) ppm.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-serine methyl ester (9):

Serine methyl ester hydrochloride (1.31 g, 8.39 mmol), carbonate coupler **3a** (2 g, 8.4 mmol), diisopropylethyl amine (1.5 mL, 8.8 mmol) and DMAP (102 mg, 0.84 mmol) were dissolved in DMF (45 mL) and stirred at 40 °C for 8 h. After adding an additional DMAP portion (924 mg, 7.56 mmol) the reaction was stirred at 40 °C for 8 h. The solvent was removed by distillation under reduced pressure and the product was isolated as colourless oil by column chromatography (eluent: pentane/ethyl acetate 3:1 to 1:1). Yield: 1.26 g, 57.3 %



^{1}H-NMR (300 MHz, DMSO-d_6):

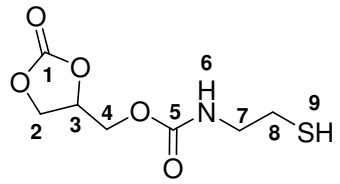
δ = 3.64 (s, 3 H, H-9), 3.66 (t, 2 H, H-10), 4.12 – 4.18 (kB, 1 H, H-7), 4.20 – 4.31 (kB, 3 H, H-2, H-4), 4.57 (t, 1 H, H-4´), 4.95 – 5.05 (m, 1 H, H-3), 7.19 (s, br, 1 H, H-6_Z), 7.63 – 7.66 (d, 1 H, H-6_E) ppm.

^{13}C-NMR (75 MHz, DMSO-d_6):

δ = 51.8 (C-9), 56.6 (C-7), 61.0 (C-10), 63.4 (C-2), 65.7 (C-4), 74.6 (C-3), 154.6 (C-1), 155.5 (C-5), 171.0 (C-8) ppm.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-cysteamine (10):

Cysteamine hydrochloride (1 g, 8.88 mmol), carbonate coupler **3a** (4 g, 8.57 mmol) and triethyl amine (1.3 mL, 9.4 mmol) were dissolved in THF (20 mL) under nitrogen atmosphere and stirred at 40 °C for 5 h. The solvent was removed by distillation under reduced pressure and the product was isolated by column chromatography (eluent: $CHCl_3$/acetone). Yield: 902.4 mg, 47.6 %



^{1}H-NMR (300 MHz, $CDCl_3$):

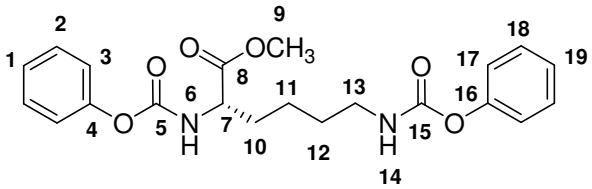
δ = 1.43 (t, 1 H, H-9), 2.57 – 2.65 (q, 2 H, H-8), 3.27 – 3.34 (q, 2 H, H-7), 4.18 – 4.33 (kB, 3 H, H-2, H-4), 4.53 (t, 1 H, H-4´), 4.88 -4.95 (m, 1 H, H-3), 5.46 (s, br, 1 H, H-6_Z), 5.79 (t, 1 H, H-6_E) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$):

δ = 24.5 (C-8), 44.1 (C-7), 63.5 (C-2), 66.1 (C-4), 74.5 (C-3), 154.9 (C-1), 155.7 (C-5) ppm.

N,N'-bis-(phenoxycarbonyl)-lysine methyl ester (11):

Lysine methyl ester dihydrochloride (1.0 g, 4.29 mmol), diphenylcarbonate (1.83 g, 8.54 mmol) and DMAP (1.26 g, 10.30 mmol) were dissolved in acetonitrile (40 mL) and stirred for 14 h at 40 °C. The solvent was evaporated; the crude product was dissolved in $CHCl_3$ and washed with 5% NaOH (3x 20 mL) and 5% HCl (3x 20 mL). The organic phase was dried over $MgSO_4$ and concentrated. Finally the white product was washed twice with hexane and dried in high vacuum. Yield: 1.08 g (68.6 %)



^{1}H-NMR (300 MHz, $CDCl_3$):

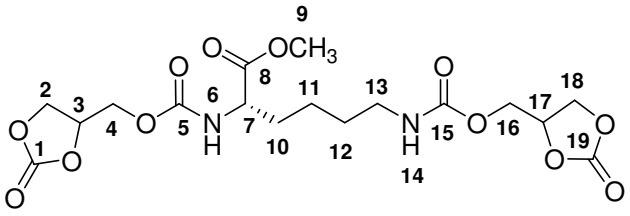
δ = 1.43–1.66 (m, 4 H, H-11, H-12), 1.71–1.83 (m, 1 H, H-10), 1.87–1.99 (m, 1 H, H-10´), 3.16–3.27 (m, 2 H, H-13), 3.78 (s, 3 H, H-9), 4.39–4.46 (m, 1 H, H-7), 5.03 (t, 1 H, H-14_Z), 5.21 (t, 1 H, H-14_E), 5.52 (d, 1 H, H-6_Z), 5.71 (d, 1 H, H-6_E), 7.11–7.21 (m, 6 H, H-1, H-3, H-3´, H-17, H-17´, H-19), 7.31–7.36 (m, 4 H, H-2, H-2´, H-18, H-18´) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$):

δ = 22.3 (C-11), 29.2 (C-12), 32.1 (C-10), 40.7 (C-13), 52.6 (C-9), 53.8 (C-7), 121.6 (C-3, C-3´, C-17, C-17´), 125.3, 125.5 (C-1, C-19), 129.3 (C-2, C-2´, C-18, C-18´), 150.8, 151.0 (C-4, C-16), 154.4, 154.8 (C-5, C-15), 172.7 (C-8) ppm.

N,N'-bis-(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-lysine methyl ester (12):

Lysine methyl ester dihydrochloride (1.3 g, 5.59mmol), carbonate coupler (**3a**, 2.66 g, 11.17 mmol) and DMAP (1.64 g, 13.38 mmol) were dissolved in acetonitrile (30 mL) and stirred for 16 h at 40 °C. DMAP hydrochloride precipitated at room temperature was removed by filtration and the solvent was evaporated. The crude product was dissolved in $CHCl_3$ and washed with 5% NaOH (3x 20 mL) and 5% HCl (3x 20 mL). The solution was dried over $MgSO_4$, concentrated and the oily product was dried in high vacuum. Yield: 340.0 mg (13.6 %, the product contains phenol and DMAP)



^{1}H-NMR (300 MHz, $CDCl_3$):

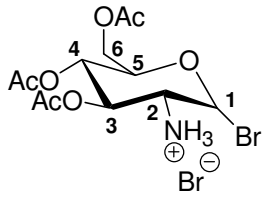
δ = 1.42 – 1.89 (m, 4 H, H-11, H-12), 1.73 – 1.76 (m, 1 H, H-10), 1.85 – 1.90 (m, 1 H, H-10´), 3.20 – 3.24 (m, 2 H, H-13), 3.78 (s, 3 H, H-9), 4.25 – 4.40 (m, 7 H, H-2, H-4', H-16', H-18, H-7), 4.59 (t, 2 H, H-4, H-16), 4.98 (m, 2 H, H-3, H-17), 5.53 (m, 1 H, H-14), 5.98 (m, 1 H, H-6) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$):

δ = 22.1 (C-11), 29.1 (C-12), 31.4 (C-10), 40.1 (C-13), 52.5 (C-9), 53.9 (C-7), 63.5 (C-2, C-18) 66.0 (C-4, C-16), 74.6 (C-3, C-17), 154.8 – 156.2 (C-1, C-5, C-15, C-19), 172.6 (C-8) ppm.

Tri-O-acetyl-2-amino-2-deoxy-β-D-glucopyranosyl bromide hydrobromide (13):

Acetyl bromide (48 mL, 648 mmol, destilled) was added to D-glucosamine hydrochloride (15.0 g, 69.6 mmol, dried 24 h in vacuo at 70 °C over P_2O_5) and the mixture was stirred for 3 days at room temperature. Residual acetyl bromide was removed in vacuo (water aspirator) and the crude product was dissolved in hot chloroform and filtered while still hot. Et_2O was added to the solution and the product precipitated after cooling. The product was filtrated, washed with cold chloroform and Et_2O to obtain a white powder. Yield: 23.9 g (67.6 %)



^{1}H-NMR ($CDCl_3$, 300 MHz):

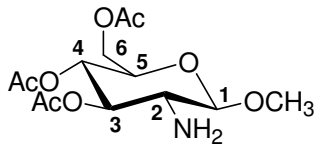
δ = 2.00 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.18 (s, 3H, OAc), 3.87-3.90 (dd, 1H, H-2), 4.06-4.09 (d, 1H, H-6'), 4.19-4.29 (m, 2H, H-5, H-6), 5.13-5.19 (t, 1H, H-4), 5.38-5.44 (t, 1H, H-3), 7.01-7.03 (d, 1H, H-1), 8.59 (s, br, 2H, NH) ppm.

^{13}C-NMR ($CDCl_3$), 75 MHz):

δ= 21.8 (OAc), 20.5 (OAc), 20.7 (OAc), 54.3 (C-2), 60.7 (C-6), 66.6 (C-4), 69.9 (C-3), 72.8 (C-5), 85.3 (C-1), 169.2 (OAc), 170.4 (OAc), 171.5 (OAc) ppm.

Methyl tri-O-acetyl-2-amino-2-deoxy-β-D-glucopyranoside (14):

Hydrobromide **13** (7.0 g, 15.6 mmol) was dissolved in dry methanol (120 mL) and dry pyridine (1.5 mL, 18.6 mmol) was added. After 75 min of stirring at r.t., toluene (45 mL) was added and the mixture was concentrated. The yellow residue was dissolved in $CHCl_3$ (300 mL) and washed with Na_2CO_3(aq) (5 %, 2x 150 mL), water (2x 150 mL) and dried over $MgSO_4$. The solvent was removed and the residue was dried in vacuo. Yield: 4.07 g (81.7 %)



^{1}H-NMR (DMSO-d_6, 300 MHz):

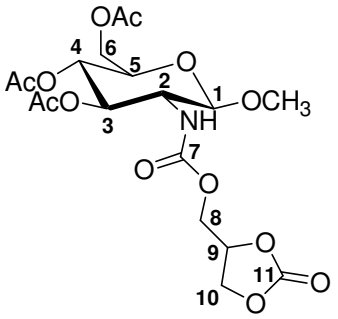
d = 1.52 (s, 2H, NH), 1.95 (s, 3H, OAc), 1.98 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.57-2.63 (dd, J=8.0, 10.0 Hz, 1H, H2), 3.41 (s, 1H, OCH3), 3.77-3.83 (ddd, J=2.3, 5.0, 10.0 Hz, 1H, H5), 3.96-4.01 (dd, J=2.5, 12.4 Hz, 1H, H6'), 4.15-4.2 (dd, J=5.1, 12.2 Hz, 1H, H6) ,4.26-4.28 (d, J=7.9 Hz, 1H, H1), 4.76 (t, J=9.6 Hz, 1H, H4), 4.92 (t, J=9.6 Hz, 1H, H3) ppm.

^{13}C-NMR (DMSO-d_6, 75 MHz):

δ = 20.9 (OAc), 21.0 (OAc), 21.1 (OAc), 56.3 (C-2), 56.8 (OCH3), 62.5 (C-6), 69.3 (C-4), 71.1(C-5), 75.2 (C-3), 104.8 (C-1), 169.9 (CO), 170.4 (CO), 170.5 (CO) ppm.

Methyl tri-O-acetyl-2((2-oxo-1,3-dioxolan-4-yl)methoxy)carbonylamino 2-deoxy-β-D-gluco-pyranoside (15) (Method A):

Carbonate coupler **3b** (521 mg 2,19 mmol) was dissolved in CH_2Cl_2 (15 mL) and stirred at 50 °C. The mixture of methyl tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranoside (**14**) (700 mg, 2.19 mmol) and 1-hydroxy benzotriazole (296 mg, 2.19 mmol) were added in CH_2Cl_2 dropwise for 30 min. The reaction was heated for 9 h at 50 °C, then stirred at room temperature. More carbonate coupler (**3b**, 260 mg, 1.09 mmol) was added after 48 h and the solution was stirred at 50 °C for 7.5 h and left at room temperature over night. The mixture was diluted with CH_2Cl_2 (30 mL), washed with 1 N NaOH, H_2O, 1 N HCl, H_2O, dried and concentrated. Column chromatography with $CHCl_3$/acetone (4:1) gave **15** as white solid. Yield: 423.1 mg, 41.6 %



^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 1.96 (s, 3H, OAc), 1.98 (s, 3H, OAc), 2.02 (s, 3H, OAc), 3.46 (s, 3H, OMe), 3.49-3.52 (m, 1H, H-2), 3.63-3.67 (m, 1H, H-5), 4.05-4.10 (dd, 1H, H-6'), 4.19-4.28 (kB, 5H, H-6, H8', H-10), 4.43-4.56 (2 H, H-1, H-8), 4.83 (m, 1H, H-9), 4.99 (t, 1H, H-4), 5.16-5.29 (2H, H-3, N*H*) ppm

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 20.6, 20.7 (OAc), 56.3 (C-2), 57.2(OMe), 62.0 (C-6), 63.4, 63.8 (C-10), 65.8 (C-8), 65.9 (C-4), 68.7 (C-4), 71.7, 72.0 (C-3, C-5), 74.3 (C-9), 101.6, 101.8 (C-1), 155.1 (C-7, C-11), 169.5, 170.8 (COAc)-ppm.

Methyl tri-O-acetyl-2((2-oxo-1,3-dioxolan-4-yl)methoxy)carbonylamino 2-deoxy-β-D-gluco-pyranoside (15) (Method B):

Methyl tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranoside (**14**) (3 g, 9.4 mmol) was dissolved in dry THF (70 mL) and dry pyridine (830 µL, 10.3 mmol). The mixture was cooled

to -5 °C and glycerol carbonate chloroformate (**5**) was added dropwise. The mixture was stirred at room temperature over night. Then pyridinium hydrochloride was removed by filtration, THF was removed by distillation, and the crude product was dissolved in chloroform, washed with H_2O (2x), brine (1x) and dried over $MgSO_4$. The solvent was evaporated and the residue dried in vacuo. Yield: 4.07 g, 93.6 %.

^{1}H-NMR (DMSO-d_6, 300 MHz):
δ = 1.93 (s, 3H, OAc), 1.97 (s, 3H, OAc), 2.02 (s, 3H, OAc), 3.37 (s, 3H, OMe), 3.45 (m, 1H, H-2), 3.77-3.80 (m, 1H, H-5), 4.01-4.04 (dd, 1H, H-6'), 4.16-4.26 (kB, 5H, H-6, H8', H-10), 4.45-4.55 (2 H, H-1, H-8), 4.83 (t, 1H, H-4), 4.98-5.07 (m, 2H, H-3, H-97.54-7.57 (1H, N*H*) ppm.

Evaluation of the couplers reactivity:

Each coupler was reacted at room temperature with an equimolar amount of valine methyl ester hydrochloride (0.6 mmol/mL) and DMAP. At different time intervals samples were analyzed by NMR spectroscopy. $CDCl_3$ and DMSO-d_6 were used as solvents. The conversion was calculated from the signal intensity of the proton at C3 of the cyclic carbonate. The intensity of this signal in the investigated couplers **3a-d** was compared to the same signal in the product N-(glycerol carbonate) valine methyl ester **7**.

Comparison of hexylamine and glycine methyl ester reactivity:

Both amines were dissolved in $CDCl_3$ at a concentration of 0.47 mmol/mL each, in the case of competitive conditions and 0.34 mmol/mL in the case of non-competitive conditions and carbonate coupler (**3a**) and triethylamine was added. At different time intervals samples were analyzed by NMR spectroscopy. The conversion for glycine methyl ester was calculated from the signal intensity of the α-CH_2-group in the starting material and in the corresponding functional coupler. For hexylamine the conversion was calculated from the methyl signal in the educt and from the methylene group vicinal to the carbamate in the product.

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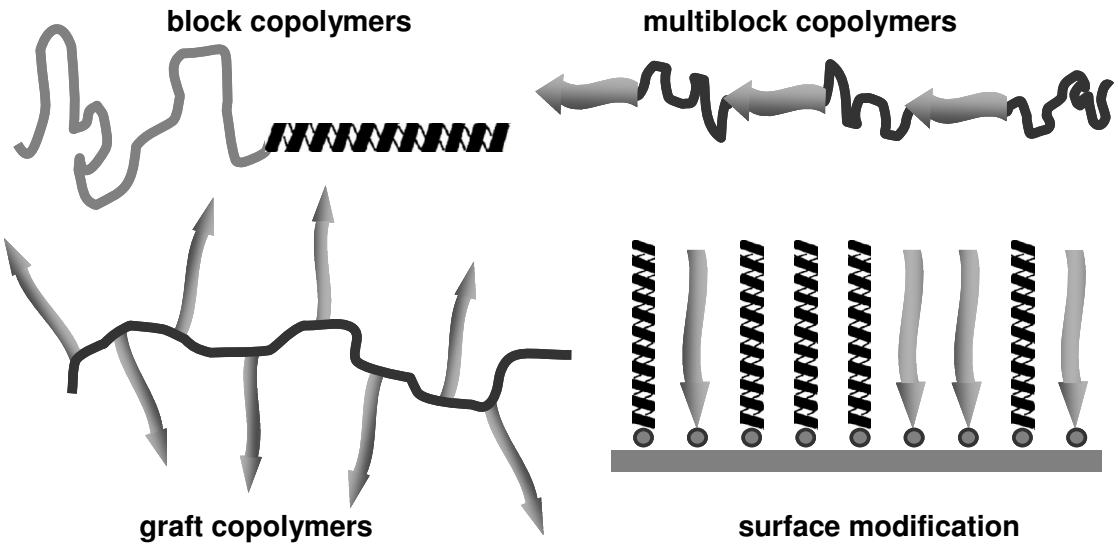
Chapter 5 Investigations on the coupling of amino acids or peptides with polymers

5.1 Introduction

Among macromolecules, peptides and proteins represent a highly sophisticated class of compounds with a broad variety of structures (α-helix, β-sheet), functions (enzymes, chaperones, scleroproteins), molecular weights and a large variation of composition. The combination of natural and synthetic macromolecules is of interest because of the combination of special properties of the components and synergistic effects. This is especially important in the field of pharmaceutical application of peptides and proteins as drugs, where PEGylation increases the *in vivo*-life time or where such conjugates are discussed as self-assembled vehicles for drug transport.[1, 2] Other applications are medical devices, where such compounds improve the surface properties or make bioanalysis and bioseparation of enzymes and other analytes possible.[3] Beside certain fields of application, peptide/protein-polymer conjugates receive attention as objects of scientific research from polymer chemists because of their ability to aggregate. Selected examples of the preparation and the structural versatility (see Scheme 19) of these hybrid materials will be given within the next paragraph.



Scheme 19 Architectures of peptide/protein conjugates

A peptide-polymer triblock copolymer was achieved by connecting the cell adhesion promoting peptide sequence GRGDS via reductive amination to electrospun nanofibres of PEG-*b*-PDLLA copolymer.[4] The finished fibres revealed cell adhesive properties, whereas the unconjugated fibres did not. A tetrapeptide-polyisoprene conjugate was used to arrange diacetylene units in solution.[5] The diacetylene unit was coupled to the free N-terminus of the hybrid polymer and could be polymerised according to the "self-assemble, then polymerize" strategy via the previous formation of a parallel β-sheet. Maleimide-functionalised PEG was used to bind monodisperse peptides obtained through protein engineering.[6, 7] Telechelic-peptides with cysteine residues on the N- and C-terminus were used to be coupled with maleimide functionalised PEG. The telechelic-peptides represent a sequence similar to the crystalline part of *Bombyx mori* silk fibroin, namely $([Ala\text{-}Gly]_3\text{-}Glu\text{-}Gly)_n$ (n=10 or 20). AFM and TEM pictures revealed the formation of fibres, which the authors explained by the formation of an antiparallel β-sheet. Nanoscale structures were also formed by AB-diblock and ABA-triblock copolymers, where (A) is a PEG block of ~2600 Da and (B) being an amphiphilic β-strand peptide sequence.[8] It could be shown that the assembling ability of the peptide is retained upon conjugation and that lamellar superstructures with alternating PEG

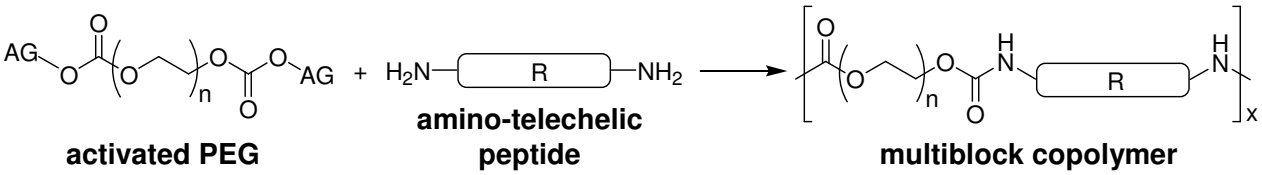
and antiparallel β-sheet domains are formed. Nature produces all kinds of structures with very simple sequences like fibroin and collagen, but with highly organised structure. In the class of scleroproteins elastin is another fascinating protein, which is in its natural form difficult to apply for any purpose. However, solid phase peptide synthesis (SPPS) and protein engineering make elastin-mimetic sequences (e.g. VPGVG) easily accessible and support the investigation on the inverse temperature transition (ITT) behaviour.[9] The lower critical solution temperature could be preserved in elastin-based side chain polymers, where the pentapeptide VPGVG was attached to 2-isocyanatoethyl methacrylate, which was afterwards polymerised.[10]

Due to the special properties of peptide-polymer conjugates it was our goal to investigate different coupling strategies and to prepare different architectures of conjugates. First, the preparation of multiblock copolymers with an elastin-mimetic block and a PEG block will be discussed. Such polymers should be able to associate via hydrophobic interactions as it was shown for amphiphilic copolymers of PEG and tyrosine-derived diphenols.[11, 12] Other peptide-PEG-multiblock copolymers were designed with an enzyme substrate sequence that on one hand offers biodegradability and on the other hand can be used to incorporate drugs to the polymer, which are released upon degradation.[13] Second, the conjugation of amino acid methyl esters to mPEG and PEG, (i) via activated PEG-carbonate; and (ii) via succinylated PEG will be described. Third, the immobilisation of alanine methyl ester and the enzyme lysozyme to acrylic polymers again via phenyl carbonate groups will be discussed.

5.2 Results and Discussion

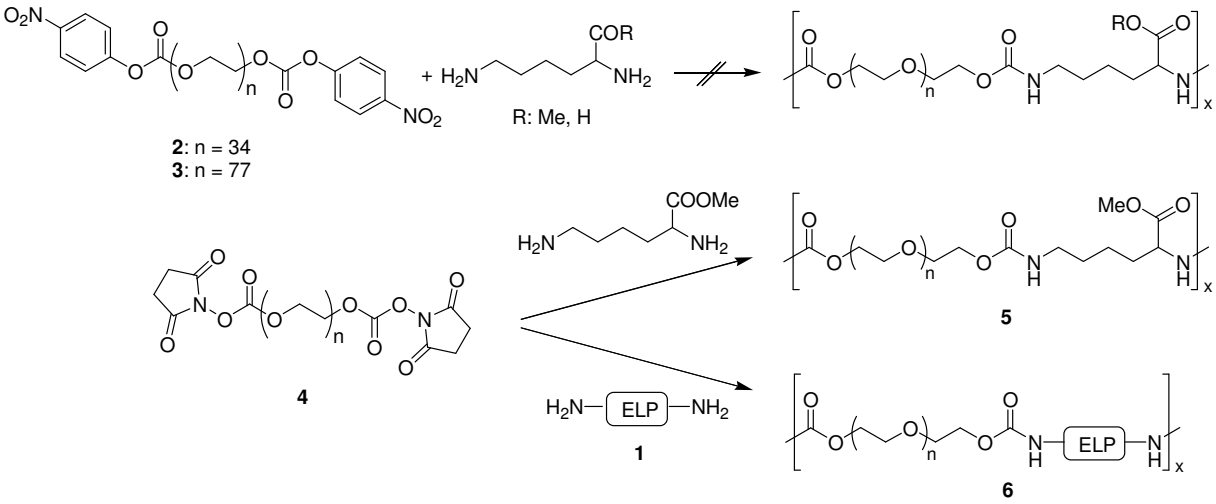
5.2.1 Preparation of multiblock copolymers with elastin-mimetic peptides

The aim was to prepare multiblock copolymers with peptide and poly(ethylene glycol) (PEG) blocks via polycondensation (see Scheme 20).



Scheme 20 Preparation of multiblock copolymers from an activated PEG (AG: activating group) and an amino-telechelic peptide

The activated PEG and the amino-telechelic components were prepared starting from hydroxy-telechelic PEG and an elastin-mimetic polymer (ELP). Model reactions were performed using lysine and lysine methyl ester as simple amino-telechelic compounds. An overview about the applied polymers and resulting conjugates is given in Scheme 21.



Scheme 21 Overview about synthetic pathways to multiblock copolymers

Synthesis of the amino-telechelic peptide

Starting with an elastin-mimetic polymer (ELP), and trypsin as active enzyme a number of peptides were obtained (Figure 28). Beside the typical elastin sequence (VPGXG), where X is isoleucine in the applied ELP, the polymer contains VPGKG blocks with lysine instead of isoleucine and therefore can be enzymatically degraded with trypsin (VPGK↓G). Due to the carboxy-terminal degradation at the lysine (K) residues (see Figure 28) amino-telechelic elastin-like peptides are obtained.

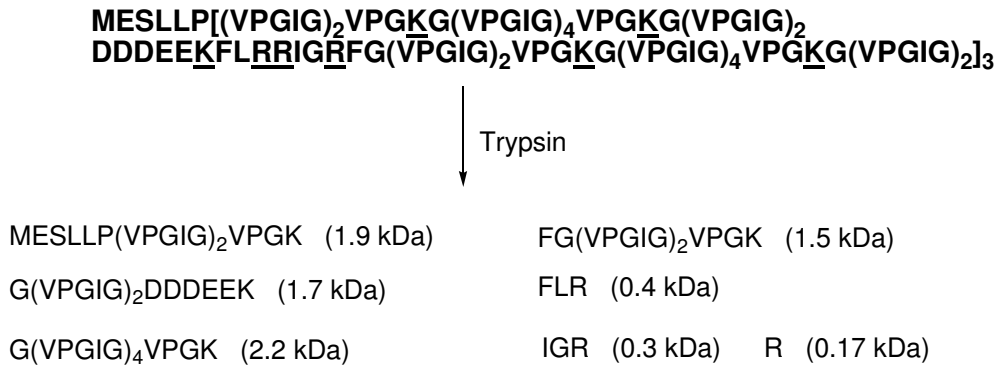


Figure 28 Degradation of the elastin-mimetic polymer with trypsin: ELP peptides **1** with their molecular weights

Trypsin is applied in a substrate-enzyme ratio of 50:1 (w/w) at its optimal operating pH and optimal operating temperature. Since trypsin is a serine protease, which also cleaves proteins after arginine (R) residues, the peptide mixture obtained also contains peptides without lysine at the carboxy-terminus. The mixture of peptides obtained were analysed by MALDI-TOF MS (see Figure 29).

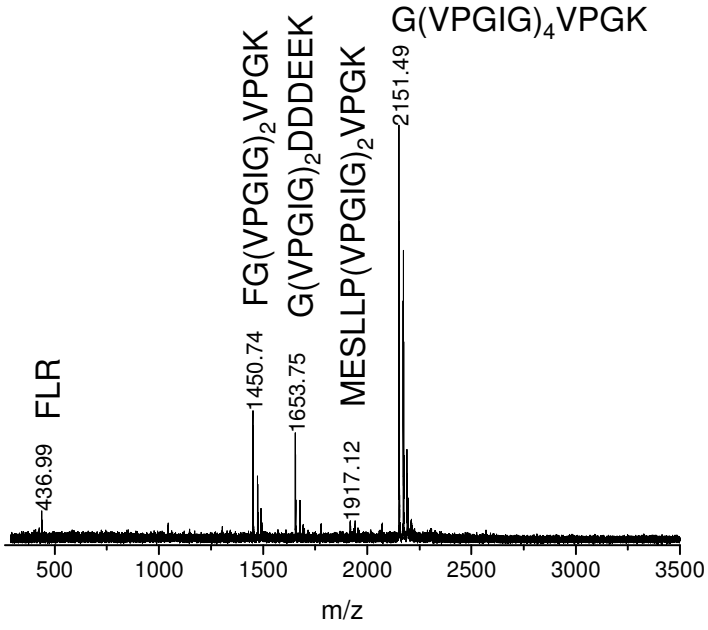


Figure 29 MALDI-TOF MS of the peptide mixture (**1**) obtained via degradation of an elastin-mimetic polymer with trypsin

Because trypsin also contains lysine residues and could therefore also react with the activated prepolymer, different attempts were made to remove the enzyme: At first, precipitation with 5 % trichloro acetic acid (TCA) - by adding the cold acid drop wise to the peptide solution - was tested. However, after intensive cooling no precipitation occurred. Filtration with a centrifuge filter was chosen as an easy and time-saving procedure. A membrane filter of poly(ether sulfone) (PES) with a molecular-weight-cut-of (MWCO) of 5 kDa was used at 10.000 rpm for 15 min and the filter was afterwards washed twice with buffer. Although PES membranes adsorb less protein as e.g. membranes made from cellulose (and the MWCO of the membrane filter is much lower than the molecular weight of trypsin (23.8 kDa)), the filtrate still contained trypsin and peptides were adsorbed on the membrane. Since the original polymer shows inverse temperature transition (ITT) behaviour it was tried to precipitate the peptides by increasing the temperature. Although it is theoretically believed that also elastin mimetic peptides have a lower critical solution temperature,[14] the obtained peptides did not precipitate upon heating. This observation is in accordance with the results of Pechar et al. on thermoresponsive self-assembly of short elastin-like polypentapeptides.[15] The last purification method used to remove the enzyme from the peptide mixture was a chromatographic method. PD-10 columns, with Sephadex 25 gel, which normally serve as desalting columns, were chosen due to the large difference of the molecular weight of trypsin and the elastin peptides. However, analyses of the obtained fractions revealed that trypsin could not be removed. Therefore the obtained peptides were purified using a Superdex 75 column and again analysed by SDS-PAGE: First purification was performed with 0.05 M NH_4HCO_3 buffer and 4 fractions were obtained, that all contained trypsin. Chromatographic purification was repeated with 0.05 M sodium phosphate buffer containing 0.15 M NaCl and on-line with desalting columns. SDS-PAGE confirmed the absence of trypsin (see Figure 30).

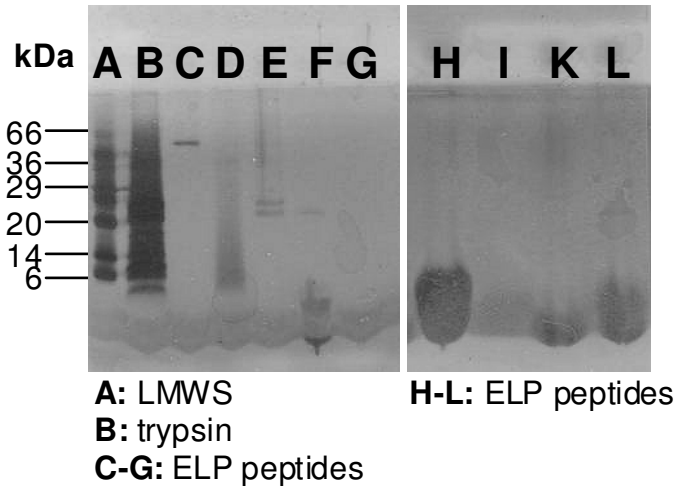


Figure 30 SDS-PAGE of purified ELP peptides: Fraction G and fractions I to fraction L are trypsin-free

During the purification attempts and the analyses by SDS-PAGE it was observed, that trypsin solution in Lämmli buffer had to be prepared in a modified manner: Trypsin usually shows two intense bands (24 kDa and 14 kDA) and some minor bands arising from autolysis.[16] Upon heating in Lämmli buffer additional bands are present, which made comparison with the neighbouring lines difficult. Therefore the trypsin reference solution was prepared in the buffer without heating, so that only certain bands were present.

Before the chromatographically purified peptides were introduced in the synthesis of multiblock copolymers lysine and lysine methyl ester were used as diamino telechelic model compounds for the peptide block. Poly(ethylene glycol), activated as 4-nitrophenyl carbonate and N-succinimidyl carbonate, served as activated PEG block.

Synthesis of the activated PEG

Di(4-nitrophenyl carbonate) PEG (Di(NPC)PEG) was prepared starting from PEG_{1500} and PEG_{3400}, which were dried at 60 °C in vacuo prior to the reaction. Both polymers were modified using 4-nitrophenyl chloroformate, whereas $Di(NPC)PEG_{1500}$ was modified in THF using pyridine as acid scavenger and $Di(NPC)PEG_{3400}$ was prepared in CH_2Cl_2 with Et_3N as

base. The resulting polymers were yellowish powders that were analysed by NMR spectroscopy and the corresponding spectra are shown in the following (see Figure 31):

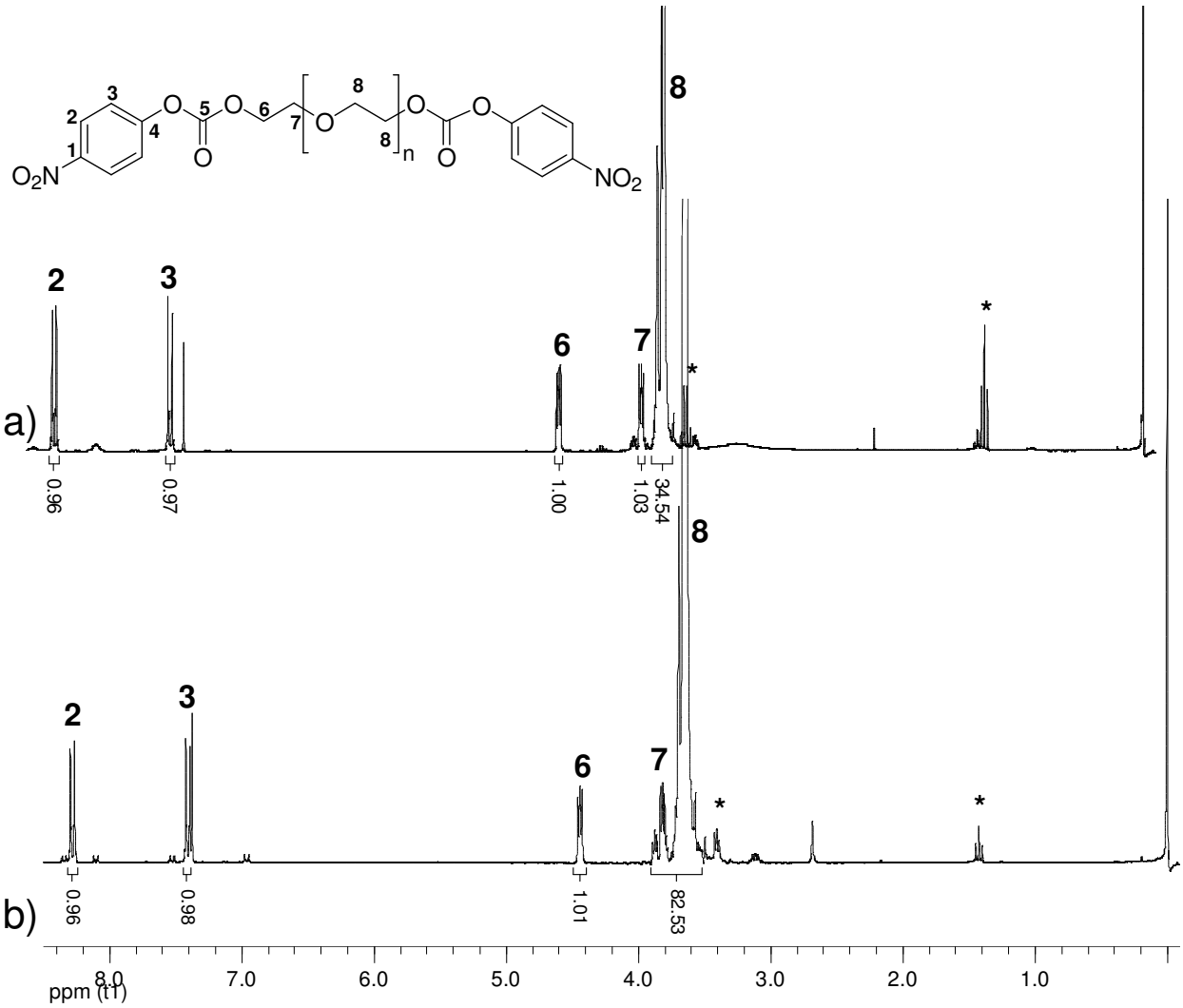


Figure 31 ^{1}H-NMR spectra of di(4-nitrophenyl carbonate)PEG, a) Di(NPC)PEG1500 **2** in $CDCl_3$; b) di(NPC)PEG3400 **3** in DMSO-d_6; *:Et_2O

The corresponding di(N-succinimidyl carbonate)PEG_{1500} **4** was prepared from PEG_{1500} in acetonitrile with di(N-succinimidyl carbonate) (DSC) using 4-dimethylamino pyridine (DMAP) as catalyst and pyridine as acid scavenger.[13] The product was isolated after precipitation in ether and NMR spectroscopy confirmed the conversion of the pendant hydroxy groups into the carbonate. The molecular weight and the molecular weight distribution of the resulting polymer were determined via MALDI-TOF MS and GPC in THF

(see Figure 32). The MALDI-TOF mass spectrum revealed the presence of a fraction of starting material. Whether this is due to unmodified material or hydrolysis during sample preparation for the analysis is unclear.

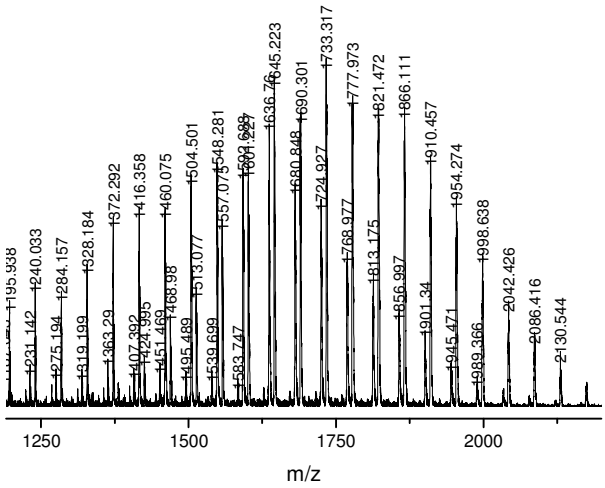
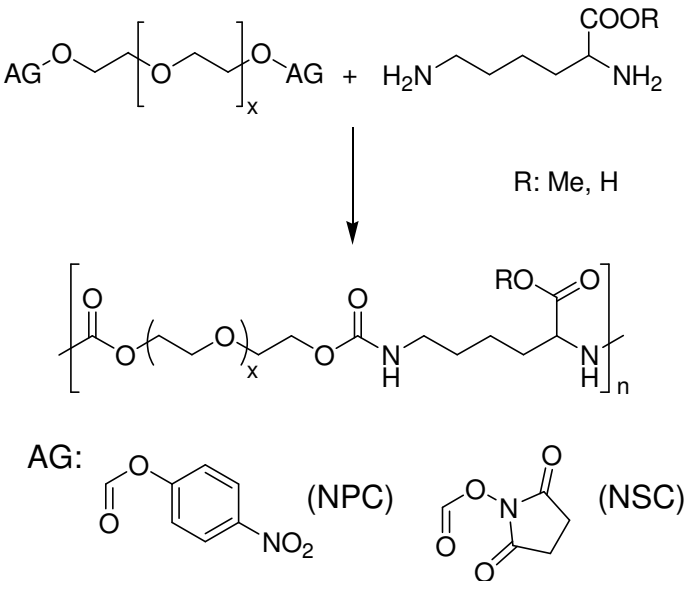


Figure 32 MALDI-TOF mass spectrum of Di(NSC)PEG1500 4

Interfacial polycondensation of activated PEGs with diamines

The preparation of multiblock copolymers was tested with lysine methyl ester dihydrochloride and lysine dihydrochloride as model compounds for α,ε-amino telechelic peptides (see Scheme 22). The reactions were performed in a mixture of dichloromethane and aqueous alkaline solution as described for the condensation of the tripeptide GluLysGlu(*OBz*) with Di(N-succinimidyl carbonate)PEG. Since multiblock copolymers with a molecular weight of 15000-30000 g/mol were obtained (starting from PEG with 2000 g/mol), this method was adapted for our purpose.[13] With lysine ethyl ester even higher molecular weights were obtained.[17] However, with Di(NPC)PEG no multiblock copolymer was observed, which was confirmed by GPC measurements in THF and via NMR spectroscopy in $CDCl_3$. The results were the same for Di(NPC)PEG_{1500} and Di(NPC)PEG_{3400}, as well as for lysine or lysine methyl ester.



Scheme 22 Reaction scheme for the preparation of multiblock copolymers from di(NPC)PEG_{1500} **2**, di(NPC)PEG_{3400} **3** or di(NSC)PEG_{1500} **4** with lysine or lysine methyl ester

Further experiments were performed with Di(NSC)PEG_{1500}, again with lysine methyl ester and lysine, but either at ambient temperature or at 40 °C. Moreover the organic solvent was varied, as well as the applied base. Reactions were performed either at interfacial conditions in CH_2Cl_2 with aqueous $NaHCO_3$ at ambient temperature or at 40 °C in CH_3CN with KOH or with Et_3N in CH_3CN or in bulk. The molecular weights of the obtained multiblock copolymers **5** from lysine methyl ester were within 18700 and 62000 g/mol (see Table 10), which was confirmed by GPC analysis in THF (see Figure 33).

Table 10 Multiblock copolymers from Di(NSC)PEG_{1500} and lysine methyl ester

entry	conditions	Mw (GPC, THF)
1	CH_2Cl_2, 4.6 eq $NaHCO_3$aq, r.t., 16h	18700-49600
2	CH_3CN, 4.2 eq KOHaq, 40°C, 22h	33000
3	CH_2Cl_2, 4.6 eq $NaHCO_3$aq, 40°C, 22h	29900
4	Et_3N, 40°C, 18h	28600
5	CH_3CN, 2.5 eq Et_3N, 40°C, 24h	61900

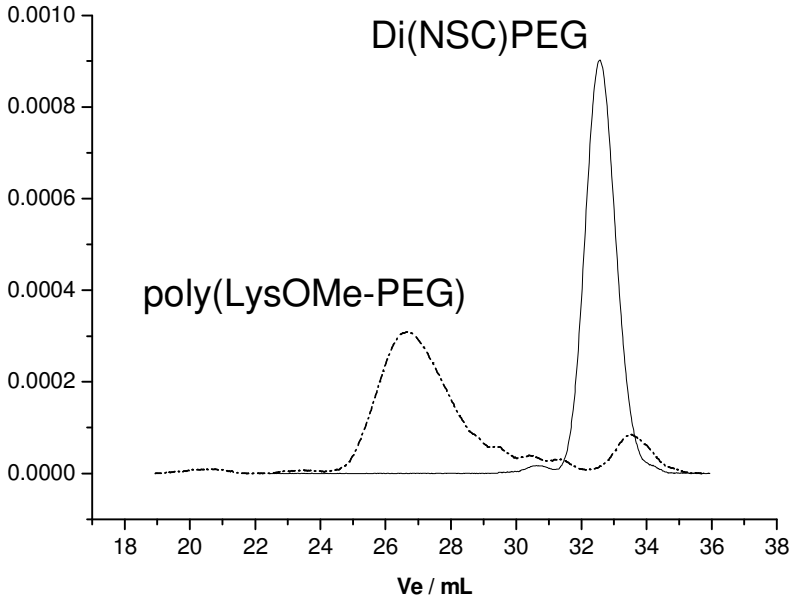


Figure 33 GPC analysis of lysine methyl ester PEG copolymer **5**, prepared via interfacial condensation reaction, Mw=49600 (entry 1)

The NMR spectrum of the product is shown in Figure 34.

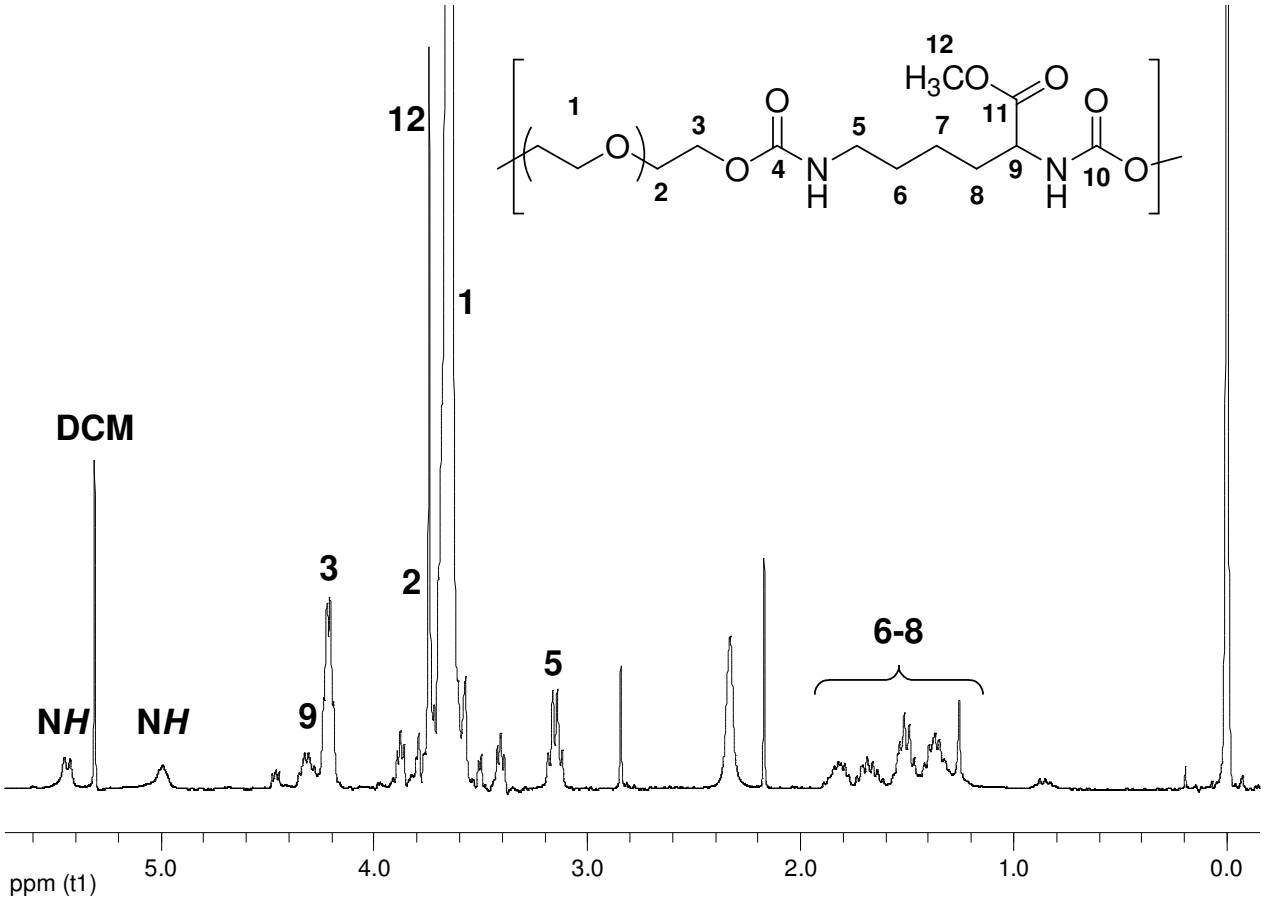


Figure 34 ^{1}H-NMR spectrum of lysine methyl ester PEG copolymer **5** in $CDCl_3$

The signals in the multiplett region between $\delta = 1.26$ ppm and $\delta = 1.82$ ppm are assigned to the methylene groups of the polymerised lysine methyl ester (H-6, H-7, H-8), as well as the signals at $\delta = 3.15$ ppm (H-5). The singulett at $\delta = 3.60$ ppm derives from the ethylene glycol units (H-1, H-2), the signal of H-3 appears at $\delta = 4.21$ ppm and the multiplett at $\delta = 4.32$ ppm is of the α-proton H-9. The signals of the urethane groups appear at $\delta = 4.99$ ppm and $\delta = 5.44$ ppm, respectively.

Reactions with lysine were also performed via interfacial condensation in CH_3CN with different bases. However, compared to the corresponding ester, the products had lower molecular weights and performance of a blind reaction without amino acid revealed that condensation of Di(NSC)PEG takes place: First, reactions were performed in CH_2Cl_2 and aqueous $NaHCO_3$ or aqueous Na_2CO_3 at room temperature. In both cases only molecular

weights between 5700 and 11150 g/mol were obtained. Moreover NMR analysis revealed that no urethane signal was present in the spectra. Therefore two blind reactions were performed: The prepolymer was stirred at room temperature either in a mixture of CH_2Cl_2 and aqueous $NaHCO_3$ or in a mixture of CH_2Cl_2 and aqueous Na_2CO_3. The product was analysed after work-up by ^{1}H-NMR spectroscopy and by GPC in THF. The chromatograms showed two fractions – one with a molecular weight similar to the starting material; the other fraction with a Mw around 5500 g/mol was a coupling product.

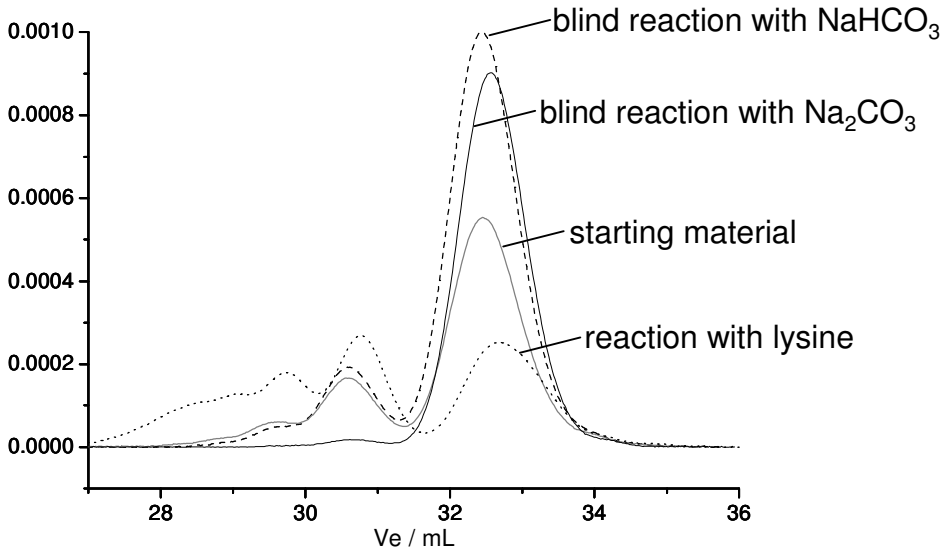


Figure 35 Comparison of GPC traces of different samples

The homopolymer was formed due to hydrolysis of the succinimidyl carbonate. The hydroxy end group of the PEG then reacted with remaining N-succinimidyl carbonate groups to form oligomers via ester bond formation.

Interfacial polycondensation of Di(NSC)PEG$_{1500}$ with ELP peptides

Purified peptides (see Figure 30, fraction K and fraction L) were applied in the polycondensation with Di(NSC)PEG$_{1500}$ in a mixture of acetonitrile and aqueous KOH at 40 °C. Products were then analysed by SDS-PAGE with the original ELP peptide mixture as reference. In a first experiment ELP peptides that still contained traces of trypsin were used.

Analysis revealed the formation of a high-molecular weight compound with more than 66 kDa (see Figure 36, left, line E). A high-molecular weight compound was also formed at r.t (see Figure 36, left, line C). The analysis of the enzyme trypsin is in line A and the analysis of the ELP peptides in line D and line F, which are not depicted as single band due the low molecular weight. In case of the formation of conjugates it could not be ruled out that the band aroused from PEGylated trypsin, even though the concentration of trypsin compared to the ELP peptides was low. The experiment was repeated with trypsin-free fractions and with trypsin alone. The products were analysed via SDS-PAGE without further purification (see Figure 36, right).

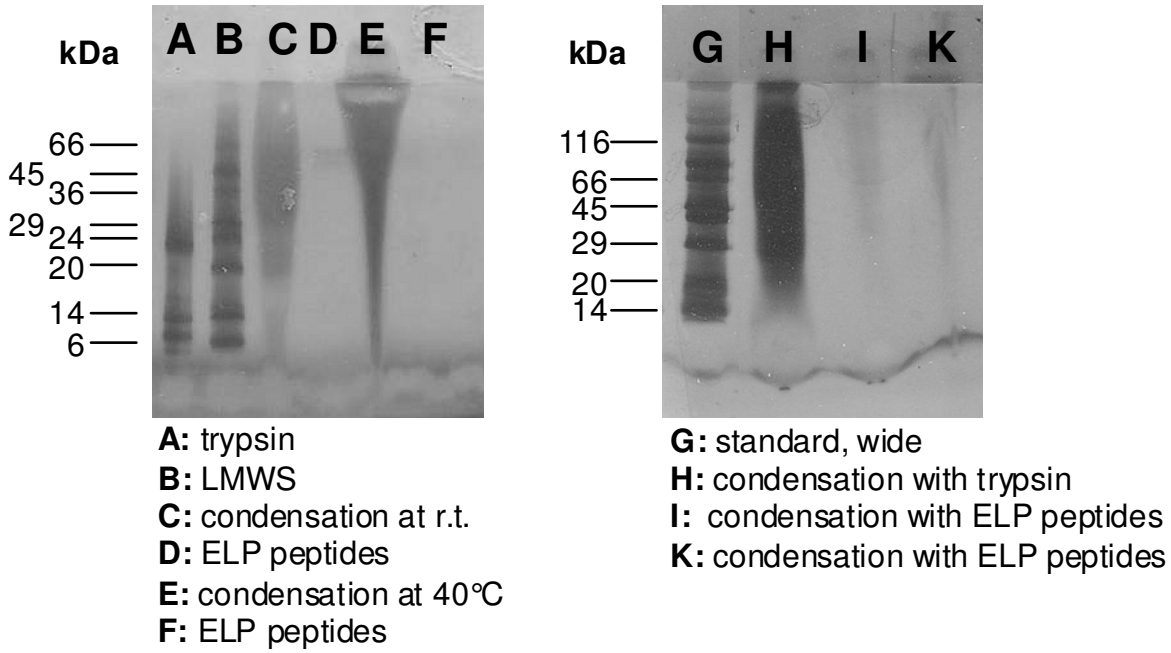


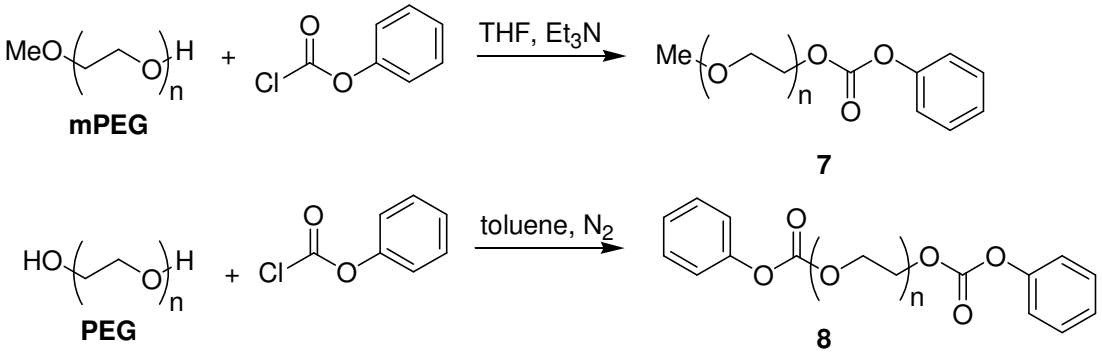
Figure 36 SDS-PAGE of products obtained from conjugation of ELP peptides (**6**, line I and line K) and trypsin (line H) with Di(NSC)PEG_{1500} (LMWS: low molecular weight standard)

The analysis of the condensation of trypsin with Di(NSC)PEG is in line H, the one from the condensation of ELP peptides in line I and line K. It can be seen that also with trypsin a conjugate is formed just as with ELP peptides albeit the bands in line I and line K are weak.

5.2.2 Endcapping of mPEG and PEG with amino acids

For the preparation of linear hybrid block copolymers poly(ethylene glycol) (PEG) was chosen as the synthetic polymer. PEG is a water-soluble polymer, which is available in different molecular weights and a variety of end-group functionalities. Severable routes for the preparation of peptide/protein-PEG-conjugates were described.[18]

The hydroxy group of $mPEG_{2000}$ and PEG_{400} was activated with phenyl chloroformate to prepare the corresponding phenyl carbonate derivatives (see Scheme 23).



Scheme 23 Activation of mPEG and PEG with phenyl chloroformiate

$mPEG_{2000}$-PC **7** was prepared in toluene by removing hydrogen chloride with a constant nitrogen flow;[19] and di(PC)-PEG_{400} **8** was prepared in THF by using Et_3N as acid scavenger.[20] The ^{1}H-NMR spectrum of $mPEG_{2000}$-PC **7** is shown in Figure 37.

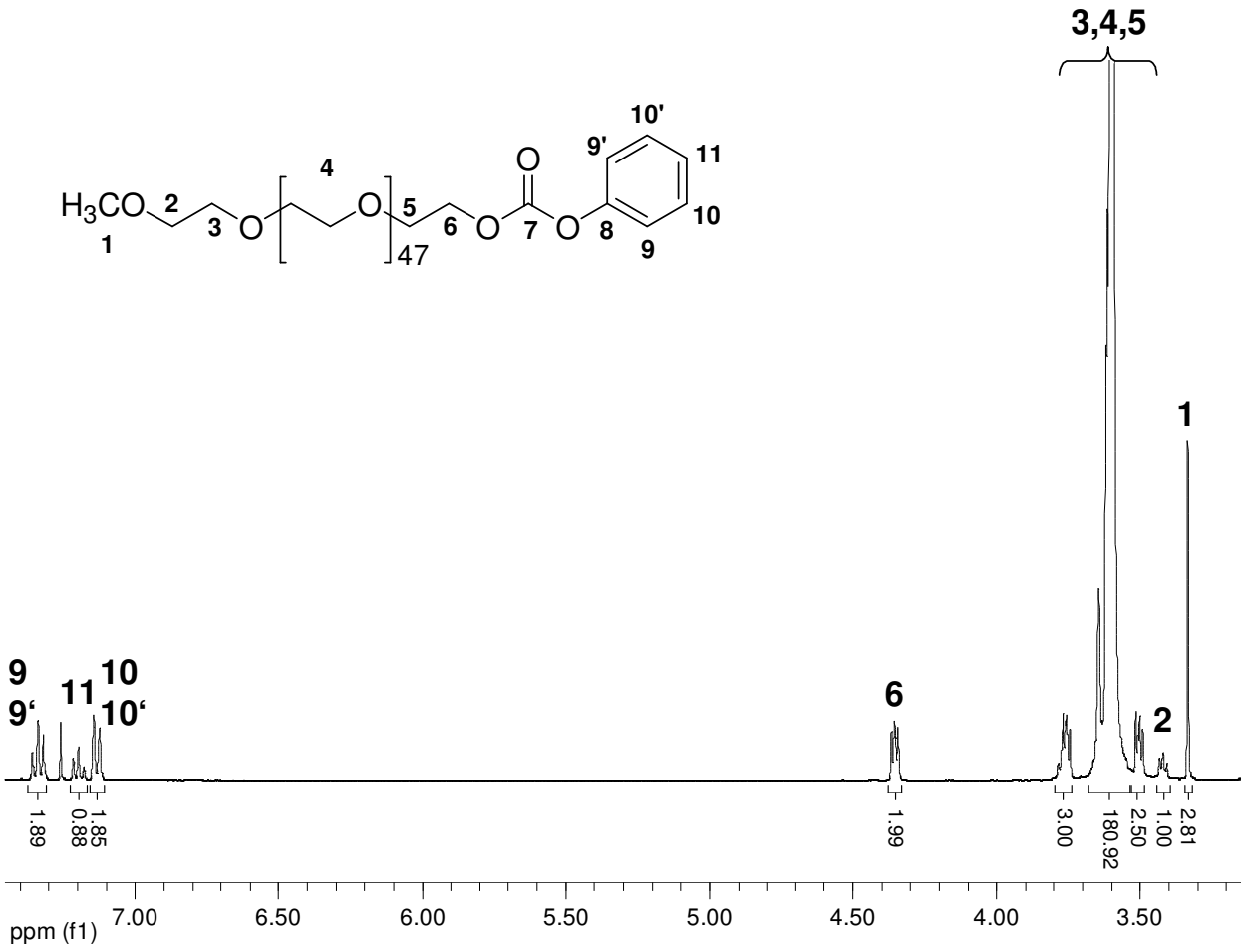


Figure 37 ^{1}H-NMR spectrum of methoxy poly ethylene glycol phenyl carbonate ($mPEG_{2000}$-PC) **7**

The singulett of the methoxy group appears at δ= 3.33 ppm, the peaks between δ= 3.42 ppm and δ= 4.35 ppm are assigned to the ethylene glycol chain and the peaks of the phenyl carbonate group are at low field (δ= 7.12 ppm and δ= 7.34 ppm). Reaction with 2 equivalents of glycine methyl ester, a derivative of the simplest amino acid and therefore the most nucleophilic one, revealed slow reaction in DMSO. At room temperature no conversion was observed, at 80°C after 4 h only 26.0 % conversion was achieved. This is much slower than 1-hexylamine that was reacted in another reaction with Di(PC)PEG_{400} **8** in $CDCl_3$ and could already be converted at room temperature after 3 h to 70 %. To evaluate the dependence of substrate and conversion on the solvent used and the base equivalents the following experiments were performed: An equimolar amount of alanine methyl ester, a derivative of

the simplest enantiomeric amino acid, and $mPEG_{2000}$-PC **7** was reacted in $CDCl_3$ at 50 °C and in DMSO-d_6 at 70 °C with 1.2 eq and 2 eq DMAP. DMAP has already been proven to be a good choice for condensation of amino acid methyl esters.[21] The results from the four reactions are depicted in Figure 38.

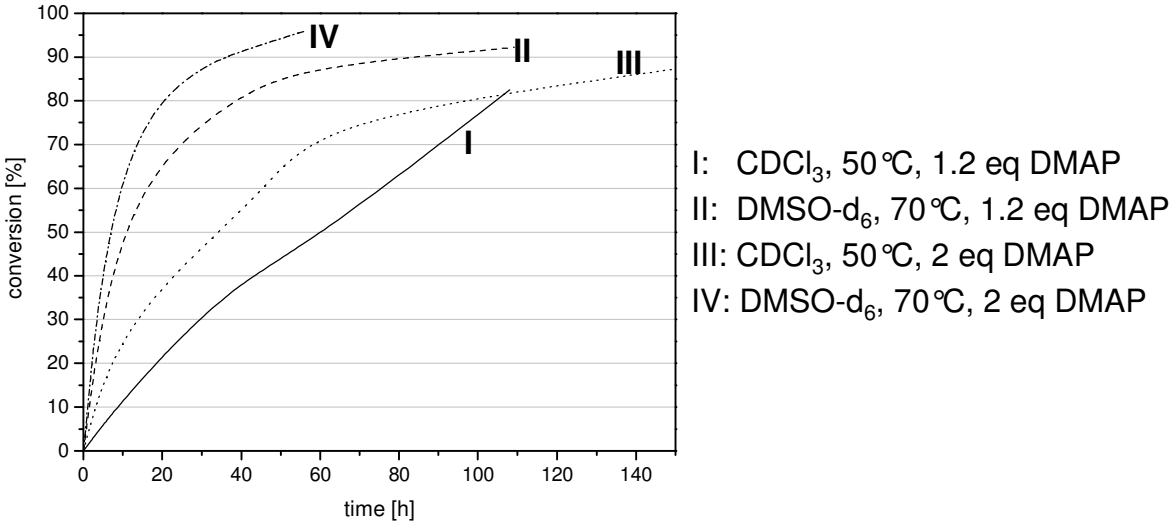


Figure 38 Conversion of alanine methyl ester with $mPEG_{2000}$-PC **7** in the presence of DMAP

The reaction proceeded the fastest in DMSO-d_6 at 70 °C with 2 eq of DMAP (curve IV). With 1.2 eq DMAP the conversion is only a little slower (curve II). Performance of the reaction in $CDCl_3$ proceeds the slowest with the lower amount of base (curve I).

Differences in the reactivity of amino acid methyl esters (AAME) were evaluated by reacting the methyl esters of glycine, alanine, valine, serine and cysteine with Di(PC)-PEG_{400} **8** in $CDCl_3$ in the presence of DMAP at 60 °C. The amount of conjugated amino acid was determined by amino acid analysis (AAA) and compared with the applied amount and the remaining amount of AAME (see Figure 39).

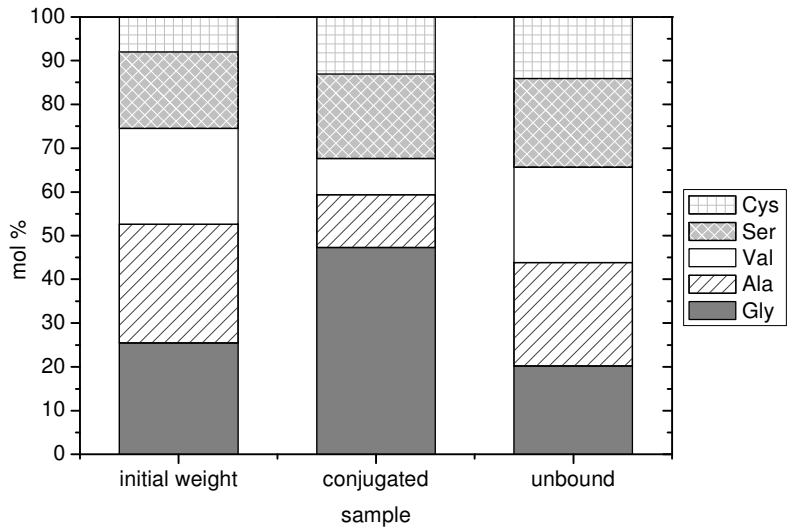
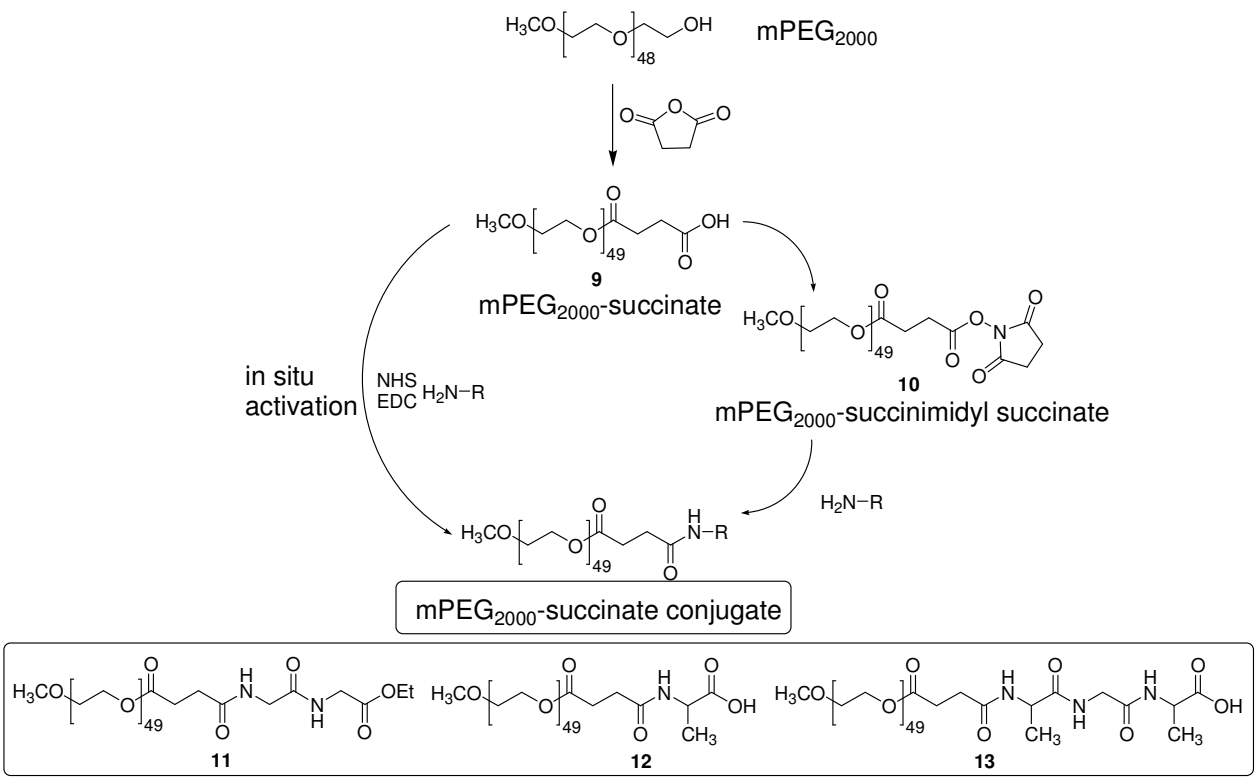


Figure 39 Conjugation of AAMEs with Di(PC)-PEG$_{400}$ **8**

The determination shows that glycine methyl ester is the component which was coupled to the highest extent to the activated PEG. This is reasonable since the nucleophilicity of the amino group is not affected by steric effects or bulky side group. Serine and cysteine methyl ester were also coupled in a relatively high extent, which might also be due to reaction of the hydroxy group and the thiol group with Di(PC)-PEG$_{400}$ **8**. Valine methyl ester was coupled to a lower extent than alanine methyl ester, which is probably arising from the larger steric hindrance by the iso-propyl group compared to the methyl group. From these results it can be concluded that a coupling strategy peptides is favourable with glycine at the N-terminus.

Another strategy was followed by introducing a carboxylic group into mPEG$_{2000}$ using succinic anhydride.[22] The obtained mPEG$_{2000}$-succinate **9** served either as starting material to prepare mPEG$_{2000}$-succinimidyl succinate – an activated ester - or could be used for in situ conjugation with amino acid derivatives (see Scheme 24).



Scheme 24 Preparation of $mPEG_{2000}$ conjugates (**11**, **12**, **13**) using $mPEG_{2000}$-succinate **9**

The first derivative $mPEG_{2000}$-succinate was prepared in THF with succinic anhydride using DMAP as base and the product was further modified via carbodiimide chemistry with N-hydroxy-succinimide (NHS) and DCC in THF. Both products were analysed via ^{1}H-NMR spectroscopy and the spectra are depicted in Figure 40.

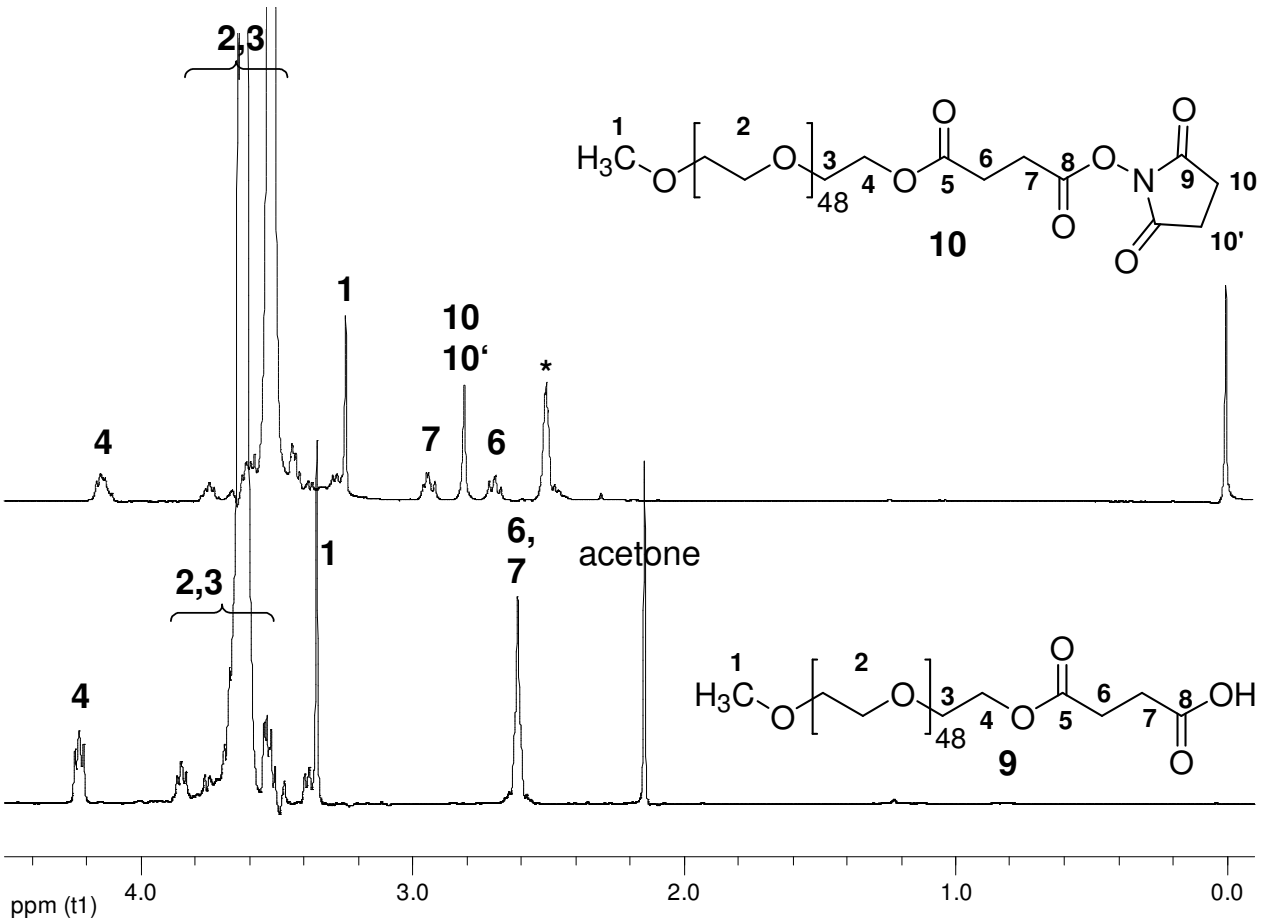


Figure 40 ^{1}H-NMR spectra of mPEG$_{2000}$-succinate **9** in $CDCl_3$ and mPEG$_{2000}$-succinimidyl succinate **10** in DMSO-d_6 (*: starting material, here **9**)

The conversion of mPEG$_{2000}$ into the corresponding succinate was complete, however the subsequent activation as NHS ester could only be perfomed to an extent of 80 %, probably due to the high tendency of the NHS ester to hydrolyse. Nonetheless the polymer was applied as precursor for the conjugation with glycinylglycine ethyl ester, alanine and the tripeptide alanine-glycine-alanine (AGA): The conjugate with GlyglyOEt was prepared in DMSO-d_6 using DMAP as acid scavenger. The product mPEG$_{2000}$-GlyglyOEt-succinate **11** was obtained as a mixture of the conjugate and mPEG$_{2000}$-succinate. The structure was analysed by means of ^{1}H-NMR spectroscopy, GPC in THF and MALDI-TOF MS, which clearly indicates the presence of two fractions (see Figure 41) with mPEG$_{2000}$-succinate **9** being the minor fraction.

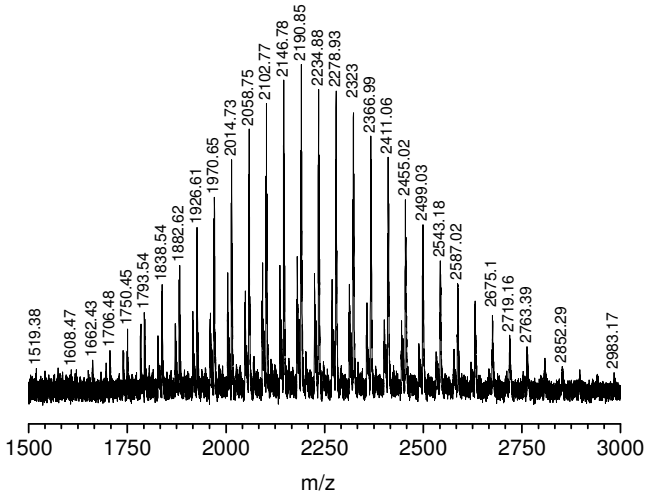


Figure 41 MALDI-TOF mass spectrum of $mPEG_{2000}$-GlyglyOEt-succinate **11** ([M+Na]=2278.9) (side product mPEG-succinate **9**)

Preparation of conjugates with alanine and AGA in aqueous Na_2CO_3-solution and a mixture of THF and aqueous Na_2CO_3, respectively, was not successful and resulted only in the hydrolysis of the NHS ester into the original succinate derivative. In order to prepare such conjugates, $mPEG_{2000}$-succinate **9** was activated in situ using the water-soluble carbodiimide EDC and again NHS. This polymer was added to the amino component, which was in one case alanine that was coupled in aqueous solution at r.t. over night. The product was isolated by extraction with $CHCl_3$. The peptide AGA was the amino component in the other case, which was brought to reaction under similar reaction conditions. Both products were analysed by ^{1}H-NMR spectroscopy (see Figure 42).

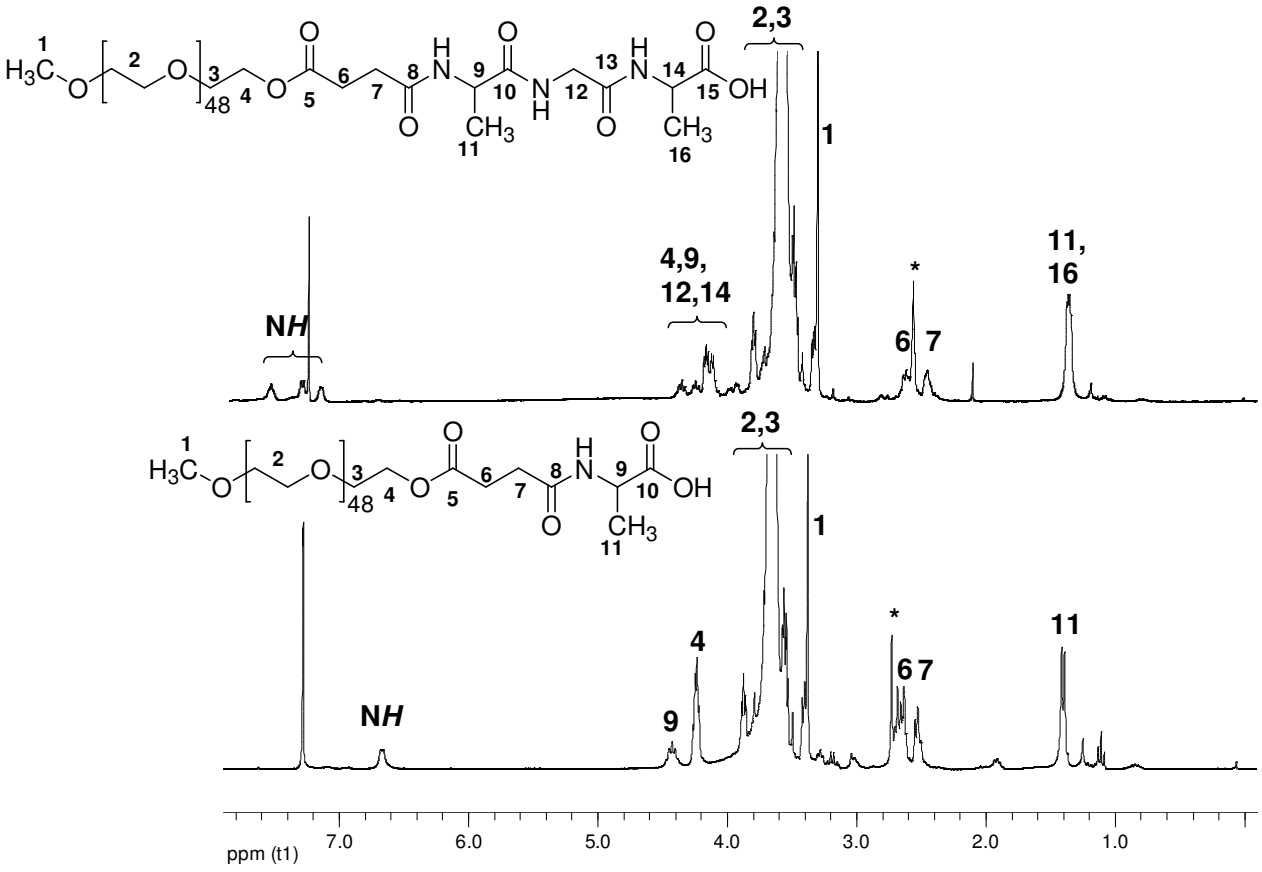


Figure 42 ^{1}H-NMR spectra of mPEG$_{2000}$-Ala-succinate **12** and mPEG$_{2000}$-AGA-succinate **13** in $CDCl_3$ (*: mPEG$_{2000}$-succinate)

The ^{1}H-NMR spectra show that it was possible to couple alanine and the peptide AGA to the mPEG-succinate via in situ activation. The signals at low chemical shift are assigned to the methyl group of the alanine residue. At $\delta = 2.5$ - 2.7 ppm appear the peaks of the methylene groups (H-6, H-7) of the succinate unit in the product (two multipletts) and in the starting material (one singulett). The singulett at $\delta = 3.3$ ppm and the broad peak at $\delta = 3.6$-3.9 ppm derive from the methoxy (H-1) and the ethylene unit (H-2) of the polymer. In the multiplett region between δ =4.15-4.4 ppm arise the signals of H-4 and the α-protons in the amino acid and the peptide block. The peak of the urethane and peptide group appear at $\delta = 6.67$ ppm and $\delta = 7.18$-7.57 ppm, respectively.

5.2.3 Grafting of amino acids and lysozyme to polymethacrylates

Polymethacrylates are a class of polymers, which are available in broad variety of structures – homopolymers and copolymers – and therefore properties. Poly(hydroxyethyl methacrylate) PHEMA is a water-soluble polymer that was converted into an amino-reactive polymer by activating the alcoholic group as phenylcarbonate group.[23] The aim here was to couple amino acids and derivatives thereof to the activated polymer. This was achieved by using alanine methyl ester hydrochloride and DMAP as base and reaction at 50°C. Even though the reaction proceeded slowly (see Figure 43), a degree of functionalisation of 93.8 % was achieved.

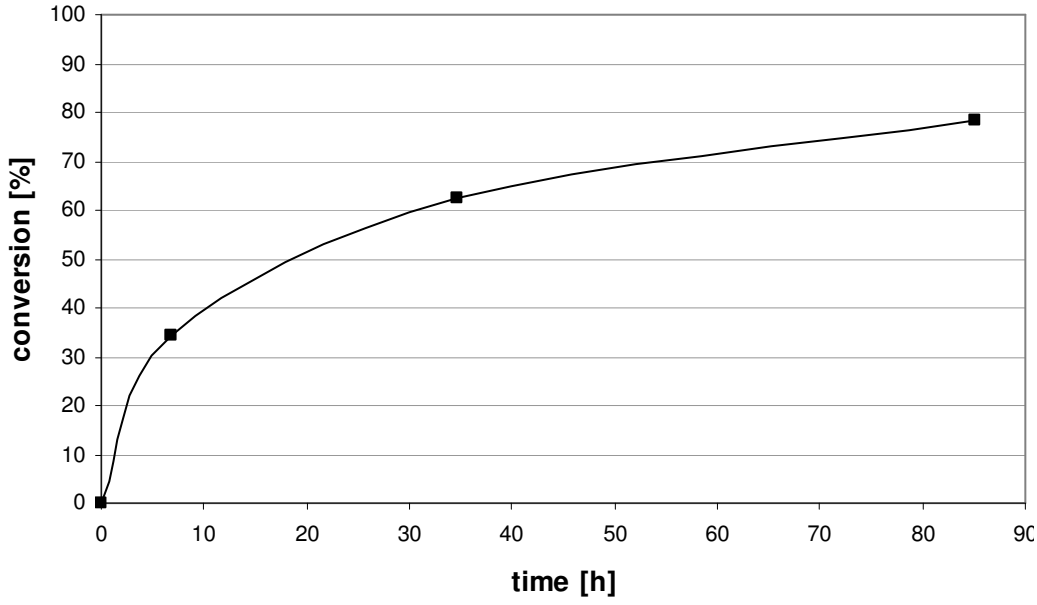


Figure 43 Conversion of alanine methyl ester in the polymer analogues reaction with poly[2-(phenoxycarbonyloxy)ethyl methacrylate] P(PCEMA) **14**

The product still contained unreacted phenyl carbonate groups, which opens the possibility to introduce further functional groups. The ^{1}H-NMR spectrum is shown in Figure 44 with the signals of the aromatic group H-14 to H-16 are at low field.

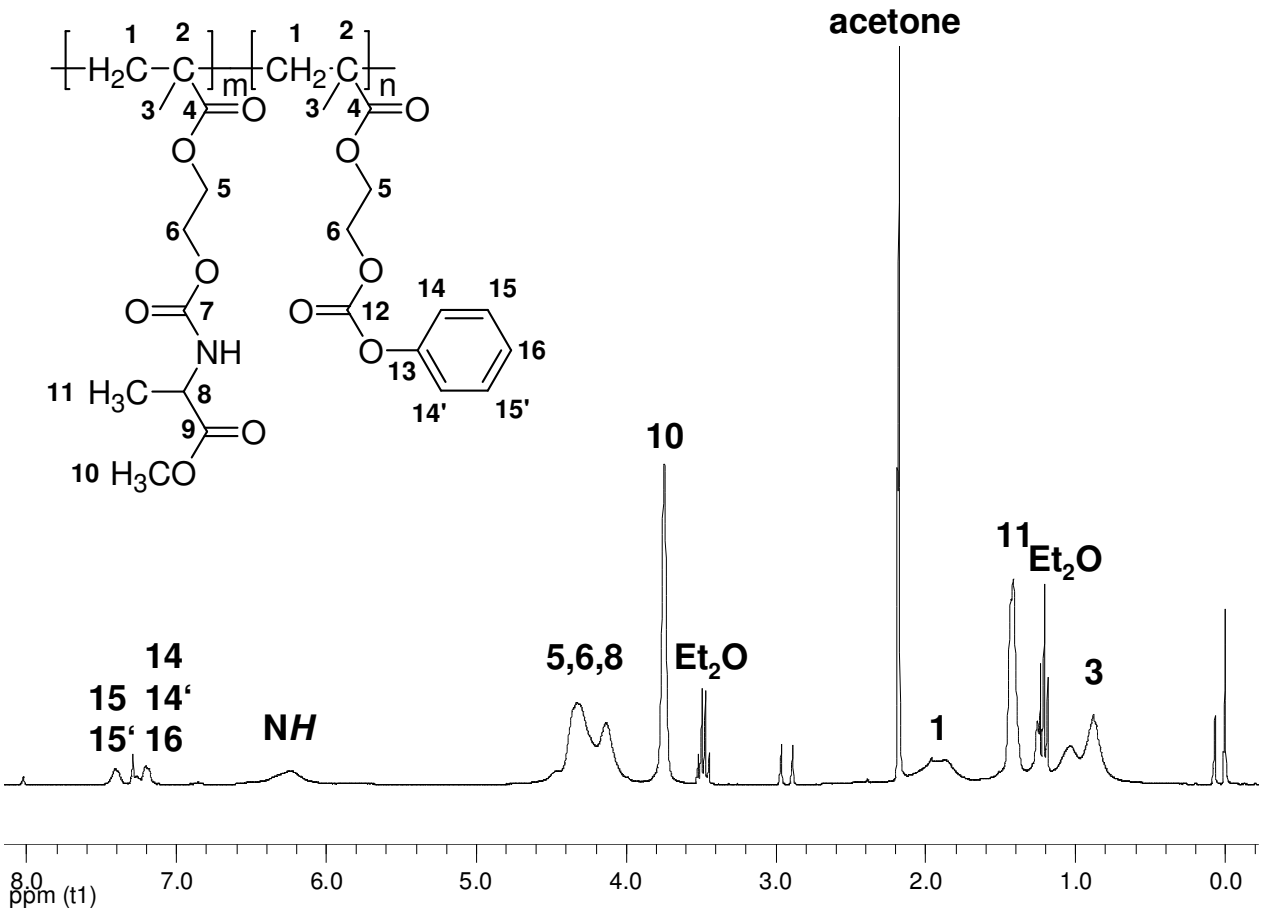


Figure 44 ^{1}H-NMR spectrum of **14** in $CDCl_3$

The signals of the polymer backbone appear as broad peaks in the region of δ=0.88-1.04 ppm (H-3) and δ=1.87-1.96 ppm (H-1). The peaks at δ=1.42 ppm, δ=3.75 ppm are attributed to the methyl (H-11) and the methoxy (H-10) group of the immobilised alanine methyl ester. The signal of the α-proton appears in the same region (δ=4.13-4.33 ppm) as the peaks of H-5 and H-6. By comparing the synthesis performed with Et_3N and DMAP, respectively, it was observed that the reaction proceeds faster in the presence of DMAP. Attempts to bind free amino acids (Ala, Gly, Ser, Tyr, Val) or short peptides (AGA, GAG) to P(PCEMA) failed due to differences in solubility, which made homogenous reactions impossible. At heterogeneous reaction conditions, using THF and H_2O, the usually water-soluble amino acid precipitated.

In order to combine properties of synthetic and natural polymers we attempted to immobilise lysozyme to an acrylic copolymer with different side-groups, one of them being an amino-reactive phenyl carbonate group equal to the functional group of P(CEMA). Lysozyme is an enzyme found in egg-white with a molecular weight of 14.4 kDa, which catalyses the degradation of 1,4-β-linked peptidoglycans in the cell wall of prokaryotes, which leads to distruction of the cell wall of the bacteria. Reactions were performed in DMF with DMAP at 60 °C. The initially heterogeneous mixture formed a gel upon stirring. Products were analysed by GPC in DMAc and by SDS-PAGE and fractions with a higher molecular weight than the starting materials were obtained (see Figure 45).

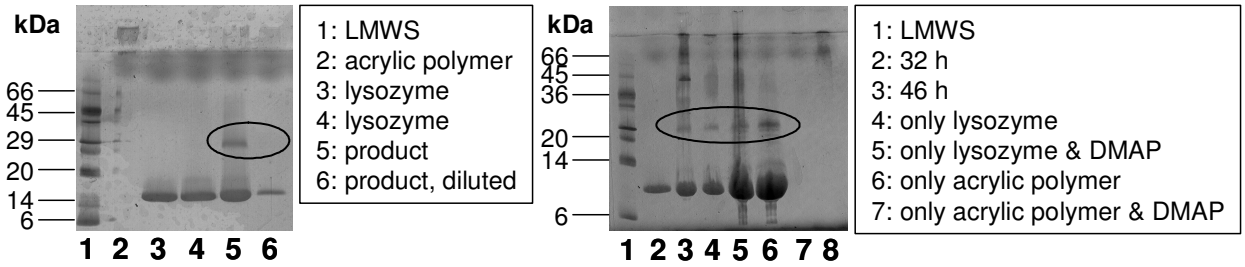


Figure 45 SDS-PAGE of products obtained upon reaction of lysozyme with poly(methacrylates); left: compared to starting material (line 2, 3, 4) and right: compared to blind reaction (line 4-6) [LMWS: low molecular weight standard]

However, a blind reaction of the enzyme at the chosen coupling conditions revealed that the protein formed a dimeric conjugate with a molecular weight of 28kDa,[24] suggesting a positive result.

5.3 Conclusion

Different architectures of polymer-peptide/amino acid conjugates were prepared:

Peptides for the preparation of multiblock copolymers were obtained via tryptic degradation of an elastin-mimetic polymer (ELP). The amino-telechelic peptides (amino-terminus and the ε-amino group of lysine at the carboxy-terminus) were purified via

chromatography and analysed via MALDI-TOF MS. Poly(ethylene glycol) (PEG) was activated using 4-nitrophenylchloroformiate or di(N-succinimidyl carbonate). Both Polymers di(NPC)PEG, with a molecular weight of 1500 Da and 3400 Da and di(NSC)PEG_{1500} were applied in the polycondensation with lysine and lysine methyl ester. Di(NPC)PEG did not react neither with lysine, nor with the corresponding methyl ester. The polycondensation of di(NSC)PEG_{1500} with lysine methyl ester resulted in the formation of a multiblock copolymer, which was confirmed via GPC analysis and ^{1}H-NMR spectroscopy. The reaction of lysine with di(NSC)PEG_{1500} resulted also in the formation of a condensation product. However, this product was identified as the condensation product of di(NSC)PEG_{1500} alone. A multiblock copolymer with PEG blocks and elastin peptide blocks was formed by the reaction of elastin-like peptides and di(NSC)PEG_{1500}, which was shown by SDS-PAGE analysis. However, due to low amounts of peptides and therefore of the condensation product, the multiblock copolymer could not be isolated.

In order to prepare block copolymers with PEG and amino acids or peptides, PEG and mPEG were activated as phenyl carbonate derivative or as succinate with subsequent activation as succinimidyl ester. Endcapping of $mPEG_{2000}$-PC with alanine methyl ester proceeded the fastest in DMSO at 70 °C using DMAP as base. Endcapping of di(PC)PEG_{400} was performed as comparative study of the reactivity of amino acid methyl esters. Glycine methyl ester was found to conjugate to the highest extent. GlyglyOEt, alanine and the tripeptide Ala-Gly-Ala could be successfully couple to $mPEG_{2000}$-succinate using N-hydroxy-succinimide (NHS) for activation. The formation of the conjugates was confirmed by ^{1}H-NMR spectroscopy.

With the aim to prepare comb-like hybrid copolymers and to immobilise lysozyme, alanine methyl ester was reacted with poly(2-phenoxycarbonyloxy)ethyl methacrylate) P(PCEMA). The reaction in $CDCl_3$ at 50 °C with DMAP resulted in a product which was functionalised

with AlaOMe to 94 % and remaining phenyl carbonate groups, which opens the possibility for further functionalisations. The enzyme lysozyme was reacted with a poly(methacrylate), which was also decorated partially with phenyl carbonate groups. However, the coupling of the enzyme lysozyme failed.

5.4 Experimental Part

5.4.1 Materials and methods

The elastin-mimetic polymer (ELP) was prepared by recombinant DNA technology and expressed by *Escherichia coli* fermentation. The material was provided from the BIOFORGE group at the University of Valladolid, Spain. poly(phenoxycarbonyloxy ethyl methacrylate) (P(PCEMA)) **14** was synthesised by Dr. R. Adelmann.[23] Poly(phenoxycarbonyloxy ethyl methacrylate-*co*-methyl methacrylate-*co*-dodecyl methacrylate-*co*-dimethylaminopropyl-methacrylate) (poly[(PCEMA)(MMA)(A12)(DAP)]) **16** was synthesised by D. Popescu.[25] Trypsin (23.8 kDa) from porcine pancreas, Type IX-S, lyophilised powder was purchased from Aldrich. NH_4HCO_3 was received from KMF, 4-dimethylaminopyridine (DMAP) and glycinyl glycine ethyl ester hydrochloride (GlyglyOEt) from Aldrich, trifluoro acetic acid, trichloro acetic acid (TFA, TCA) and Di(N-succinimidyl carbonate) (DSC) from Fluka, amino acid methyl ester hydrochlorides (AAME), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and dicyclohexylcarbodiimide (DCC) were purchased from IRIS. Alaninylglycinylalanine (AGA) was obtained from Bachem. All chemicals were used as received. Tetrahydrofuran (THF) was distilled from potassium under a nitrogen atmosphere. Amino acid analyses (AAA) were performed after hydrolysis with 6 N HCl in a closed glass vial at 110 °C for 24 h and subsequent removal of excess of hydrochloric acid by repeated distillation with water following the method of *Spackman, Stein* and *Moore*.[26] For MALDI-TOF MS analyses α-cyano-4-hydroxy cinnamic acid (HCCA),

2,5-dihydroxybenzoic acid (DHB), sinapinic acid (SA) or dithranol served as matrix, a mixture (2:1, v/v) of aqueous trifluoric acid (TFA, 0.1 %) and acetonitrile served as solvent. Samples were dissolved at a concentration of 100 pmol/µL and mixed with the matrix solution (20 mg/mL) in a ratio of 1:1. The final solution (0.5 µL) was applied to the sample target. Centrifugal filter (Vivaspin 6) with poly(ethersulfone) (PES) membrane and MWCO 5000 were purchased from vivascience. PD-10 Desalting columns were obtained from Amersham Bioscience. Standards for SDS-PAGE (SigmaMarker low molecular weight and Standard wide) were purchased from Aldrich.

5.4.2 Instruments

FTIR spectra were recorded with KBr pellets on a Nicolet FT-IR spectrophotometer Nexus 470. NMR Spectra were recorded on a Varian VXR 300 or a Bruker DPX-300 FT-NMR spectrometer at 300 MHz and 75 MHz, respectively. Chloroform-*d* ($CDCl_3$) and dimethyl sulfoxide-d_6 (DMSO-d_6) were used as solvents, and tetramethylsilane served as internal standard. AAA was performed on an analyser Alpha-Plus II, Pharmacia. MALDI-TOF MS analyses were performed on a Biflex™ III, Bruker Daltonics (Bremen), equipped with a nitrogen laser (3ns, 337 nm) and a scout-sample source. Gel permeation chromatography was carried out using PMMA calibration (polymer standards from PL). GPC analyses were carried out in tetrahydrofuran (THF) or *N,N*-dimethylacetamide (DMAc) as the eluent. GPC analyses performed with THF as the eluent were carried out using a high pressure liquid chromatography pump (PL-LC 1120 HPLC) and both a refractive index detector (ERC-7515A) and a UV detector (ERC-7215, λ = 254 nm) at 35°C. The eluting solvent was THF with 250 mg/L 2,6-di-tert-butyl-4-methylphenol (Aldrich) and a flow rate of 1.0 mL/min. Four columns with MZ-DVB gel were applied: length of each column 300 mm, diameter 8 mm, diameter of the gel particles 5 µm, nominal pore widths 50, 100, 1000, 10 000 Å. GPC analyses using DMAc as the eluting solvent were carried out using a high temperature GPC

device at 80°C (Polymer Laboratories PL-GPC210 with a Bischoff HPLC contact pump) and a refractive index detector (Polymer Laboratories). The eluting solvent was used with 2.44 g/L LiCl and a flow rate of 0.8 mL/min. Four columns with MZ-DVB gel were applied: length of each column 300 mm, diameter 8 mm, diameter of gel particles 5 µm, nominal pore width 100, 100, 1000, 10 000 Å. For peak deconvolution, peak fitting software (hs NTeqGPC V 6.2.12, Hard- und Software mbH, Dr. W. Schupp) was used. Column chromatography for peptide purification was performed on an Äkta Prime (Amersham) with a Superdex 75 column (60 cm) at a flow rate of 1 mL/min. Eluent buffers were prepared prior to use. Sodium dodecyl sulphate-polyacrylamide gel electrophoreses (SDS-PAGE) were performed on a PhastSystem (Pharmacia) using ready-to-use gels PhastGel$_{TM}$Homogeneous 20 and PhastGel$_{TM}$SDS Buffer Stripes (Pharmacia). Samples were dissolved in *Lämmli* buffer.[27] Gels were stained with silver nitrate according to *Ansorge*.[25]

5.4.3 Synthesis of multiblock copolymers

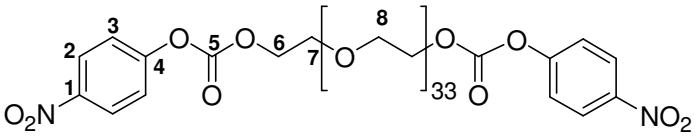
Tryptic degradation of ELP

ELP (100 mg) was dissolved in ammonium bicarbonate buffer (16 mL, pH 8.13) and treated with 4 mL of a solution of trypsin (2.08 mg) in ammonium bicarbonate buffer (4.16 mL, pH 8.13) and incubated for 6 h at 37 °C under 130 rpm agitation.

Synthesis of di(4-nitrophenyl carbonate)poly ethylene glycol$_{1500}$ 2

PEG 1500 (dried in high vacuum at 60 °C) (7.52 g, 5.01 mmol) was dissolved in dry THF (50 mL) and pyridine (444 µL, 5.51 mmol). The solution was cooled to 0 °C and 4-nitrophenyl chloroformate (5.05 g , 25.1 mmol) dissolved in dry THF (10 mL) was drop wise added.. The solution was stirred for 30 min at 0 °C, then 17 h at room temperature. Pyridinium hydrochloride was removed by filtration and the product was isolated by precipitation in Et_2O, filtrated and dried in vacuo.

Yield: 8.15g (88.8 %)



2

GPC (THF):

M_n: 2400

M_w: 2450

M_w/M_n: 1.02

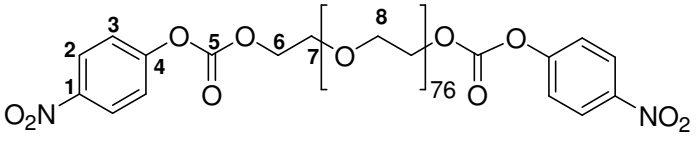
^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 3.66 (m, 138H, H-8), 3.83 (m, 4H, H-7), 4.44 (m, 4H, H-6), 7.41 (d, 4H, H-3), 8.29 (d, 4H, H-2) ppm.

Synthesis of di(4-nitrophenyl carbonate)poly ethylene glycol$_{3400}$ 3

PEG 3400 (dried in high vacuum at 60 °C) (3.67 g, 1.08 mmol) was dissolved in dry CH_2Cl_2 (50 mL), cooled to 0 °C and 4-nitrophenyl chloroformate (1.09 g, 5.4 mmol) was added, finally Et_3N (730 µL, 6.4 mmol). The solution was stirred for 90 min at 0 °C, then 17 h at room temperature. After removing of the solvent, ethyl acetate was added, the white precipitate was filtrated and the filtrate dropped into diethyl ether to precipitate the polymer. The product was filtrated and dried in high vacuum.

Yield: 2.21 g (54.8 %)



3

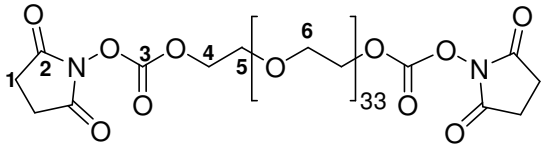
^{1}H-NMR (DMSO-d_6, 300 MHz):

δ = 3.65 (m, 330H, H-8), 4.44 (d, 4H, H-6), 7.41 (d, 4H, H-3), 8.29 (d, 4H, H-2) ppm.

Synthesis of di(N-hydroxy succinimide carbonate)poly ethylene glycol$_{1500}$ 4

Poly (ethylene glycol)$_{1500}$ (5 g, 3 mmol) (dried in high vacuum at 60 °C) was dissolved in pyridine (25 mL, dry) together with 4-dimethylaminopyridine (DMAP, 163.6 mg; 1.3 mmol) and mixed with a suspension of di(N-succinimidyl carbonate) (3.42 g, 13.3 mmol) in dry

acetonitrile (28 mL). The solution was stirred at room temperature for 12 h and protected from light. Solvents removed under vacuum, the residue was dissolved in CH_2Cl_2 and isolated by precipitation in cold ether. This procedure was repeated three times yielding 4.3 g (72.3 %) of a waxy solid.



4

^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 2.85 (s, 8H, H-1), 3.66 (d, 138H, H-6), 3.79 (m, 4H, H-5), 4.47 (m, 4H, H-4) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 25.4 (C-1), 68.3 (C-4), 70.2-70.9 (C-5,-6), 151.6 (C-3), 168.6 (C-2) ppm.

GPC (THF):

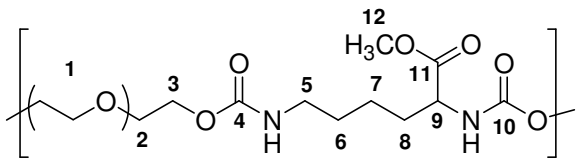
M_n: 2200

M_w: 2240

M_w/M_n: 1.02

General procedure for the interfacial poly condensation of amino-telechelic monomers and PEG prepolymers 5

Di(NSC)PEG_{1500} (200 mg, 112.1 mmol) was dissolved in CH_2Cl_2 (3 mL). Lysine methyl ester hydrochloride (26.17 mg, 112.3 mmol) and $NaHCO_3$ (43.4 mg, 516 mmol) were dissolved in H_2O (3 mL) and mixed with the polymer solution. The mixture was stirred at room temperature for 16 h. Samples were taken after 60 min and 120 min. The pH was brought to 2 by adding 0.1 N HCl, the organic solution was washed with brine, dried with $MgSO_4$, filtrated and the solvent was evaporated. The product was dried in vacuo. Yield: 95.5 mg



5

^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 1.26-1.82 (m, 6H), 3.14-3.16 (m, 2H, H-5), 3.67 (s, 4H, H-1), 3.79 (s, 3H, H-12), 3.98 (m, 2H, H-2), 4.21 (m, 2H, H-3), 4.31-4.33 (m, 1H, H-9), 4.99 (m, 1H, N*H*), 5.44 (m, 1H, N*H*) ppm.

GPC (THF):

M_n: 36800; M_w: 49600; M_w/M_n: 1.35

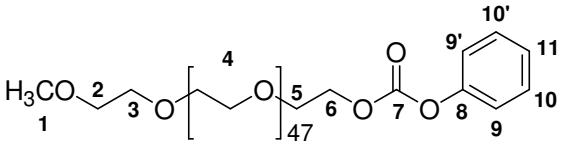
Interfacial poly condensation of ELP peptides and di(NSC)PEG$_{1500}$ 6

ELP peptides (17.09 mg, 7.30 µmol NH_2) and KOH (1.72 mg, 0.031 mmol) were dissolved in H_2O (113 µL). di(NSC)PEG$_{1500}$ (13.0 mg, 7.30 µmol) was dissolved in CH_3CN and was added to the aqeous solution. The mixture was stirred at 40 °C for 22 h. The solvent was removed by distillation and the crude product was analysed by SDS-PAGE.

5.4.4 Coupling to mPEG and PEG

Synthesis of phenyl carbonate methoxy poly(ethylene glycol)$_{2000}$ 7

MPEG$_{2000}$ (25.03 g, 12.5 mmol) was dried by azeotropic distillation with toluene (250 mL). After cooling phenyl chloroformate (6.3 mL, 50 mmol) was added and the solution was refluxed for 3 d. HCl was removed in a continuous nitrogen flow. Toluene was removed by distillation; the crude product was dissolved in CH_2Cl_2, K_2CO_3 was added and the mixture was stirred for 90 min. The solution was filtrated and the product was obtained by precipitation in cold hexane. Residual phenol and diphenyl carbonate was removed by precipitation in Et_2O from a toluene solution. Yield: 23.06 g (87.0 %) of a white solid.



7

^{1}H-NMR ($CDCl_3$, 300 MHz):

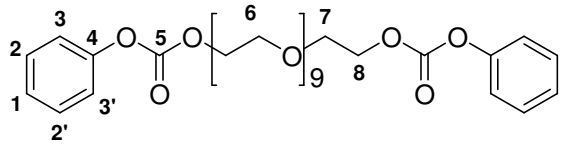
δ = 3.33 (s, 3H, H-1), 3.42 (t, 2H, H-2), 3.50 (m, 2H, H-3), 3.61 (m, 188H, H-4), 3.77 (m, 2H, H-5), 4.35 (t, 2H, H-6), 7.14 (t, 2H, H-10, H-10'), 7.20 (t, 1H, H-11), 7.34 (t, 2H, H-9, H-9') ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 59.1 (C-1), 67.7 (C-5), 68.8 (C-3), 70.4-70.7 (C-4, C-6), 72.0 (C-2), 121.0 (C-9, C-9'), 126.0 (C-11), 129.4 (C-10, C-10') ppm.

Synthesis of di(phenyl carbonate)poly(ethylene glycol)$_{400}$ 8

PEG_{400} (11 g, 0.028 mol) was dried 6 h in high vacuum and then dissolved in CH_2Cl_2 (100 mL), Et_3N (17.5 mL, 0.126 mol) was added and the solution was cooled to 0 °C. Phenyl chloroformate (9.5 mL, 0.076 mol) was added drop wise over 60 min. After stirring for 24 h at r.t. the mixture was poured onto ice, the organic layer was separated and washed twice with cold water (75 mL), twice with 10 % aqueous HCl (75 mL), twice with saturated $NaHCO_3$ (75 mL) and with water (100 mL). The organic phase was dried with $MgSO_4$, filtrated and CH_2Cl_2 was removed in vacuo. To remove residual phenol and diphenyl carbonate the product was dissolved in $CHCl_3$ (75 mL), washed three times each with 5 % aqueous NaOH and with 5 % aqueous HCl, dried with $MgSO_4$, filtrated and the solvent removed in vacuo. Yield: 14.99 g (85.2 %) of slightly coloured oil.



8

δ = 3.64-3.69 (m, 28H, H-6), 3.87 (m, 4H, H-7), 4.27-4.39 (m, 4H, H-8), 7.19-7.28 (m, 6H, H-1, H-3, H-3'), 7.34-7.39 (m, 4H, H-2, H-2')ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 67.0 (C-7), 67.6-70.6 (C-6), 72.5 (C-8), 121.0 (C-3, C-3'), 126.0 (C-1), 129.4 (C-3, C-3') ppm.

Influence of solvent, temperature and amount of base on the reaction of alanine methyl ester with mPEG-PC 7

Alanine methyl ester hydrochloride, DMAP and mPEG-PC **7** were dissolved in $CDCl_3$ and stirred at 50 °C. The solution was analysed by means of ^{13}C-NMR spectroscopy after 6.5 h, 11 h, 15 h and 23 h. The conversion was determined from the average intensity of three

phenol signals (o-, m-, p-H) in comparison to the corresponding signals in the starting material mPEG-PC **7** (H-1, H-2, H-3).

The further experiments (Table 11, entry II, IIIand IV) were performed according to the described method.

Table 11 Reaction conditions and applied amount of reactants

Entry	solvent	temperature	Concentration of [mg/mL]			amount of DMAP
			AlaOMe	mPEG-PC **7**	DMAP	
I	$CDCl_3$	50 °C	19.2	288.2	20.3	1.2 eq
II	DMSO-d_6	70 °C	13.1	197.6	13.8	1.2 eq
III	$CDCl_3$	50 °C	10.0	285.4	33.5	2 eq
IV	DMSO-d_6	70 °C	13.6	203.6	23.9	2 eq

Comparison of the reactivity of amino acid methyl esters

Two reactions were performed by dissolving amino acid methyl ester hydrochlorides (same molar amount of amino group) in $CDCl_3$ with a slight excess of DMAP. In the first reaction the methyl ester of glycine, alanine, valine, serine and cysteine were brought to reaction with Di(PC)-PEG_{400}. In the second reaction lysine was additionally added. The applied amount of Di(PC)-PEG_{400} corresponded with the molar amount of one amino aid methyl ester type. The amounts are summarised in

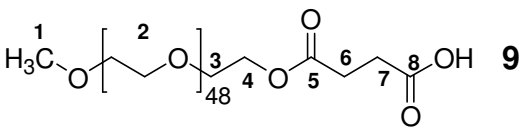
Table 12. AAA was performed from the applied amino acid methyl ester solution, again after 16 h stirring at 60 °C and aqueous work-up (3x10 mL 1N NaOH, 3x10 mL 1N HCl, drying with Na_2SO_4, concentrating). The product as well as the aqueous solution was analysed by AAA.

Table 12 Applied amount of reactants

Methyl ester of	Reaction 1		Reaction 2	
	m [mg]	n [mmol]	m [mg]	n [mmol]
glycine	50.95	0.40	50.37	0.40
alanine	57.47	0.40	56.03	0.40
valine	68.38	0.40	67.52	0.40
serine	63.50	0.40	62.41	0.40
cysteine	69.76	0.40	69.0	0.40
lysine	--	--	46.8	0.20
Di(PC)PEG_{400} **8**	128.4	0.20	128.5	0.20
DMAP	269.6	2.21	367.83	3.01
$CDCl_3$	12 mL		12 mL	

Synthesis of $mPEG_{2000}$-succinate 9

$mPEG_{2000}$ (10 g, 5 mmol) was dried at 60 °C in vacuo for 3 h and dissolved in dry THF (100 mL). Succinic anhydride (1.26 g, 12.54 mmol) and DMAP (0.61 g, 5 mmol) were added and the mixture was refluxed for 7.5 h and stirred at r.t. for 14 h. The suspension was concentrated, dissolved in $CHCl_3$ (50 mL) and the product was isolated by precipitation in Et_2O. This work-up procedure was repeated thrice. Residues of DMAP were removed by washing the chloroform solution with 1N aqueous HCl (10 mL). The organic phase was dried with Na_2SO_4, filtrated, concentrated and the product was dried in vacuo. Yield: 5.18 g (49.52 %)

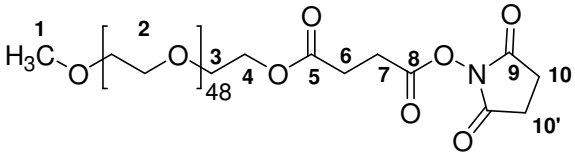


^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 2.61 (s, 4H, H-6, H-7), 3.35 (s, 3H, H-1), 3.54-3.85 (m, 194H, H-2, H-3), 4.23 (m, 2H, H-4) ppm.

Synthesis of $mPEG_{2000}$-succinimidyl succinate 10

$mPEG_{2000}$-succinate (**9**, 2 g, 0.874 mmol), NHS (100.6 mmol, 0.874 mmol) and DCC (225.8 mg, 1.09 mmol) were dissolved in THF (20 mL) and stirred for 6 h at r.t. The precipitate was filtrated and the product was isolated by precipitation in Et_2O, filtration and drying in vacuo. Yield: 1.78 g (84.9 %)



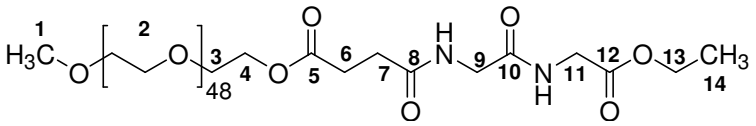
10

^{1}H-NMR (DMSO-d_6, 300 MHz):

δ = 2.50 (s, 4H, H-10, H-10'), 2.69 (m, 2H, H-6), 2.80 (s, 4H, H-$6_{mPEG\text{-}succinate}$, H-$7_{mPEG\text{-}succinate}$), 2.94 (m, 2H, H-7), 3.24 (s, 3H, H-1), 3.24-3.74 (m, 194H, H-2, H-3), 4.14 (m, 2H, H-4)ppm.

Conjugation of GlyglyOEt with $mPEG_{2000}$-succinimidyl succinate 10

$mPEG_{2000}$-succinimidyl succinate (**10**, 201.5 mg, 0.084 mmol), GlyglyOEt hydrochloride (17.4 mg, 0.089 mmol) and DMAP (12.4 mg, 0.101 mmol) were dissolved in DMSO-d_6 (1 mL) an stirred at 60 °C for 2 h. The crude product was isolated by precipitation in Et_2O, dissolved in $CHCl_3$ and washed thrice with brine. The organic phase was dried with $MgSO_4$, filtrated and precipitated in Et_2O. The white solid was dried in vacuo. Yield: 174.9 mg (85.2 %)



11

^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 1.28 (m, 3H, H-14), 2.54 (m, 2H, H-7), 2.74 (m, 2H, H-6), 3.38 (s, 3H, H-1), 3.65-3.88 (m, 194H, H-2, H-3), 4.0 (m, 4H, H-9, H-11), 4.17-4.25 (m, 4H, H-4, H-13), 6.71-7.21 (m, 3H, N*H*) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 14.2 (C-14), 29.4 (C-7), 30.8 (C-6), 41.2 (C-11), 43.1 (C-9), 59.0 (C-13), 61.2 (C-1), 63.8 (C-4), 68.9 (C-3), 70.5-71.9 (C-2), 169.7 (C-12), 172.2 (C-10), 172.9 (C-5) ppm.

GPC (THF):

M_n: 3100 ; M_w: 3200; M_w/M_n: 1.03

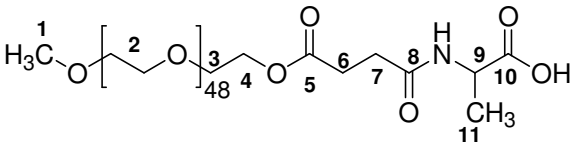
MALDI-TOF MS (Matrix: DHB)

$[M+Na]^+$: 2190.8

$[mPEG\text{-}succinate + Na]^+$: 2048.2

Conjugation of alanine with $mPEG_{2000}$-succinate 9

$mPEG_{2000}$-succinate (**9**, 300.0 mg, 0.131 mmol) was dissolved in H_2O (750 µL), degassed and EDC (62.8 mg, 0.328 mmol) and NHS (18.25 mg, 0.159 mmol) were added under constant nitrogen flow and stirred for 20 min. Alanine (11.9 mg, 0.133 mmol) was dissolved in 0.1 N aqueous Na_2CO_3 (150 µL). The polymer solution was drop wise added to the alanine solution and stirred for 20 h at r.t. The product was isolated by extraction with $CHCl_3$, drying with $MgSO_4$, filtration and precipitation in Et_2O. This procedure was repeated to remove residual side products. Yield: 164.2 mg (53.1 %)



12

^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 1.39-1.41 (d, 3H, H-9), 2.52-2.54 (t, 2H, H-6), 2.62-2.68 (kB, 2H, H-5), 3.37 (s, 3H, H-1), 3.63 (s, 196H, H-2'), 3.87 (t, 2H, H-2), 4.22 (m, 2H, H-3), 4.38-4.45 (m, 1H, H-8), 6.51-6.53 (d, 1H, N*H*) ppm.

^{13}C-NMR ($CDCl_3$, 300 MHz):

δ = 18.4 (C-9), 29.5 (C-5), 30.9 (C-6), 42.9 (C-8), 59.0 (C-1), 63.8 (C-3), 69.9 (C-2), 70.6 (C-2'), 71.9 ($\underline{C}H_2OMe$) ppm.

Conjugation of AGA with $mPEG_{2000}$-succinate

$mPEG_{2000}$-succinate (**9**, 301.4 mg, 0.132 mmol) was dissolved in H_2O (1.2 mL), degassed and EDC (63.5 mg, 0.331 mmol) and NHS (18.33 mg, 0.159 mmol) were added under constant nitrogen flow and stirred for 15 min. AGA (28.7 mg, 0.132 mmol) was dissolved in 0.1 N aqueous Na_2CO_3 (150 µL). The polymer solution was drop wise added to the peptide solution and stirred for 20 h at r.t. The product was isolated by extraction with $CHCl_3$. The

organic phase was washed thrice with 1N aqueous HCl, dried with Na_2SO_4, filtrated and the product was precipitated in Et_2O. Yield: 172.1 mg (52.5 %).

^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 1.36-1.38 (m, 6 H, H-9, H-14), 2.47 (m, 2H, H-6), 2.63-2.66 (m, 2H, H-5), 3.32 (s, 3H, H-1), 3.59 (s, 196 H), H-2'), 3.82 (t, 2H, H-2), 4.13-4.40 (kB, 6H, H-3, H-8, H-11, H-13) ppm.

GPC in THF:

M_n=2900; M_w=3000; M_w/M_n=1.03

MALDI-TOF MS (Matrix: DHB)

$[M+Na]^+$=2027.5

$[mPEG\text{-}Succinat + Na]^+$=2048.5

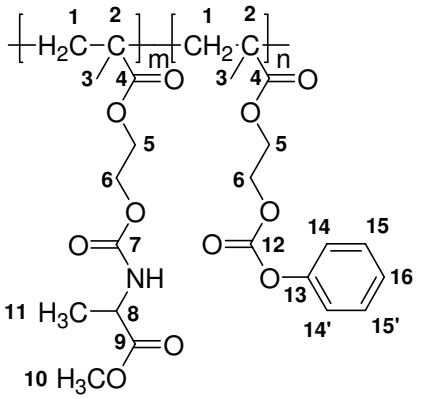
$[mPEG + Na]^+$=2036.6

5.4.5 Grafting of amino acids and lysozyme to PCEMA

Coupling of alanine methyl ester to poly[2-(phenoxycarbonyloxy)ethyl methacrylate]

P(PCEMA) 14

P(PCEMA) (**13**, 75.5 mg, 0.30 mmol), alanine methyl ester hydrochloride (42.5 mg, 0.30 mmol) and DMAP (44.9 mg, 0.37 mmol) were dissolved in $CDCl_3$ (0.6 mL) and heated to 50 °C. NMR analyses were performed after 402 min, 2072 min, 5100 min and 6195 min. Solution was than washed thrice with 5 % aqueous NaOH, 5 % aqueous HCl, dried with $MgSO_4$, filtrated and dried in vacuo. This procedure was repeated to remove residual phenol and the product was washed with Et_2O and again dried in vacuo. Yield: 40.3 mg (51.7 %) of a white solid.



14

^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 0.88-1.04 (br, 6H, H-3), 1.42 (m, 3H, H-11), 1.87-1.96 (br, 4H, H-1), 3.75 (s, 3H, H-10), 4.13-4.33 (m, 9H, H-5, H-6, H-8), 6.24 (br, 1H, N*H*), 7.20 (m, 3H, H-13, H-13', H-15), 7.41 (br, 2H, H-14, H-14') ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 17.9 (C-11), 29.6 (C-2), 44.6 (C-1), 49.6 (C-10), 52.4 (C-8), 62.0 (C-5), 63.7 (C-6), 121.0 (C-14, C-14'), 126.3 (C-16), 129.6 (C-15, C-15'), 151.0 (C-12), 153.5 (C-13), 155.6 (C-7), 173.5 (C-9), 176.8 (C-4) ppm.

Coupling of lysozyme to poly(methacrylate) 15

The poly(methacrylate) (**15**, 350.3 mg) and DMAP (90 µg) were dissolved in DMF (3.5 mL) and lysozyme (107.1 mg) was added. The mixture was stirred at 60 °C. During stirring the mixture changed from a white suspension to a homogeneous colourless gel. After 45 h DMF was removed by distillation and the crude product was analyzed via SDS-PAGE. Control reactions were performed according to the Table 13 at 60 °C:

Table 13 Control reactions for the coupling of lysozyme to poly(methacrylate) **16**

	Reactant mass			Volume	Reaction time	
entry	**acrylic copolymer [mg]**	**lysozyme [mg]**	**DMAP (0.84 µg/mg enzyme)**	**DMF [µL]**	**[h]**	
1	54.27	16.65	13.99 µg	529	2	white gel
2	55.29	16.95	14.24 µg	539	4	white gel
3	51.67	15.85	13.31 µg	504	8	white gel
4	53.17	16.30	13.69 µg	518	16	white gel
5	53.65	16.44	13.81 µg	523	32	white gel
6	52.45	16.09	13.52 µg	512	46	white gel
7	--	15.4	--	500	46	suspension
8	--	15.6	13.10 µg	587	46	suspension
9	59.87	--	--	500	46	clear gel
10	50.74	--	13.04 µg	494	46	clear gel

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Appendix A Chitosan derivatisation with functional couplers

A.1 Introduction

Chitosan (poly(β-(1→4)-D-glucosamine) is a naturally occurring polyamine, which is found in the cell wall of some fungi.[1] However, the usual resource of chitosan is the polysaccharide chitin, which is after cellulose the second most abundant organic compound in nature. The amounts produced per year of these two biopolymers are estimated to be around 10^{11} tons.[2] Chitosan is obtained from chitin by deacetylation with sodium hydroxide. This procedure leads to a derivative, which is than called chitosan if the degree of deacetylation (DD) is higher than 50 % (see Figure 46 for structural details).

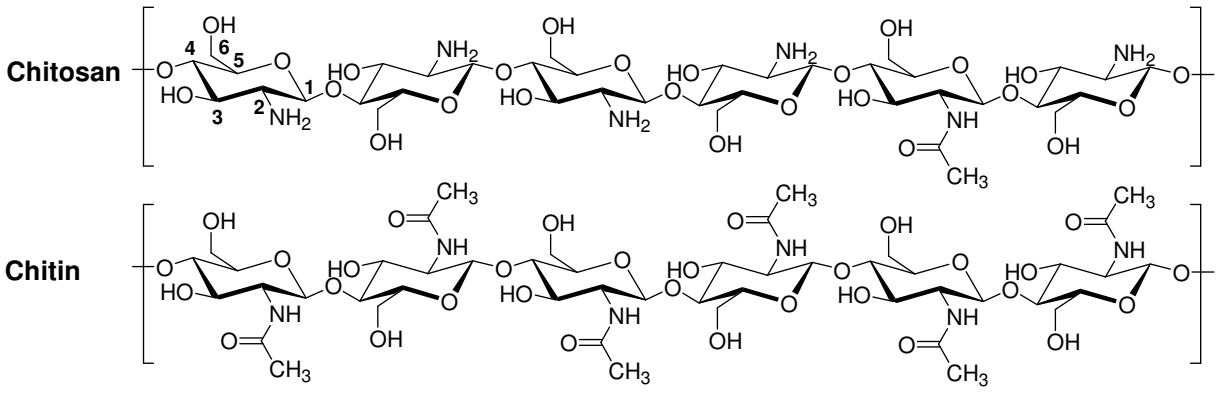

Figure 46 Chemical structure of chitosan and chitin

Functionalisation of chitosan is a topic of growing interest since a few years, in terms of production of water-soluble derivatives with improved properties. At our institute an approach for the functionalisation of amines – monodisperse amines and polyamines – has been developed, which is called the "carbonate coupler approach" (see Figure 47).[3] The carbonate coupler **1** is a bifunctional reagent that reacts with primary amines (F^1-NH_2) via substitution under release of an activating group to yield the so-called "functional coupler" **2**. This

compound reacts at elevated temperature (F^1-NH_2) under ring-opening of the cyclic carbonate moiety with another primary amine. The two carbonate groups can be addressed selectively.

A similar compound is the quaternary ammonium carbonate **3**, which reacts also under ring-opening with primary amines (F^2-NH_2). All products bear either a secondary **4a** or a primary hydroxy group **4b** due to the direction of the ring-opening. The ratio of secondary to primary hydroxy group is normally 7:3.[4]

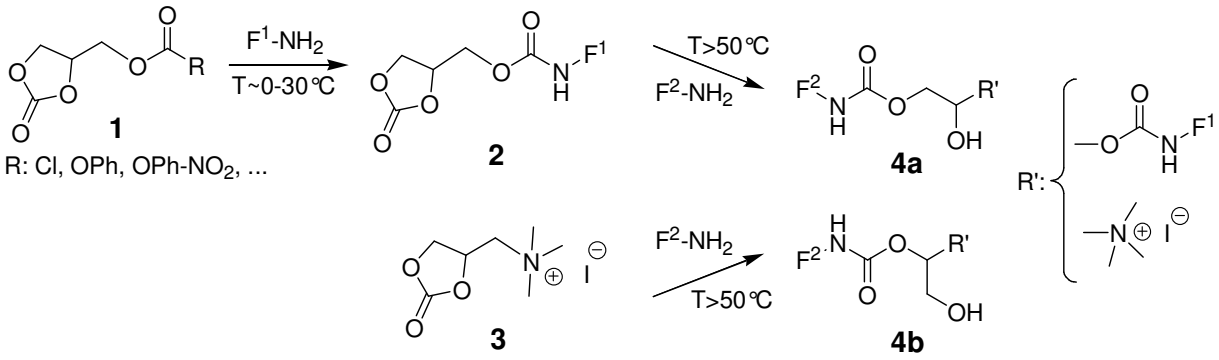

Figure 47 The "carbonate coupler approach" for the modification of amines

Our aim was the functionalisation of the amino groups of chitosan following the previously presented approach. The results from the model reaction with ethylene carbonate and from the reaction with quaternary ammonium carbonate **3** will be shown in the following paragraphs.

A.1.1 Model reactions with ethylene carbonate

First experiments were carried out with ethylene carbonate (EC) and chitosan as a model reaction for the functional couplers. Two different commercially available chitosan derivatives were applied; Chitoclear is a derivative with a molecular weight of 70.000 g/mol and a degree of deacetylation of 86 % and glycol chitosan, a derivative, which bears a hydroxy ethylene unit at C6 and has a DD of 88 %. This modification makes it – in contrast to unmodified chitosan – well soluble in aqueous solutions and mixtures of water with e.g. acetonitrile and methanol over the whole pH range. Reactions were performed in water, in bulk, in dimethylformamide (DMF) and at different temperatures.

Initially Chitoclear was brought to reaction with ethylene carbonate in three different ratios, either at room temperature (r.t.) or at 50 °C and each for 68 h (see Table 14).

Table 14 Reactions of ethylene carbonate (**EC**) with Chitoclear (**C**) in aqueous solution

entry	ratio (C:EC)	reaction conditions
1	1:1	r.t., stirring
2	1:2	r.t., stirring
3	1:0.5	r.t., stirring
4	1:1	50 °C, agitation 130 rpm
5	1:2	50 °C, agitation 130 rpm
6	1:0.5	50 °C, agitation 130 rpm

Each mixture was analysed by NMR spectroscopy directly after reaction and after purification by dialysis. The ^{1}H-NMR spectrum directly measured after the reaction showed two additional singuletts at $\delta = 3.40$ ppm and $\delta = 4.36$ ppm which were assigned to the ethylene glycol chain attached to the chitosan backbone. What seemed initially as a successful conversion turned out to be an obvious error after purification via dialysis. as a wrong interpretation due to signals of the hydrolysed ethylene carbonate. Neither at r.t. with the highest amount of EC, nor at 50 °C with the same ratio of chitosan to EC a conversion was observed (see Figure 48). The dialysis leads obviously to the elimination of the excess of hydrolysed ethylene carbonate, which affects the decrease of intensity of the two additional singuletts at δ=3.40 and 4.36 ppm.

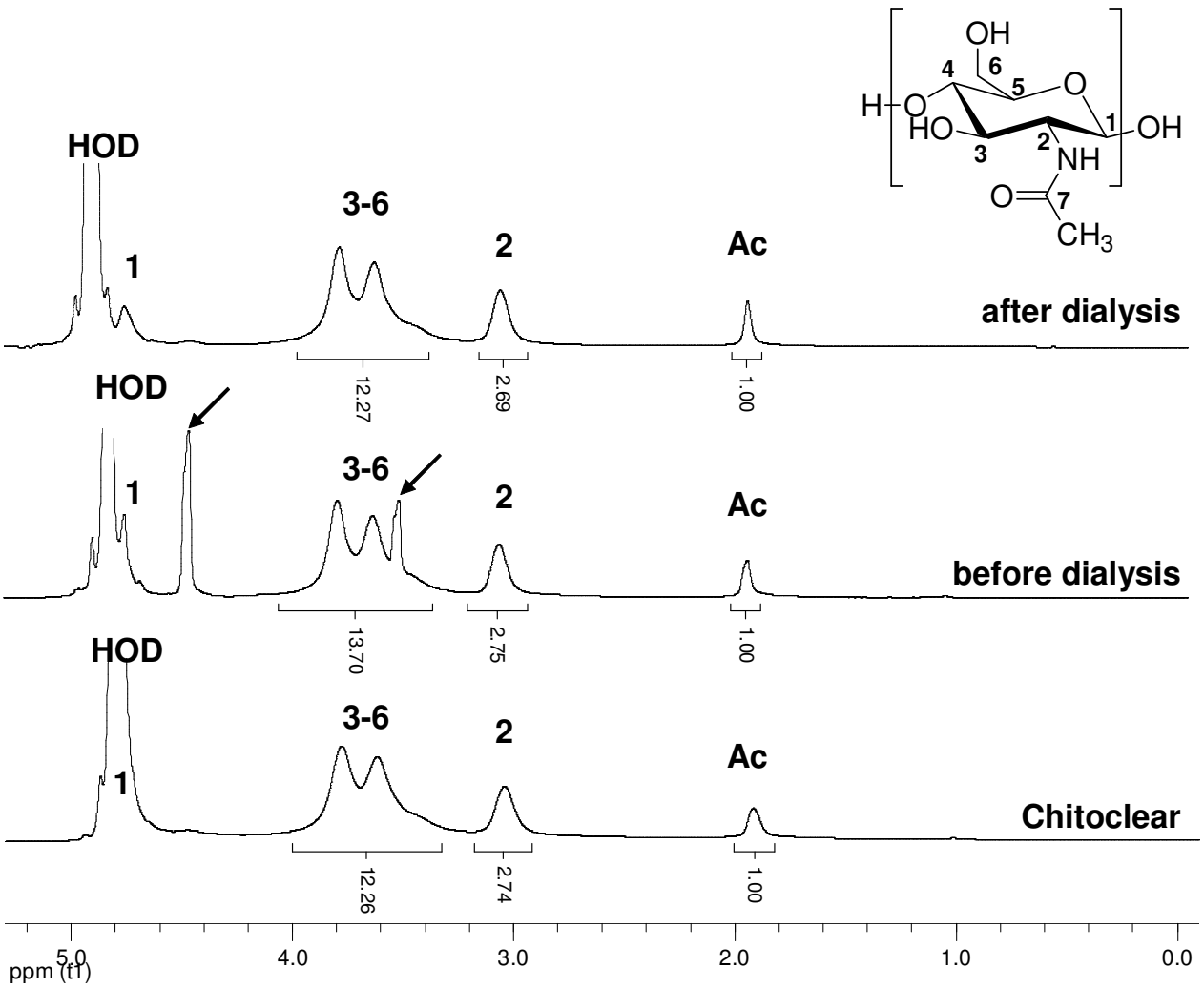

Figure 48 ^{1}H-NMR spectra in D_2O of initial Chitoclear in comparison with the product obtained after reaction with ethylene carbonate (analysis before and after purification by dialysis); see Table 14, entry 2

Glycol chitosan was reacted with EC at room temperature in a ratio of 1:1 (based on the amount of deacetylated amino groups) for 23 h. The reaction conditions were not as stringent as for the previously described procedure, but due to its better solubility glycol chitosan should react more easily. The analysis was performed in the same manner as for Chitoclear, which means the reaction mixture was lyophilised, analysed, than dialysed and analysed again. Like before, no conversion was observed.

A reaction in bulk at 100 °C, which is above the melting point of ethylene carbonate (M_p= 34-37 °C) was only performed with Chitoclear for 65 h, following the already described

cycle of analysis and purification. Again, the chitosan could not be modified at these rather harsh conditions.

The last model experiment was carried out with glycol chitosan in DMF and, like the reaction mentioned before, also at a temperature of 100 °C for 28.5 h. In this case a product was obtained, which was not soluble in H_2O anymore. Hence, the product was analysed by IR spectroscopy (see Figure 49). In the spectrum no bands occur, which can be related to a carbamatisation; the signal broadening in (b) is caused by residual ethylene carbonate (ν=1805 cm^{-1}) occurring at the typical wavenumber of a cyclic carbonate.

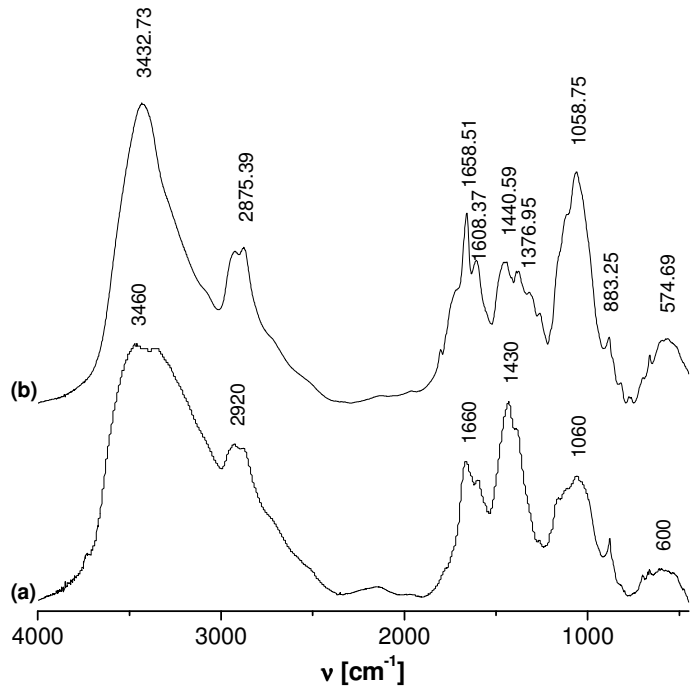

Figure 49 IR spectrum of initial glycol chitosan (a) in comparison with the IR spectrum of the product mixture(b)

Carbamate bands, which would assure a conversion usually emerge at ~1550 cm^{-1} (amid II) and around 1700 cm^{-1} (amid I).

A.1.2 Reactions with quaternary ammonium carbonate

Even though the model reactions with ethylene carbonate were not very promising, reactions were also performed with quaternary ammonium carbonate (quatcoupler) **3**. This was done to rule out any doubts with respect to differences in reactivity.

Similar to the first row of experiments, reactions with the quatcoupler were performed in water and in organic solvents.

Chitoclear and glycol chitosan were both reacted in aqueous solution at r.t., the first for 3.5 d, the latter for 23 h. Both mixtures - (i) Chitoclear and (ii) glycol chitosan - have been analysed by NMR spectroscopy directly after the reaction and after purification via dialysis. Again the first analysis was encouraging, but analysis after the dialysis showed that the newly appearing signals, which were first assigned to the desired derivatives, were related to unreacted starting material or its hydrolysis product (see Figure 50 and Figure 51).

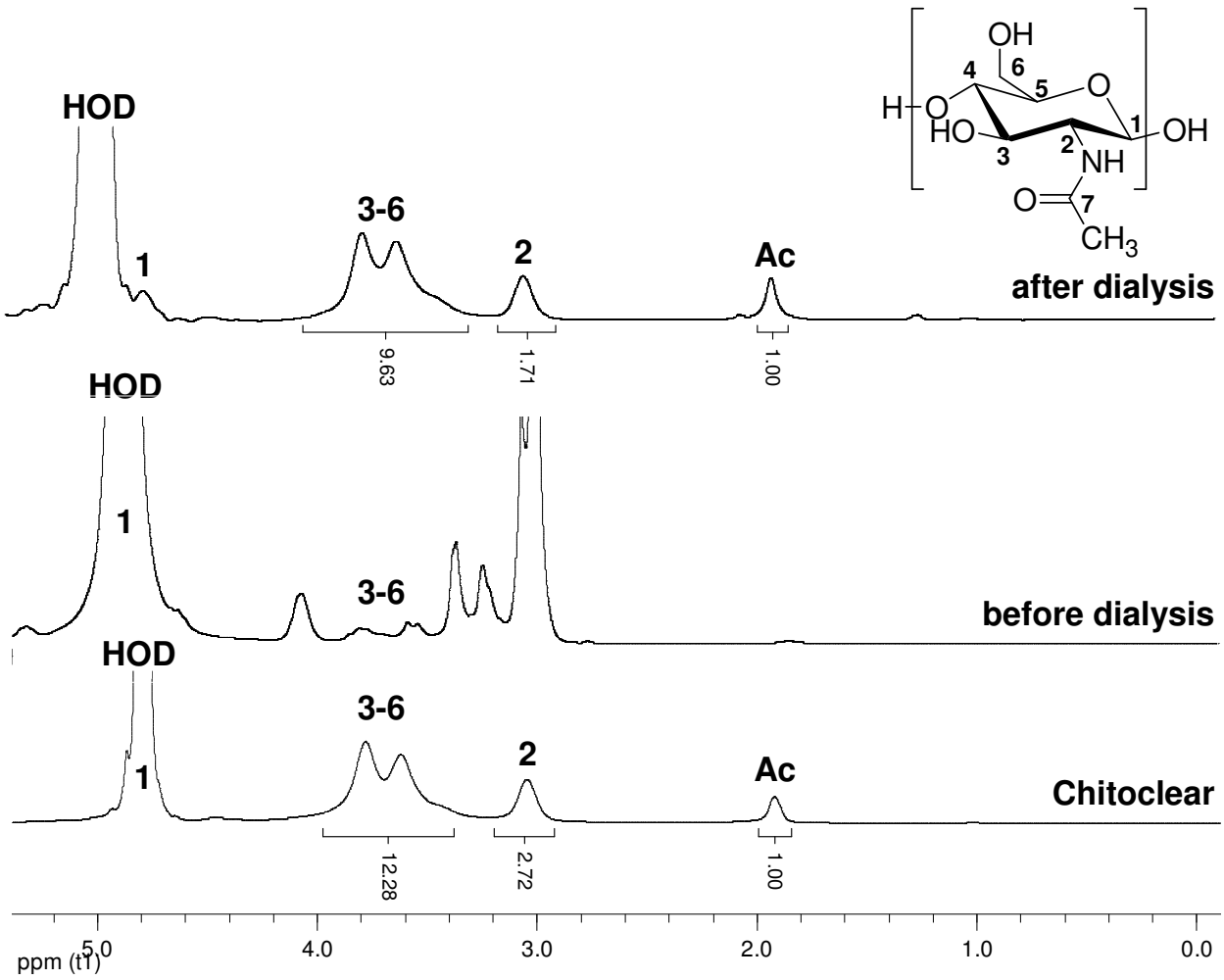

Figure 50 ^{1}H-NMR spectra in D_2O of Chitoclear and of the product obtained after reaction with quaternary ammonium carbonate in water at r.t. (analysis before and after purification by dialysis)

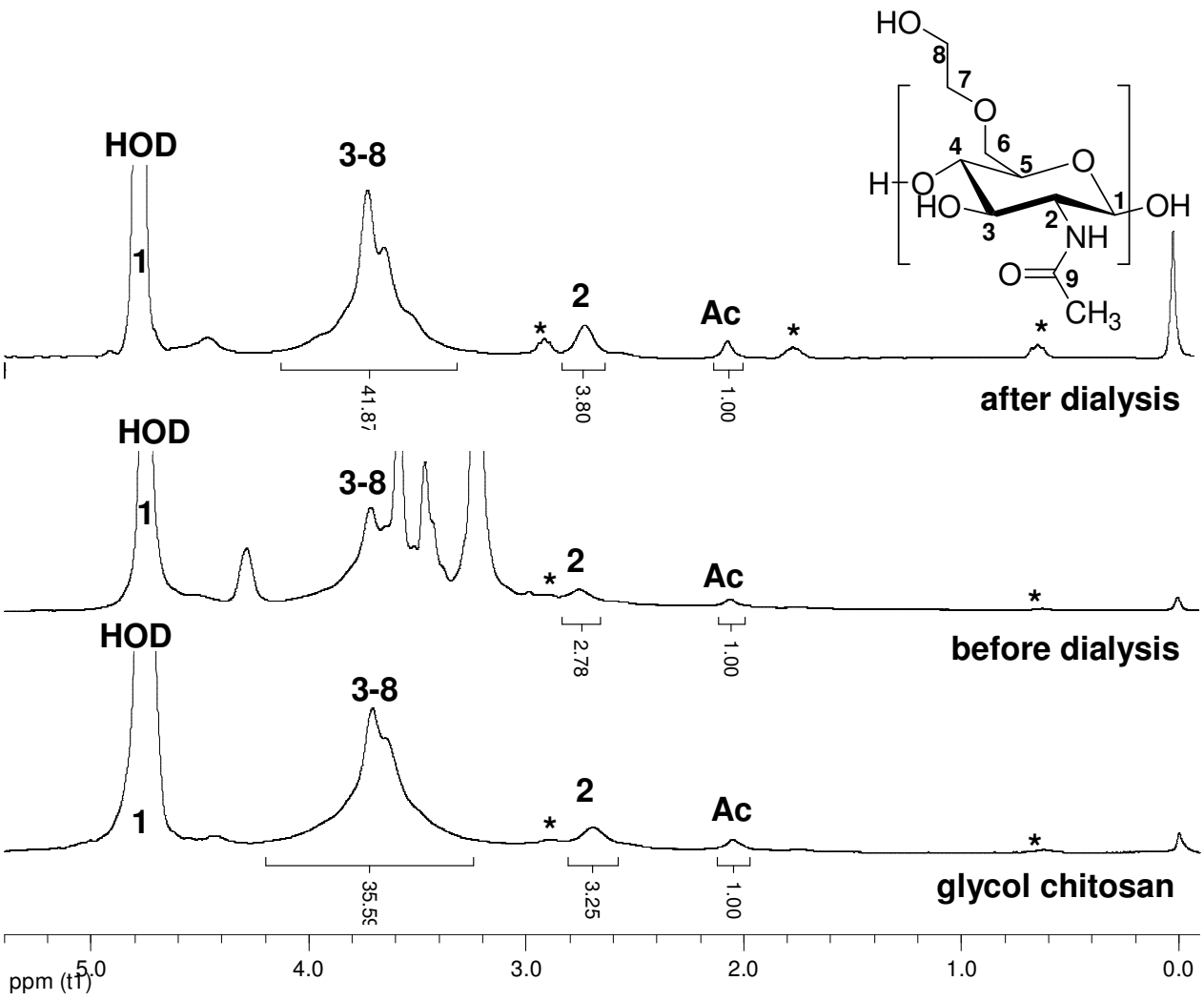

Figure 51 ^{1}H-NMR spectra in D_2O of glycol chitosan and of the product achieved after reaction with quaternary ammonium carbonate at r.t. (analysis before and after purification by dialysis; standard: 3-trimethylsilyl-1-propane sulfonate)

Reaction in aqueous solution was also performed with glycol chitosan at elevated temperature, namely at 60 °C for 43 h. Here, for the first time, modification of the chitosan with the quatcoupler occurred (see Figure 52).

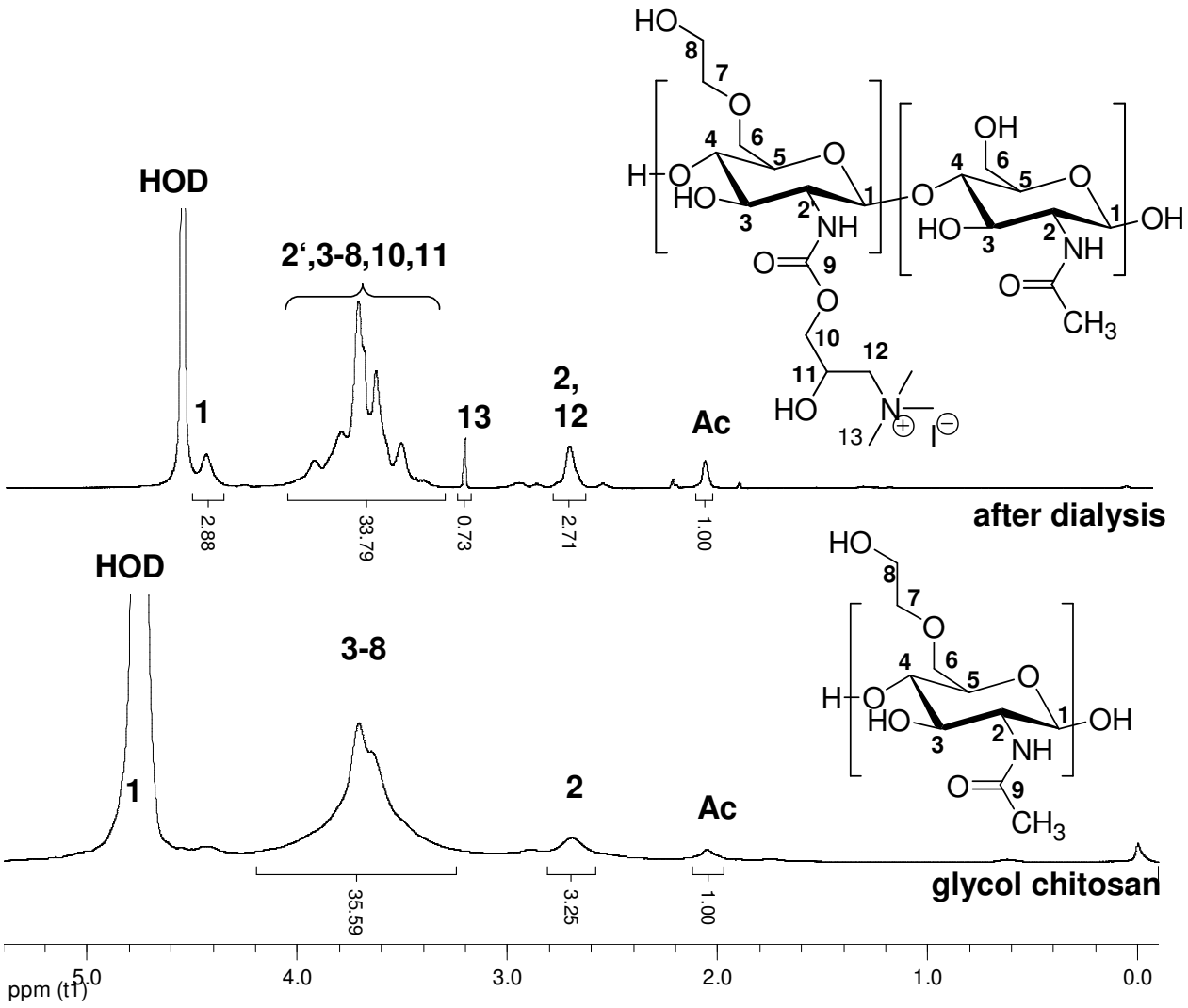

Figure 52 ^{1}H-NMR spectra in D_2O of initial glycol chitosan and of the product achieved after reaction with quaternary ammonium carbonate at 60 °C (initial glycol chitosan measured at 300 MHz, the product at 600 MHz)

The singulett at δ=3.20 ppm is related to the methyl groups of the trimethyl ammonium group (H-13). However, the degree of functionalisation (DF) was determined to be 2.9 %: For this the intensity of the peak of the trimethyl ammonium group (I_{H-13}, 9H) was divided by three and related to the intensity of the singulett of the acetyl group (I_{Ac}, 3H) at δ=2.05 ppm and the known DD.

$DF = (I_{H-13}/3)I_{Ac} \times (100-DD) = 0.243/1 \times 12\ \% = 2.92\ \%$

The NMR analysis in D_2O revealed also a turbid solution at a concentration of 17.5 mg/mL.

Modification in organic solvent, with Chitoclear in DMSO at 60 °C for 65 h and with glycol chitosan in DMF at 100 °C for 28.5 h, again failed. The latter reaction resulted, like the similar reaction of glycol, chitosan with ethylene carbonate, in an insoluble product. The analysis was again performed in this case by IR spectroscopy (see Figure 53).

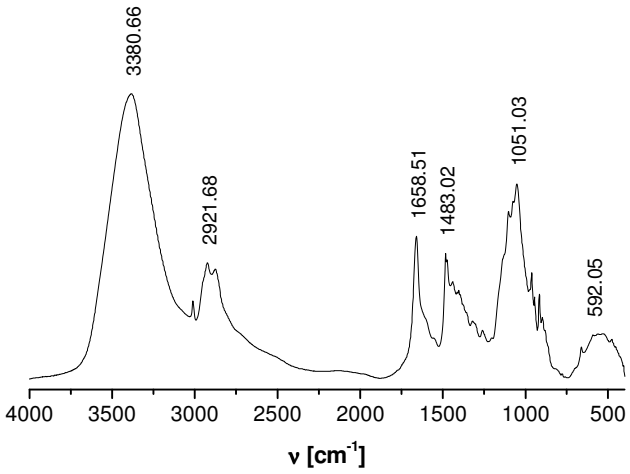

Figure 53 IR spectrum of the insoluble product, obtained by reaction of glycol chitosan with the quatcoupler

No characteristic bands of any carbamate group appear in the spectrum, thus, revealing that no conversion occured. The insolubility might derive from a degradation process, especially at the glycol branch, since the reaction of Chitoclear at the same temperature (in DMSO) did not lead to an insoluble product.

A.2 Conclusion

Based on the results obtained the following conclusions were drawn: Different conditions were tested for the modification of the amino groups in chitosan with ethylene carbonate or substituted ethylene carbonates. The commercially available Chitoclear and glycol chitosan

were reacted in aqueous mixture, in bulk and in organic solvent with ethylene carbonate as a model compound. However, none of the performed reactions resulted in the modification of the chitosan derivatives.

The same was observed for the reaction of chitosan with quaternary ammonium carbonate (quatcoupler). Only in one reaction, namely with glycol chitosan in aqueous solution at 60 °C a low conversion with a degree of functionalisation of 2.9 % was observed. But the obtained product was also not fully soluble in water, which probably derives from the same side-reaction that leads to insoluble products in the case of performance in DMF at 100 °C. The low nucleophilicity of the chitosan amino group, similar to other natural amines (see chapter 4), requires other techniques for the modification than the direct use of functional couplers.

A.3 Experimental Part

A.3.1 Materials

Glycol Chitosan (DP ≥ 400), degree of deacetylation (DD) 88 %, determined via ^{1}H-NMR spectroscopy in D_2O was purchased from Aldrich and Chitoclear (M = 70.000 g/mol, DD 86%) from Primex. Quaternary ammonium carbonate was synthesised by N. Pasquier.[5] Deuterium chloride (35.0 %) and deuterium oxide (99.9 %) were purchased from Aldrich. Ethylene carbonate was purchased from Fluka.

A.3.2 Instruments

FTIR spectra were recorded with KBr pellets on a Nicolet FT-IR spectrophotometer Nexus 470. NMR Spectra were recorded on a Varian VXR 300, a Bruker DPX-300 FT-NMR spectrometer at 300 MHz and 75 MHz or on a Bruker AV 600 FT-NMR Spektrometer at 600 MHz, respectively.

A.3.3 Synthesis

Modification of chitosan with ethylene carbonate – reaction in aqueous solution

Chitosan (600 mg, *Chitoclear*) was hydrated in 4.8 mL 1N HCl and dissolved by addition of H_2O to obtain a 5 % solution of chitosan. The pH was slowly increased to 6.5 by adding 0.1 N NaOH under continuously stirring until a final volume of ~ 14 mL. The mixture was divided in 6 portions (à 97.7 mg, 0.585 mmol) and ethylene carbonate was added in different ratios:

Table 15 Reaction details

entry	Ratio (C:EC)	ethylene carbonate [mg]	Reaction conditions
1	1:1	97.8	r.t., stirring
2	1:2	195.4	r.t., stirring
3	1:0.5	48.9	r.t., stirring
4	1:1	97.7	50 °C, agitation 130 rpm
5	1:2	195.3	50 °C, agitation 130 rpm
6	1:0.5	49.0	50 °C, agitation 130 rpm

The mixtures were lyophilized after 68 h and then analysed by NMR-spectroscopy in D_2O/DCl. Afterwards the solutions were dialysed against water for 5 d, lyophilized and again analysed via NMR. The yield was not determined.

Modification of glycol chitosan with ethylene carbonate – reaction in aqueous solution

Glycol Chitosan (100.8 mg, 0.48 mmol) and ethylene carbonate (40.0 mg, 0.45 mmol) were dissolved in deuterium oxide (1.5 mL) and stirred at r.t. for 23 h. The slightly yellow solution was analysed by NMR spectroscopy. Afterwards the solution was dialysed against water for 1.5 d, lyophilised and again analysed via NMR.

Yield: 51.9 mg (43.7 %) yellow powder

Modification of chitosan with ethylene carbonate – reaction in bulk

Chitosan (125.3 mg, 0.750 mmol, *Chitoclear*) was stirred with ethylene carbonate (625 mg, 7.10 mmol) for 65 h at 100 °C. The mixture was filtrated and chitosan was washed with warm ethanol, dried in high vacuum and analyzed by NMR spectroscopy. The product was dissolved in HCl/H_2O and dialyzed against water for 3 d, lyophilised and again analysed via NMR. The yield was not determined.

Modification of glycol chitosan with ethylene carbonate – reaction in DMF

Glycol Chitosan (202.7 mg, 0.96 mmol) and ethylene carbonate (162.6 mg, 1.85 mmol) were suspended in DMF (2 mL) and stirred at 100 °C for 28.5 h. Et_2O was added and the mixture was filtrated, washed with Et_2O and dried in high vacuum. The sample was only analysed via IR spectroscopy, due to its low solubility in aqueous solution.

Yield: 172.3 mg (72.1 %) yellow powder

Modification of chitosan with quaternary ammonium carbonate – reaction in aqueous solution

Chitosan (220 mg, 1.32 mmol, *Chitoclear*) was hydrated in 1.8 mL 1 N HCl and dissolved by addition of 3 mL 0.1 N NaOH. The pH was slowly increased to pH 6 by addition of 1 N NaOH under continuously stirring. During this process the initial solution became a gel with chitosan particles. Quaternary ammonium carbonate (400 mg, 1.39 mmol) was added to the mixture and was stirred at room temperature for 3.5 d. The mixture was lyophilised and analysed via NMR spectroscopy. Afterwards the mixture was dialysed against water and again analysed via NMR.

Modification of glycol chitosan with quaternary ammonium carbonate – reaction in aqueous solution

Quaternary ammonium carbonate (101 mg, 0.46 mmol) and glycol chitosan (101.0 mg, 0.48 mmol) were dissolved in deuterium oxide (1.5 mL) and stirred at room temperature for

23 h. The mixture was analysed by NMR spectroscopy, than dialysed against water for 2d, lyophilised and again analysed via NMR.

Yield: 60.3 mg (31.2 %)

Modification of glycol chitosan with quaternary ammonium carbonate – reaction in aqueous solution

Glycol chitosan (25.1 mg, 118.9 mmol) was dissolved in a solution of $NaHCO_3$ (20.3 mg, 242 mmol) in water (1.4 mL). A suspension of quaternary ammonium carbonate (35.2 mg, 122 mmol) in 1,4-dioxane (0.5 mL) was added. The mixture was stirred for 43 h at 60 °C, than dialysed against water, lyophilised and again analysed via NMR. Yield: 68.9 mg (144 %)

Modification of glycol chitosan with quaternary ammonium carbonate – reaction in DMSO-d_6

Chitosan (101.9 mg, 0.610 mmol) was added to a solution of quaternary ammonium carbonate (324.3 mg, 1.13 mmol) in DMSO-d_6 (2.0 mL). The mixture was stirred for 65 h at 60 °C, filtrated, washed with DMSO and analysed by NMR spectroscopy. Afterwards the product was dialysed against water, lyophilised and again analysed via NMR. Yield: 40.3 mg (31.7 %)

Modification of glycol chitosan with quaternary ammonium carbonate – reaction in DMF

Glycol Chitosan (100.4 mg, 0.475 mmol) and quaternary ammonium coupler (65.3 mg, 0.227 mmol) were suspended in DMF (1 mL) and heated at 100 °C for 28.5 h. Et_2O was added and the mixture was filtrated. The sample was only analysed via IR spectroscopy, due to its low solubility in aqueous solution.

Yield: 134.5 mg (70.0 %) yellow powder

A.4 References

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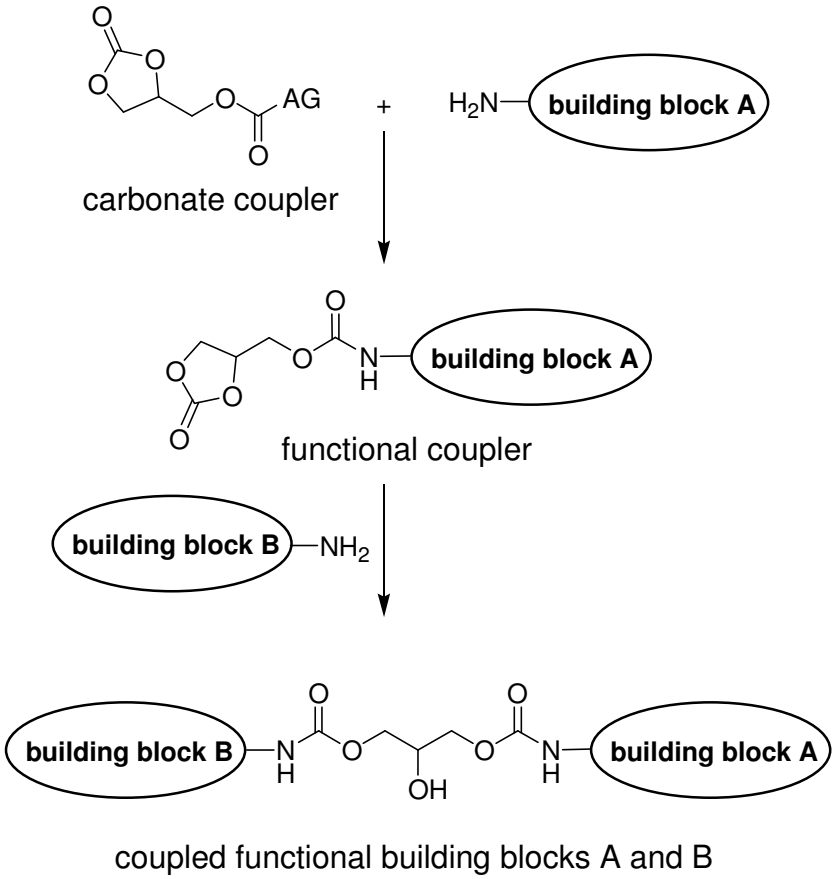
Appendix B Synthesis and application of functional cyclic carbonates from amino acids

B.1 Introduction

Polymer-peptide conjugates are materials which combine the properties of the natural and the synthetic component.[1] The most prominent field of application of such materials is the PEGylation of proteinic drugs to overcome the pharmaceutical issue of a fast degradation.[2] Different methods for coupling reactions of peptides and polymers have been described that include poly(ethylene glycols) with activated esters and carbonates, or aldehyde groups in the reaction with the α- or ε-amino groups of amino acids.[3] Classical peptides synthesis was applied for the preparation of silk-inspired peptide-oligothiophene conjugates. A pentapeptide (GAGAG) – synthesised via solid phase peptide synthesis and still immobilised on the used *Wang* resin – was coupled to a carboxy-terminated oligothiophene[4] Another method for the coupling of amino group containing materials is based on the so-called carbonate coupler approach.[5] The carbonate coupler approach is a versatile method to prepare new materials via a nucleophilic addition reaction to the cyclic carbonate that proceeds release-free (see Scheme 25).



Scheme 25 The carbonate coupler approach

In the first, so-called functional couplers are prepared from amines that bear further functional groups with interesting properties (building block A). Such compounds are obtained via the reaction of the chosen amine with the carbonate coupler – a heterobifunctional coupling agent with two reactive carbonate groups that can be addressed selectively. The activating group AG can be chlorine, phenol, 4-nitrophenol, acetone oxime or imidazole. The preparation of different carbonate couplers, starting from glycerol carbonate was shown before.[6] The functional couplers are prepared by a substitution reaction via release of AG. Highly nucleophilic amines can be converted at temperatures around 0 °C, amines like amino acids and derivatives thereof react at 40 °C. At these temperatures no reaction with the cyclic carbonate moiety occurs. The second step is the nucleophilic addition of another amine (building block B) at elevated temperatures (> 60 °C) with the functional

coupler. In each case the amine can derive from the group of aliphatic monodisperse amines or polyamines. Within the product two different compounds are combined, thus leading to multifunctional oligomers or polymers with interesting surface properties or antimicrobial activity: Following the described approach chitosan (natural polyamine) biocides have been prepared, which show high antimicrobial activity due to their surface active properties against *E. coli* and *B. subtilis* and no haemolytic activity.[7] Beside the modification of natural occurring polyamines, synthetic polyamines like poly(ethylene imine) (PEI) and polydimethylsiloxanes (PDMS) have been modified by using functional couplers.[8, 9] In the case of PEI branched polymers with ionic and hydrophobic side chain were obtained. With PDMS linear products with cationic end groups were achieved, that interact with negatively charged surfaces.

The application of the carbonate coupler approach for the preparation of hybrid materials of amino acids and polymers has not been described so far. The benefit of this strategy is the versatile application of any polymer having amino goups, like PEI, Jeffamines, poly vinyl amine and amino-functionalised poly(ethylene glycol)s (PEG). Furthermore, the coupling reaction via the cyclic carbonate occurs according to an addition reacton, therefore no contamination with side products occurs.

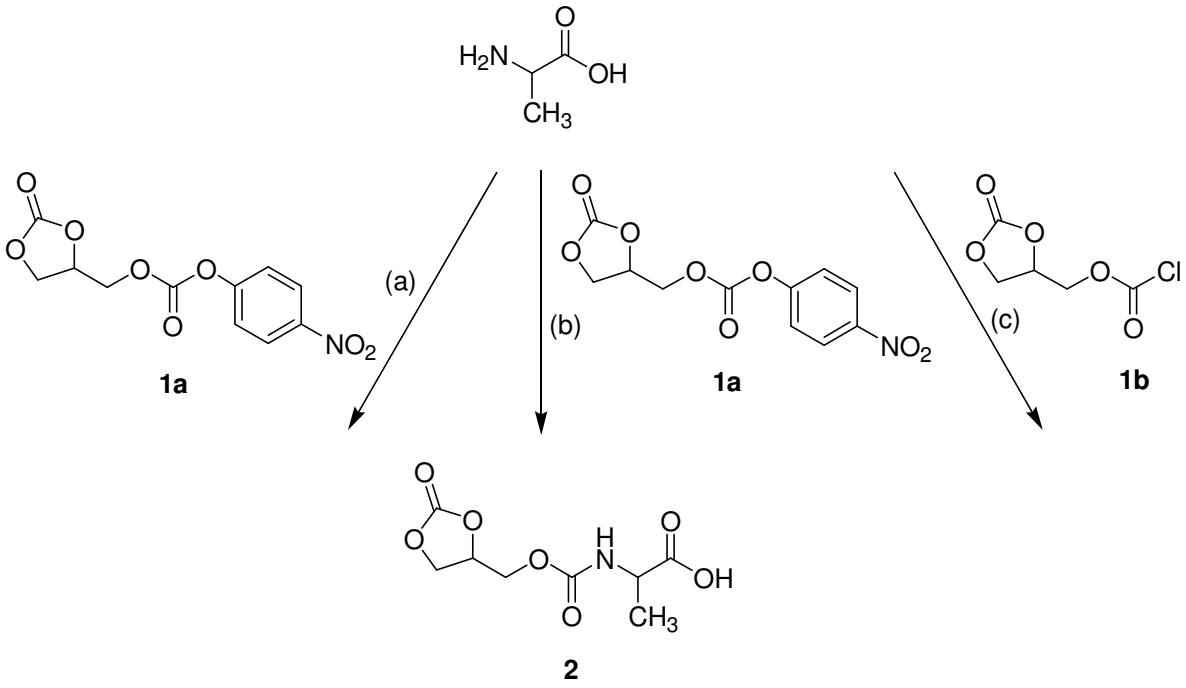
B.2 Results and Discussion

The aim of this study was the functionalisation of amino acids or peptides at the N-terminus via the carbonate coupler approach. By this means amino acid building blocks should be prepared which can be used to functionalise synthetic compounds (polyamines) with natural compounds and which also represent a simple model for proteins. First attempts to prepare (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-amino acids are described. Afterwards

the nucleophilic addition of aliphatic and linear polymeric amines to functional couplers is discussed.

B.2.1 Attempts to prepare (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-amino acids

Different attempts were made to prepare amino acid derivatives, which bear a cyclic carbonate group at the amino group like (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-alanine (**2**, see Scheme 26). The so-called carbonate coupler **1** is the reagent for inserting the cyclic carbonate moiety, with R being either 4-nitrophenyl or the more reactive halogen chlorine.



Scheme 26 Preparation of (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-alanine **3** via three different procedures: (a) DBU, CH_3CN, r.t.; (b) ^tBuOH, NaOH, rflx.; (c) NaOH, 1,4-dioxane, 0 °C and r.t.

The difficulty in preparing the shown derivative **3** lies in the difference in solubility of the starting materials. The carbonate coupler is well soluble in polar organic solvents, like DMSO or CH_3CN and also in less polar solvents. Amino acids are well soluble in water or in mixtures of water with alcohols or CH_3CN. However, with water hydrolysis of the cyclic

carbonate is observed and with alcoholic components reaction with the phenyl carbonate might be an issue. Therefore it was tested whether amino acids can be dissolved as salt of the highly nucleophilic base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in organic solvents.[10] Alanine could be well dissolved in CH_3CN by addition of DBU. The solution was than reacted with (2-oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate (**1a**) and analyses of the raw product revealed the formation of the functional alanine coupler (**2**). However, the product could not be isolated as free acid, only as salt of DBU. The same result was observed in the reaction with glycine by dissolution in the presence of DBU. The ionic interaction displays such a strong bond, that the acid can not be liberated by applying an ion exchange resin or by acidification and extraction of the product. Therefore the method applied for the protection of amino acids as *t*-BOC carbamates with *t*-butyl *p*-nitrophenyl carbonate was adapted:[11] The amino acid (alanine or glycine) and with (2-oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate (**1a**) were reacted in a mixture of aqueous sodium hydroxide and *tert*-butanol. Conversion was achieved under these conditions, trials to isolate the product from the reaction mixture failed.

Modification of amino acids to their (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-derivative was also performed with glycerol carbonate chloroformate (**1b**). This is the most reactive species among the carbonate couplers, therefore successful conversion was also expected for this compound: According to another method for the protection of amino acids alanine was dissolved in aqueous sodium hydroxide and treated with glycerol carbonate chloroformate (**1b**).[12] The mixture was stirred at 0 °C for 45 min, acidified with hydrochloric acid and extracted with ethyl acetate. The NMR analysis of the extracted oil revealed the formation of the desired product. The mixture contained glycerol carbonate and di(glycerol carbonate) carbonate, which is formed by the reaction of glycerol carbonate and glycerol carbonate chloroformate. Even though the raw product contained 69.0 % of 2-oxo-1,3-dioxolan-4-

ylmethoxycarbonyl)-alanine (**2**) the product could not be purified via column chromatography. The NMR spectrum of a fraction with high amount of product is shown in Figure 54. The signals appear at a similar chemical shift as the corresponding product obtained from alanine methyl ester (see the experimental part for comparison).[6]

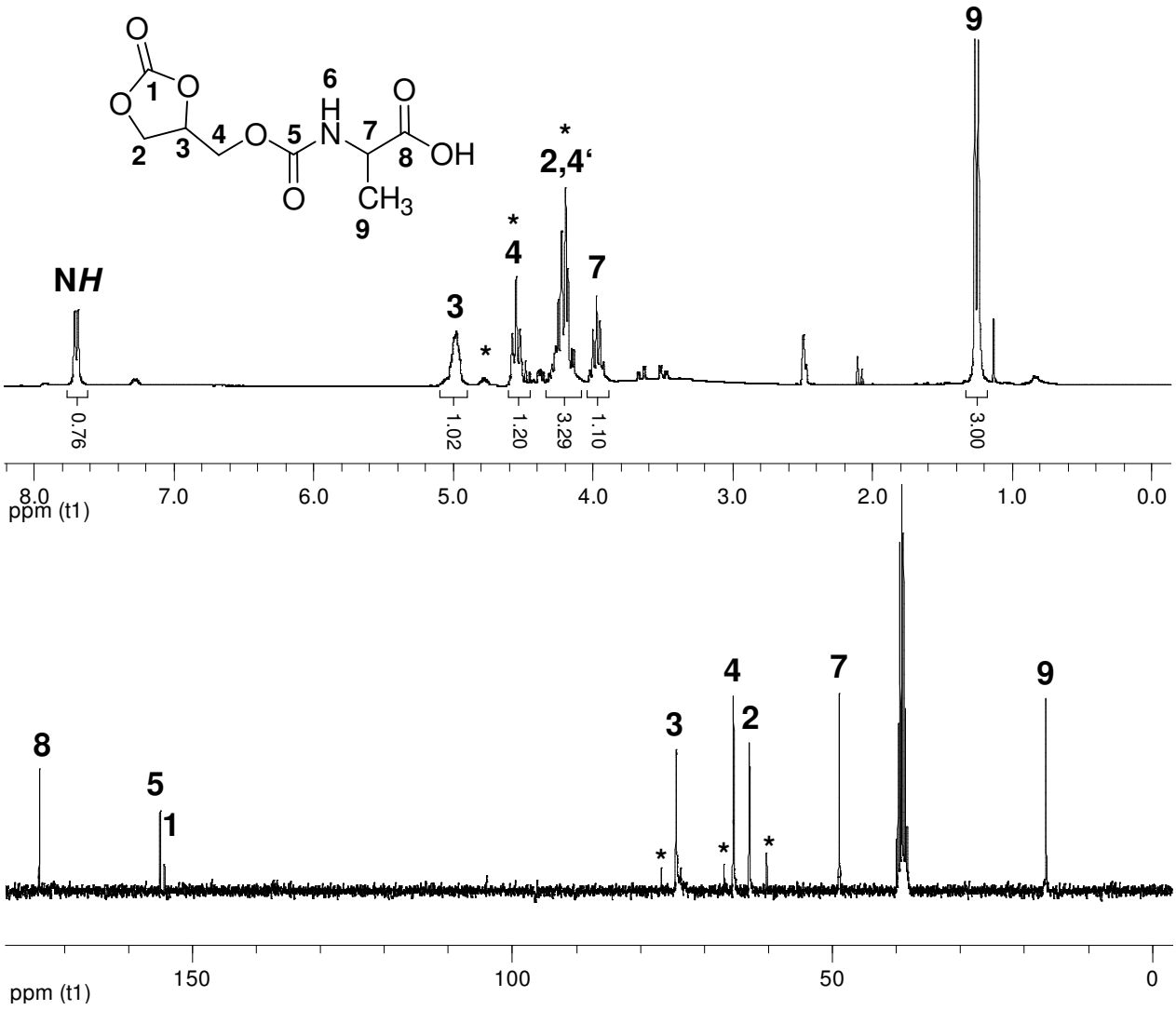


Figure 54 ^{1}H- and ^{13}C-NMR spectrum of 2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-alanine **2** in DMSO-d_6 (* glycerol carbonate)

B.2.2 Modification of natural peptides

In order to functionalise natural occurring peptides with synthetic polyamines, fibroin, the major protein of *Bombyx mori* silk, was enzymatically degraded with chymotrypsin.[13] This enzyme degrades the amino acid chain at the carboxy-terminus of tyrosin, phenylalanine and

tryptophan residues. During the degradation process two fractions, a precipitate (Cp) and a soluble (Cs) fraction are formed. The precipitate contains the crystalline part with a repetition of the typical hexapeptide sequence SGAGAG with the serine residue at every sixth position. Since chloroformates are not only reactive towards amines, but also react with hydroxy groups, the Cp fraction was reacted with glycerol carbonate chloroformate **1b**. Amino acid analysis (ASA) revealed a serine content of 16.5 mol% or 1.69 mmol/g. It was expected that a full conversion of all serine residues into the corresponding carbonate will result in weight increase of 19.8 %. IR analysis of the obtained solid showed the characteristic band of the cyclic carbonate at 1790.8 cm^{-1} (see Figure 55), however with very low intensity. Moreover no weight increase was observed, even though the work-up procedure was simple and loss of material was not expected.

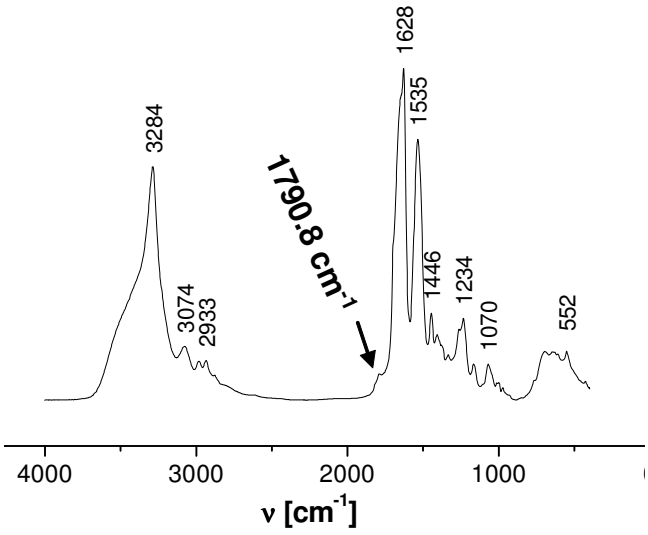
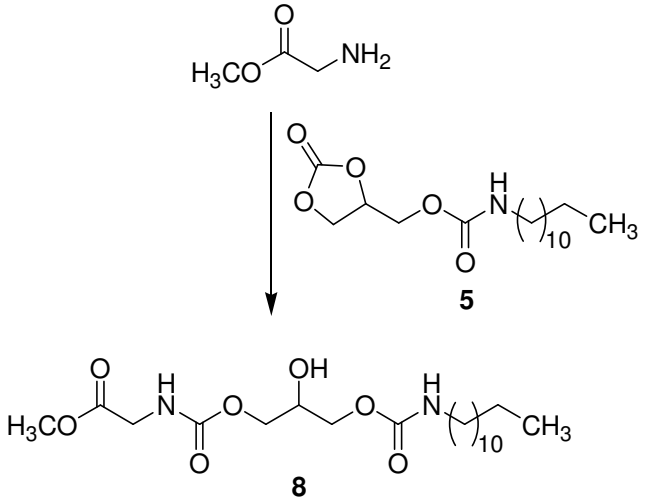


Figure 55 IR spectrum of the chymotryptic precipitate of silk peptides after reaction with glycerol carbonate chloroformate

Serine residues could therefore be functionalised, but not with high efficiency. This probably results from the heterogeneous reaction and not due to steric issues, since the hydroxy residues are avaiable due to their conformational position within the β-sheet.[14]

B.2.3 Nucleophhilic addition reaction

According to the carbonate coupler approach, two amino-reactive compounds can be coupled and the order to combine amino acids and synthetic amines was believed to be interchangeable. To proof this, a functional coupler from 1-dodecylamine, 2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-dodecylamine **5** was prepared, which was afterwards reacted with glycine methyl ester (see Scheme 27).



Scheme 27 Nucleophilic addition of glycine methl ester to (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-dodecylamine **5**

Glycine methyl ester, functional dodeylamine coupler (**5**) and DMAP were dissolved in DMSO-d_6. The solution was heated to 40°C and the conversion was checked after 6 h by ^{1}H-NMR spectroscopy. No addition to the ethylene carbonate group could be detected, also not after further 8 h at 80 °C and not after additional 7 h at 80 °C and addition of 1 eq of DMAP.

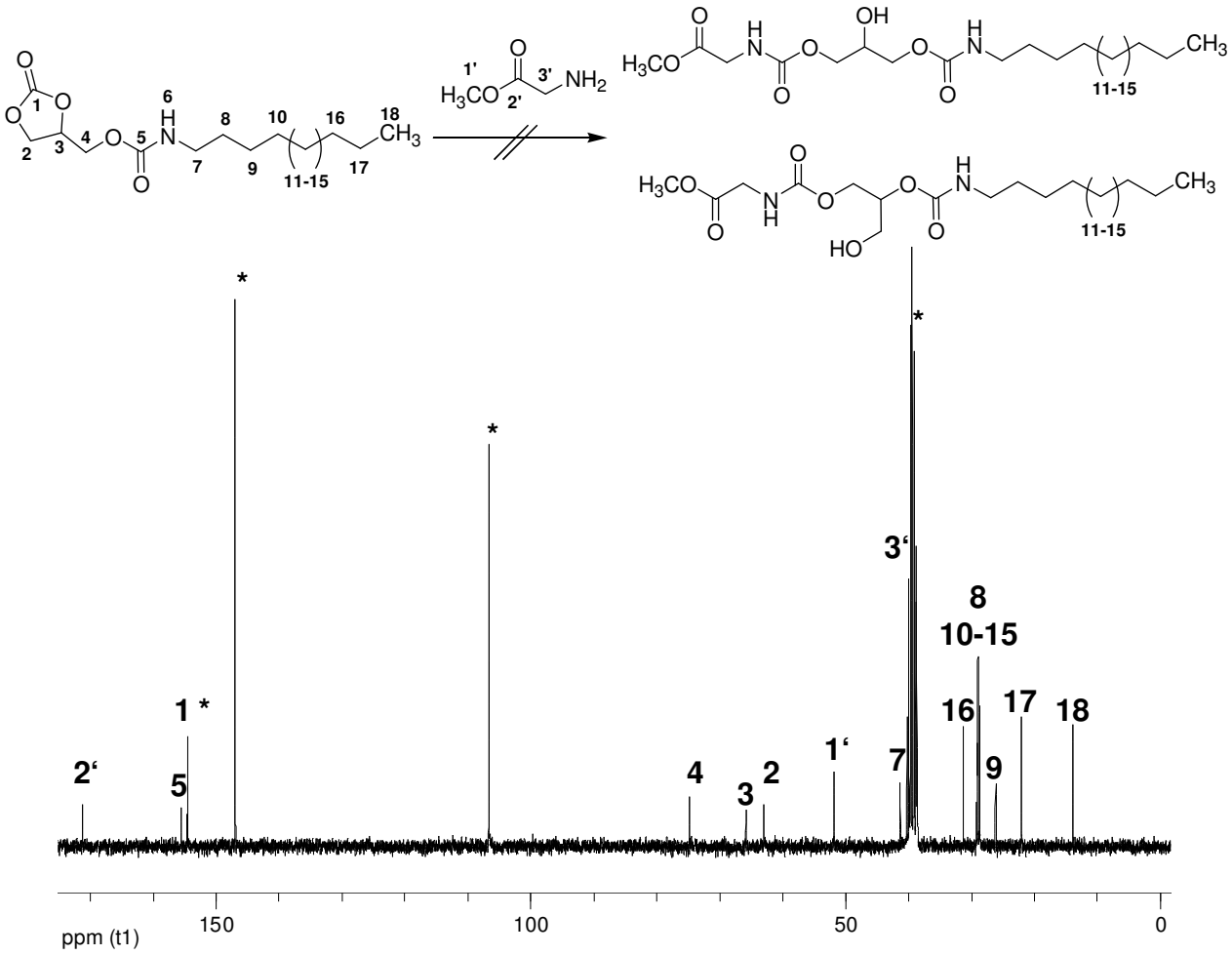
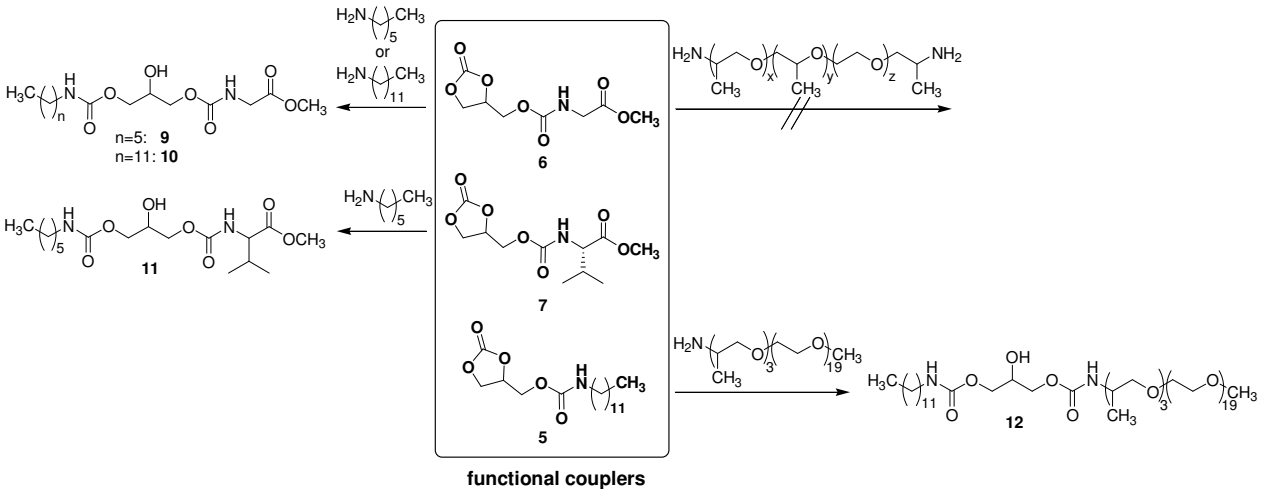


Figure 56 ^{13}C-NMR spectrum after unsuccessful reaction of functional coupler **5** with glycine methyl ester: the cyclic carbonate moiety is still present (*DMAP)

Even though glycine methyl ester is the most simple and therefore the most reactive amino acid ester, the nucleophilicity is too low for the nucleophilic addition to the cyclic carbonate unit.

B.2.4 Conjugation of functional couplers with aliphatic and polymeric amines

In the following paragraph the reverse reaction order – nucleophilic addition of aliphatic amines with functional amino acid couplers – is discussed (see Scheme 28). Moreover, results from the nucleophilic addition with linear polymeric amines are presented.



Scheme 28 Nucleophilic addition reactions of functional couplers with aliphatic and polymeric amines

Functional glycine methyl ester coupler (**6**) was modified using hexylamine and dodecylamine; with functional valine methyl ester coupler (**7**) and hexylamine an optically active product was obtained. The first two products were synthesized in acetonitrile at 50 °C, the latter in $CHCl_3$ at 40 °C. The NMR spectrum of product **9** is shown in Figure 57: The signals of hexylamine appear at $\delta = 0.89$, $\delta = 1.30$, $\delta = 1.50$ and $\delta = 3.14$ ppm. The signals between $\delta = 3.76$ and $\delta = 4.30$ ppm are attributed to the protons of the methyl ether group (H-16), the methylene group from glycine (H-14), the signals from the central unit (H-9,-10,-11,-11',-14) and the signals at $\delta = 4.91$, $\delta = 5.36$ and $\delta = 5.96$ ppm are attributed to H-10' and the urethane groups (H-7,-7' and H-13,-13'), which confirm the successful synthesis of the glycine-hexylamine hybrid **9**.

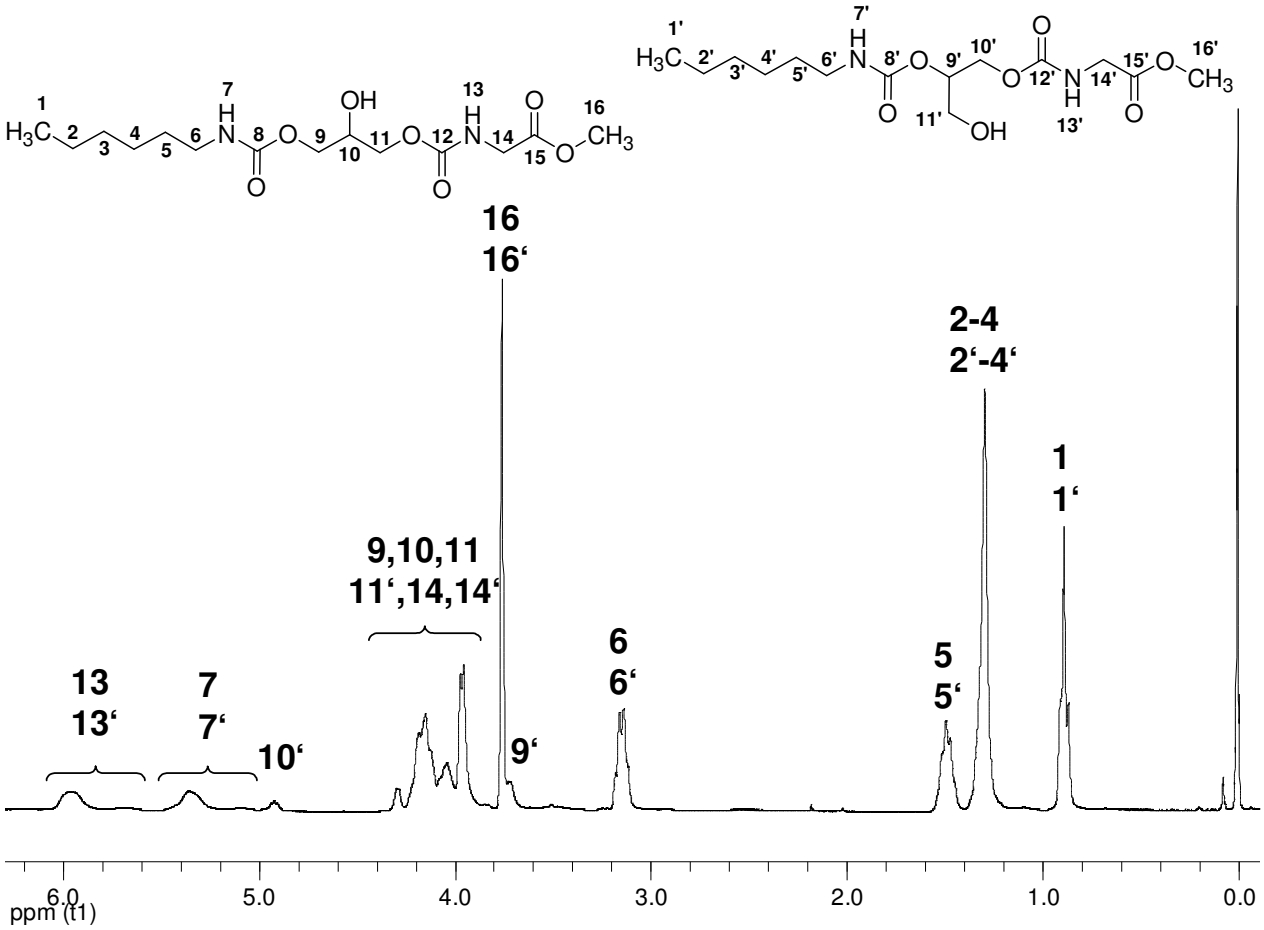


Figure 57 ^{1}H-NMR spectrum of **9** in $CDCl_3$

The ^{13}C-NMR spectrum of the analogous conjugate, prepared from 2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-valine methyl ester (**7**) and hexylamine is shown in the following figure (Figure 58).

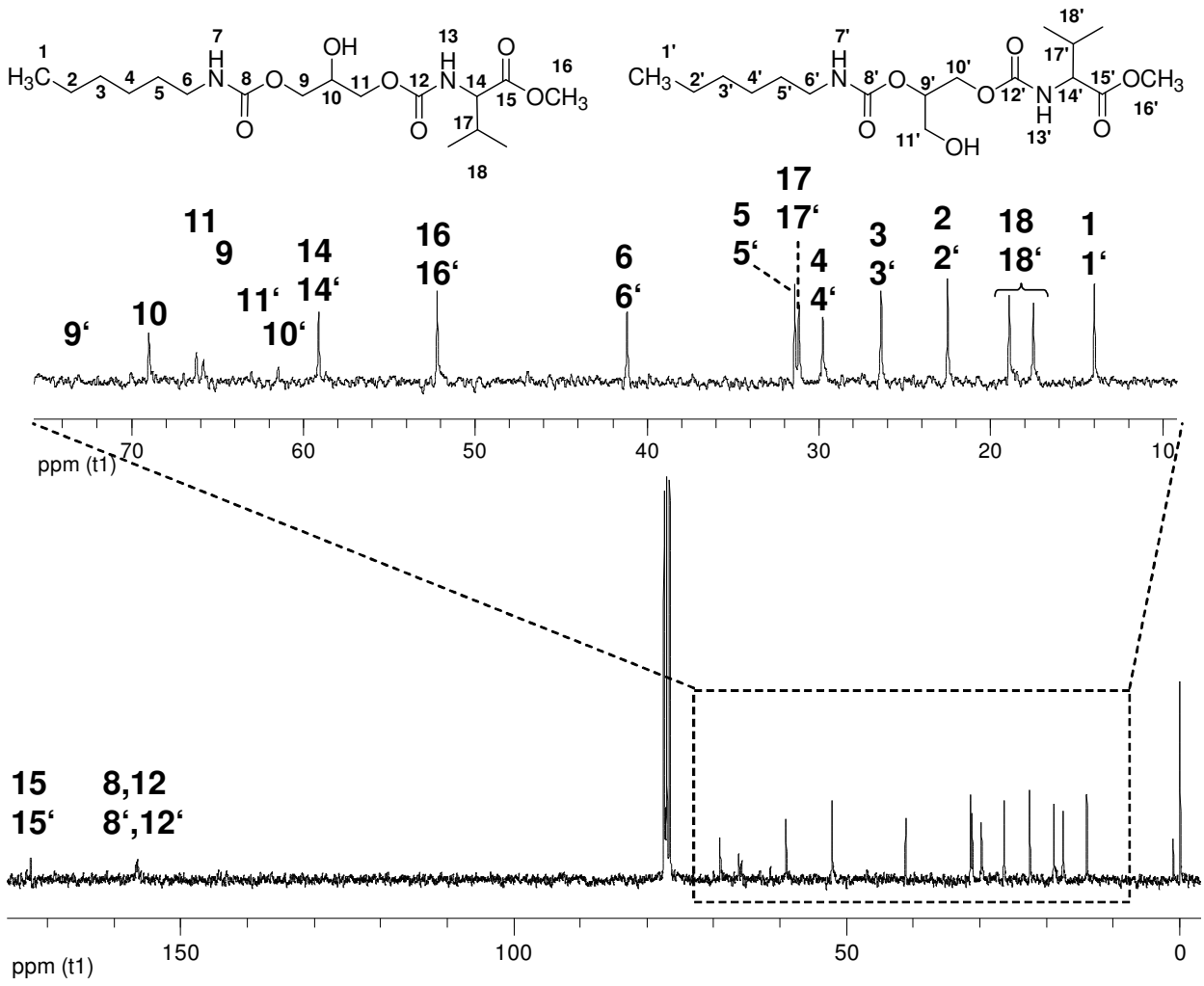


Figure 58 ^{13}C-NMR spectrum of **11** in $CDCl_3$

The signals between $\delta = 60$ and $\delta = 75$ pmm clearly indicate the presence of the two isomers, the primary and the secondary alcohol, arising from the nucleophilic addition reaction.

This nucleophilic addition reation was also performed with polyamines to test the ability to prepare functional polymers with a polymeric block and a second block deriving from functional couplers. First, dodecylamine functional coupler was reacted with a monofunctional Jeffamine (XTJ-506, M= 1000 g/ml) in DMSO-d_6 and in bulk with an equimolar amount of reagents and with an excess of the polyamine, respectively. Reactions in solution were performed at 60 °C for 4 d and since no significant conversion could be detected by NMR spectroscopy, the solution was stepwise preheated: 9.5 h at 80 °C, 9.5 h at

100 °C and 10 h at 120 C. A low conversion could only be detected after the last step. The reaction was repeated in bulk at 80 °C and the successful conversion was again followed by NMR spectroscopy (see Figure 59).

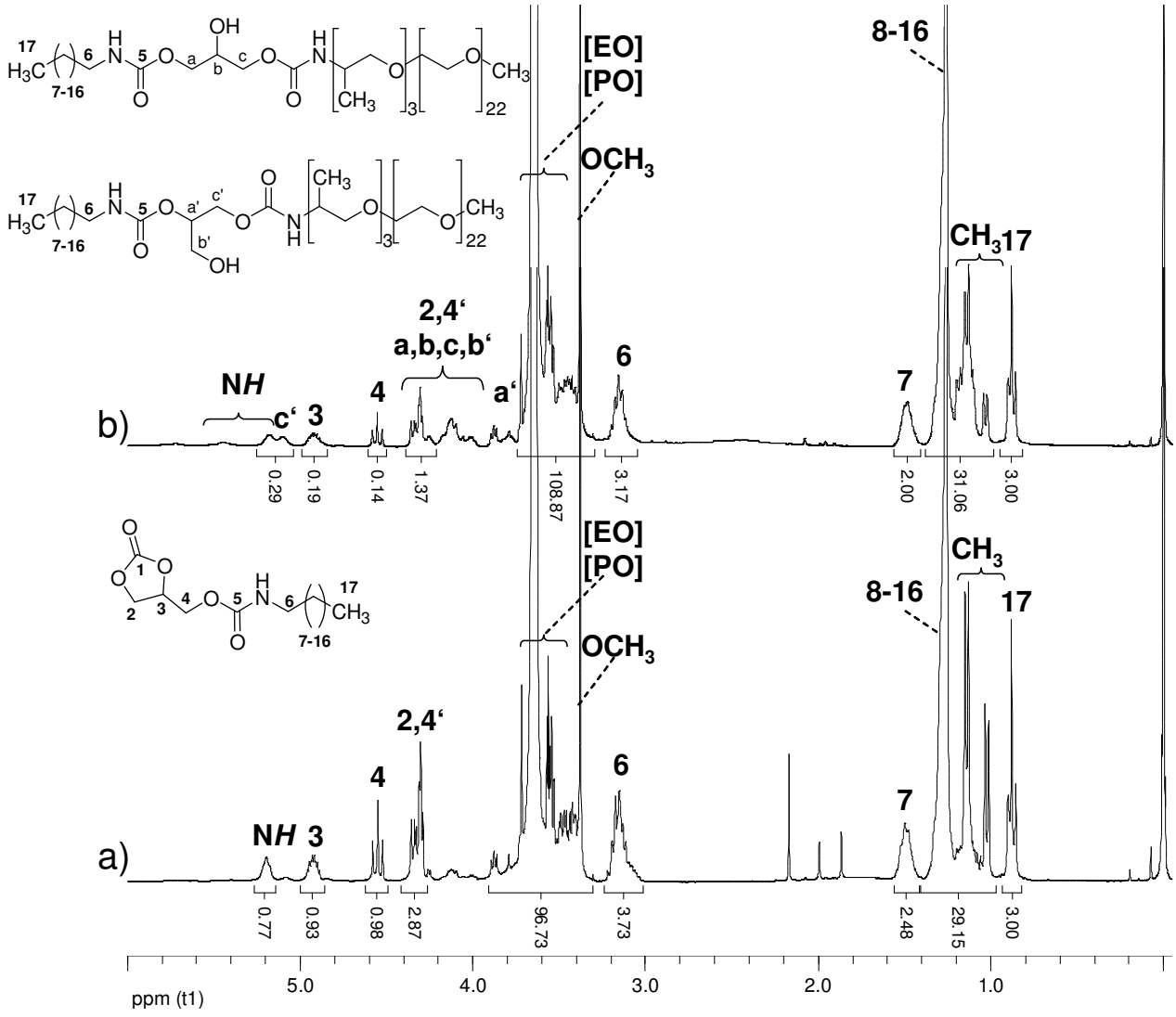


Figure 59 ^{1}H-NMR spectra in $CDCl_3$ of the nucleophilic addition of Jeffamine XTJ-506 to functional dodecylamine coupler **5**: a) after 7 h at 80 °C 4d); b) after 27 h at 80 °C

The signals of the dodecylamine functional coupler are still present in both spectra (signal H-3, H-2, H-4), however, the intensity of the signals decreased after 27 hours, confirming the conversion with the Jeffamine. Nevertheless under applied reaction conditions a full conversion was not achieved.

Reaction of functional glycine methyl ester coupler (**6**) was performed with the Jeffamine ED-2003, a bifunctional polyamine with a molecular weight of M=2000 g/mol, in bulk at 60 °C. However, after 51.5 h stirring no conversion could be detected via NMR spectroscopy.

B.3 Conclusion

Based on the results obtained the following conclusions were drawn: Functional couplers from amino acids could be prepared, according to the route described for the synthesis of 2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-alanine (**2**). However, the isolation and purification of the product failed.

Even though the carbonate coupler approach is theoretically a versatile and flexible way to prepare conjugates, the second step – the nucleophilic addition of an amine and the functional coupler – can not be performed with amino acid derivatives, due to their low nucleophilicity. In contrast, functional amino acid couplers could be modified using aliphatic amines like hexylamine and dodecylamine. With polymeric amines, the investigated reaction conditions were not suitable for the preparation of hybrid polymers from functional couplers and linear mono- or bifunctional jeffamines. No or only low conversion could be observed. Probably the low amount of reactive groups in a viscous mixture avoids the successful reaction. These problems might be avoided by applying an excess of the functional coupler or by application of poly(ethylene imine), which was already shown before.[15]

B.4 Experimental Part

B.4.1 Materials

Starting materials and reagents were used as received unless specified. Glycerol (*Acros*), 1,4-diazabicyclo[2.2.2]octane (DABCO, *ABCR*), glycerol carbonate chloroformate (**1c**, 90%, *Aldrich*), phenyl chloroformate (*Aldrich*), dimethyl carbonate (*Acros*), 4-nitrophenyl

chloroformate (*ABCR*), 4-dimethylaminopyridine (DMAP, *Aldrich*), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, *Fluka*), 1-dodecylamine (*Acros*), 1-hexylamine (*Acros*), NH_4HCO_3 (*KMF*), amino acids (*Fluka*) and L-amino acid methyl ester hydrochlorides (*Iris*) were used as received. Jeffamines (ED-2003 and XTJ 506) were a gift from *Huntsman*. Glycine methyl ester hydrochloride was synthesized according to standard procedures.[12] Tetrahydrofuran (THF, *KMF*) was dried by refluxing over potassium, methanol by refluxing over sodium; the solvents were distilled prior to use. Pyridine, triethylamine and acetonitrile were purchased from *Fluka* in anhydrous form and used without further purification. Degummed silk was obtained from *Huppertz GmbH* and chymotrypsin was from *Aldrich*. *Ajisawas solution* consists of 56 mL aqueous calcium chloride solution (1 mol $CaCl_2$, 8 mol H_2O) and 45 mL ethanol p.a (*Merck*).[16] Dialyse tube VISKING (MWCO 12.000) was obtained from *Serva*. Thin-layer chromatography was performed on precoated plates (TLC aluminium sheets with fluorescence indicator, *Macherey-Nagel* (Düren) with detection by UV light or developing with common reagents and subsequent heating: 5% molybdato phosphoric acid in ethanol was used for all cyclic carbonate containing compounds. Chromatographic purification was carried out on silica gel (particle size 60-200 µm, *Acros Organics*).

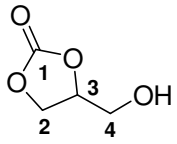
B.4.2 Instruments

NMR Spectra were recorded on a Varian VXR 300 or a Bruker DPX-300 FT-NMR spectrometer at 300 MHz and 75 MHz, respectively. Amino acid analyses (AAA) was performed on an analysator type Alpha-Plus II (Fa. Pharmacia) according to the method of *Spackman, Stein* and *Moore* after hydrolysis with 6 N HCl at 110 °C and 24 h in a closed vial. Excess of hydrochloric acid was repeatedly removed by evaporation with water.[17] FTIR spectra were recorded as KBr pellets on a Nicolet FT-IR spectrophotometer Nexus 470.

B.4.3 Syntheses

4-Hydroxymethyl-[1,3]dioxolan-2-one (Glycerol Carbonate):

Glycerol (54.54 g, 592 mol), dimethyl carbonate (149.84 g, 1663 mmol) and DABCO (667.5 g, 5.96 mmol) were heated at 75 °C for 16 h. After distillation of methanol and excess dimethyl carbonate, glycerol carbonate was used without further purification. Yield was quantitative.



^{1}H-NMR (300 MHz, DMSO-d_6):

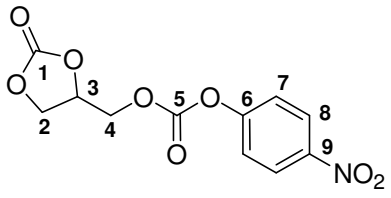
δ = 3.49 – 3.55 (m, 1 H, CH_2OH), 3.66 – 3.73 (m, 1 H, CH_2OH), 4.28 – 4.32 (m, 1 H, CH_2OCO$_2$), 4.46 – 4.52 (m, 1 H, CH_2OCO), 4.76 – 4.83 (m, 1 H, C*H*), 5.29 (s, br, O*H*) ppm.

^{13}C-NMR (75 MHz, DMSO-d_6):

δ = 60.5 (*C*H$_2$OH), 65.7 (*C*H$_2$OCO$_2$), 77.0 (*C*H), 155.2 (*C*=O) ppm.

(2-oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate 1a:

Glycerol carbonate (15 g, 127 mol) was dissolved in dry THF (150 mL) and pyridine (11.5 mL, 141 mmol) was added. The solution was cooled to -5 °C and 4-nitrophenyl chloroformate (28.2 g, 140 mmol), dissolved in dry THF (50 mL), was added slowly. The reaction was stirred at room temperature for 16 h. Pyridinium hydrochloride was removed by filtration and the solvent was removed under reduced pressure. The product was obtained by recrystallization from $CHCl_3$/Et_2O (7:1). Yield: 21.4 g, 59.6 %.



^{1}H-NMR (DMSO-d_6, 300 MHz):

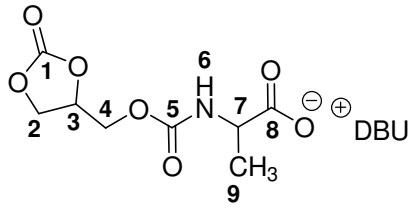
δ = 4.40-4.68 (kB, 4H, H-2, H-4), 5.13-5.20 (m, 1H, H-3), 7.59-7.62 (m, 2H, H-7), 8.32-8.36 (m, 2H, H-8) ppm.

^{13}C-NMR (DMSO-d_6, 75 MHz):

δ = 65.7 (C-2), 68.0 (C-4), 73.8 (C-3), 122.5 (C-7), 125.4 (C-8), 145.2 (C-9), 151.6 (C-5), 154.5 (C-1), 155.1 (C-6) ppm.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-alanine 2 (Method A)

Alanine (1.02 g, 11.41 mmol) was suspended in acetonitrile (5 mL) and cooled in an ice/water bath. 1,8-Diazabicyclo[5.4.0]undec-7-en (DBU, 3.5 mL, 23.4 mmol) was added drop wise and (2-oxo-1,3-dioxolan-4-yl)methyl 4-nitrophenyl carbonate **1a** (3.56 g, 12.6 mmol) was added at room temperature. Upon stirring alanine dissolved and the mixture became orange. The solution was again cooled and then let come to room temperature. within 23.5 h. A mixture of ice and water (10 mL) was added and the pH was brought from 8 to pH 2 by addition of conc. HCl. The mixture was extracted five times with ethyl acetate, the organic phase was dried over Na_2SO_4, filtrated and the solvent was evaporated. The residue from the organic phase was dried in vacuo and the aqueous phase was lyophilised. NMR-analysis revealed that the desired product remained in the aqueous phase. Yield: 5.34 g, yellow oil



^{1}H-NMR (DMSO-d_6, 300 MHz):

δ = 1.41-1.44 (d, 3H, H-9), 4.17–4.23 (m, 2H, H-2, H-4), 4.44 (m, 2H, H-7), 4.57 (m, 1H, H-4'), 4.99 (m, 1H, H-3), 7.58 (d, 1H, H-6) ppm.

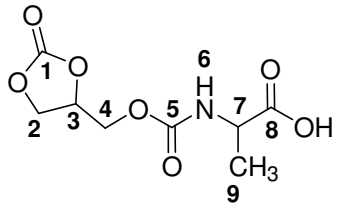
^{13}C-NMR (DMSO-d_6, 75 MHz):

δ = 18.2 (C-9), 53.5 (C-7), 63.1 (C-2), 65.9 (C-4), 74.8 (C-3), 154.7 (C-1), 155.7 (C-5), 172.4 (C-8) ppm.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-alanine 2 (Method B)

Alanine (2 g, 22.5 mmol) was dissolved in 1N $NaOH_{aq}$ (6 mL) and cooled in an ice-water bath. Glycerol carbonate chloroformate (**1b**, 6.8 g, 33.7 mmol) in 1N $NaOH_{aq}$ (6.2 mL) was added. After 15 min an additional portion of glycerol carbonate chloroformate (2.25 g,

11.2 mmol) in 1N $NaOH_{aq}$ (3.1 mL) was added. The mixture was stirred for 45 min at 0 °C. 2N HCl_{aq} (10 ml) was added and the solution was extracted with ethyl acetate. The organic phase was dried over $MgSO_4$, filtrated and the solvent was removed by evaporation (yield of raw product: 6.3 g, 69.0 % product). The product was isolated by chromatographic purification with acetone:chloroform (1:1) as eluent. The obtained fraction still contained di(glycerol carbonate) carbonate and glycerol carbonate as side products. Total yield: 1.74 g



^{1}H-NMR (DMSO-d_6, 300 MHz):

δ = 1.24-1.54 (d, 3H, H-9), 3.95–4.00 (m, 2H, H-7), 4.18-4.23 (m, 2H, H-2, H-4), 4.55 (m, 1H, H-4'), 4.99 (m, 1H, H-3), 7.69 (d, 1H, H-6) ppm.

^{13}C-NMR (DMSO-d_6, 75 MHz):

δ = 16.7 (C-9), 49.0 (C-7), 63.0 (C-2), 65.4 (C-4), 74.4 (C-3), 154.4 (C-1), 155.1 (C-5), 173.9 (C-8) ppm.

Chymotryptic degradation of fibroin 3

Small pieces of pure fibroin (5.02 g) were suspended in *Ajisawas solution* (100 mL) and stirred for 1 h at 75 °C to dissolve the protein. The solution was dialysed against water until the test for chloride was negative. The aqueous solution was centrifuged at 3500 rpm for 15 min. The solution (290 mL, concentration: ~15 mg/L) was mixed with chymotrypsin (enzyme:substrate ratio 1:100) and diluted to a final volume of 400 mL with 0.05 M NH_4HCO_3 buffer (pH = 8.0) and incubated at 37 °C, 50 rpm in a shaking oven for 15 h. The precipitate (Cp) is isolated by centrifugation (3000 rpm, 20 min) and intensively washed with water, which is combined with the soluble fraction (Cs). Both fractions were lyophilised. Yield: 2.54 g Cp (54.5 % of initial fibroin), 1.4 g Cs (29.6 % of initial fibroin)

Amino acid analysis (mol %):

	C	D	T	S	E	P	G	A	V	M	I	L	Y	F	K	H	R
Cp	0	0.4	0.8	16.5	0.3	0	41.9	35.4	0.8	0	0.2	0.2	2.7	0.6	0.1	0	0.1
Cs	0.1	4.3	2.1	7.8	2.8	1.3	36.3	22.1	5.3	0.3	1.7	1.6	10.3	1.5	1.0	0.5	1.2

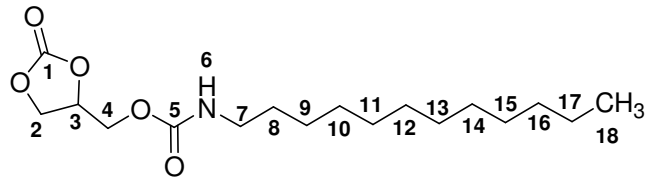
Carbonate functionalisation of Cp 4:

Glycerol carbonate chloroformate (**1b**, 432.8 mg, 2.40 mmol) and Cp (100.6 mg, 1.7 mmol serine residues/g) were suspended in dry acetonitrile (1.2 mL), triethylamine (dry, 670 µL, 4.8 mmoL) was added and stirred for 3 d at r.t. The solid was filtrated, washed with diethyl ether and dried in high vacuum. Yield: 95.61 mg

IR (KBr):
1790.8 (cycl. carbonate) cm^{-1}.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-1-dodecylamine 5:

(2-Oxo-1,3-dioxolan-4-yl)methyl phenyl carbonate **(1b**, 8.1 g, 34.1 mmol) was dissolved in THF (50 mL) and cooled to 0 °C. A solution of 1-dodecylamine (6.3 g, 34.0 mmol) dissolved in THF (60 mL) was added drop wise and the mixture was stirred over night. The solvent was removed by evaporation and the product was isolated by recrystallisation in $CHCl_3$, filtration and drying in high vacuum. Yield: 7.96 g (71.1 %)

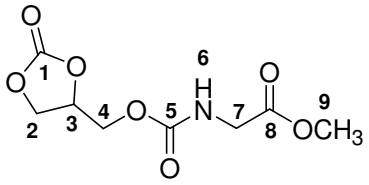


^{1}H-NMR ($CDCl_3$, 300 MHz):
δ = 0.88 (t, 3 H, H-18), 1.26 (s, 18 H, H-9 – H-17), 1.46–1.54 (m, 2 H, H-8), 3.18 (q, 2 H, H-7), 4.26–4.37 (m, 3 H, H-2, H-4), 4.54 (t, 1 H, H-4´), 4.86–4.94 (m, 2 H, H-3, H-6) ppm.
^{13}C-NMR ($CDCl_3$, 75 MHz):
δ = 14.1 (C-18), 22.7 (C-17), 26.7 (C-9), 29.3 – 29.8 (C-8, C-10 – C-15), 31.9 (C-16), 41.3 (C-7), 63.3 (C-2), 65.9 (C-4), 74.3 (C-3), 155.6 (C-1), 155.8 (C-5) ppm.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-glycine methyl ester 6:

Glycine methyl ester hydrochloride (5 g, 39.9 mmol), (2-oxo-1,3-dioxolan-4-yl)methyl phenyl carbonate (**1b**, 9.5 g, 39.8 mmol) and DMAP (5.85 g, 47.8 mmol) were dissolved in

acetonitrile (80 mL) and stirred at 40 °C for 4 h. The product was isolated as colorless oil by column chromatography (eluent: pentane/ethyl acetate 3:1 to 1:1). Yield: 7.3 g, 78.4 %



^{1}H-NMR (300 MHz, $CDCl_3$):

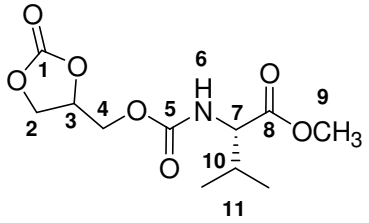
δ = 3.74 (s, 3 H, H-9), 3.93 (d, 2 H, H-7), 4.26 – 4.41 (kB, 3 H, H-2, H-4), 4.60 (t, 1 H, H-4'), 4.96 – 5.03 (m, 1 H, H-3), 5.87 (t, 1 H, H-6_Z), 6.20 (t, 1 H, H-6_E) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$):

δ = 42.6 (C-7), 52.3 (C-9), 63.9 (C-2), 66.1 (C-4), 74.7 (C-3), 155.1 (C-1), 156.1 (C-5), 170.6 (C-8) ppm.

(2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-valine methyl ester 7:

Valine methyl ester hydrochloride (1,52 g, 9.07 mmol), (2-oxo-1,3-dioxolan-4-yl)methyl phenyl carbonate (**1b**, 2.16 g, 9.07 mmol) and DMAP (1.33 g, 10.9 mmol) were dissolved in acetonitrile (25 mL) and stirred at 40 °C for 23 h. Acetonitrile was removed by distillation under reduced pressure and the crude product was dissolved in $CHCl_3$ (10 mL). After washing the organic phase with diluted sodium hydroxide and diluted hydrochloric acid, the product was isolated as colorless oil by column chromatography (eluent: pentane/ethyl acetate 3:1 to 1:1). Yield: 1.38 g, 55.3 %, yellowish oil.



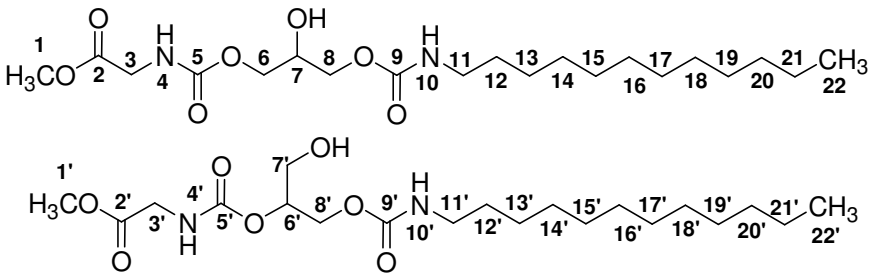
^{1}H-NMR (300 MHz, $CDCl_3$):

δ = 0.87 (d, 3 H, H-11), 0.93 (d, 3 H, H-11´), 2.13 (m, 1 H, H-10), 3.71 (s, 3 H, H-9), 4.18 – 4.37 (kB, 4 H, H-2, H-4, H-7), 4.53 (t, 1 H, H-4´), 4.91 – 4.97 (m, 1 H, H-3), 5.38 (d, 1H, H-6_Z), 5.61 (d, 1H, H-6_E) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$):

δ = 17.6 (C-11), 18.9 (C-11´), 31.1 (C-10), 52.3 (C-9), 59.3 (C-7), 63.7 (C-2), 66.0 (C-4), 74.3 (C-3), 154.7 (C-1), 155.6 (C-5), 172.2 (C-8) ppm.

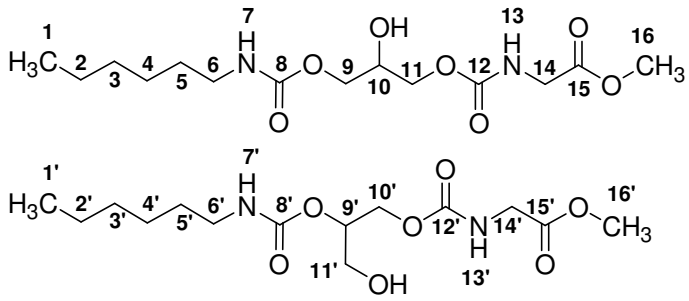
(3-Dodecylcarbamoyloxy-2-hydroxy-propoxycarbonylamino)-acetic acid methyl ester 8:



Glycine methyl ester hydrochloride (10.1 mg, 0.80 mmol), (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-1-dodecylamine (**5**, 26.5 mg, 0.80 mmol) and DMAP (11.8 mg, 96.4 mmol) were dissolved in DMSO-d_6 (500 µL), heated at 40 °C for 6 h and analysed by NMR spectroscopy. No conversion was observed. The mixture was further heated at 80 °C for 8 h and after addition of a portion DMAP (11.5 mg, 94.1 mmol) again heated for 7 h at 80 °C. NMR spectroscopy confirmed that no conversion occurred.

(3-Hexylcarbamoyloxy-2-hydroxy-propoxycarbonylamino)-acetic acid methyl ester 9

(2-Oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-glycine methyl ester (**6**, 1.16 g, 4.98 mmol) and 1-hexylamine (660 µL, 4.99 mmol) were dissolved in acetonitrile (10 mL) and stirred at 50 °C for 6.5 h and 3 d at r.t. The solvent was evaporated and the product was dried in vacuo. Yield: 1.10 g (65.8 %) colourless oil.



^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 0.88 (t, 6 H, H-1, H-1´), 1.29 (s, 12 H, H-2, H-2´, H-3, H-3´, H-4, H-4´), 1.49 (m, 2 H, H-5, H-5´), 3.14 (q, 4 H, H-6, H-6´), 3.72 (d, 2 H, H-9´), 3.75 (s, 6 H, H-16, H-16´), 3.95 –

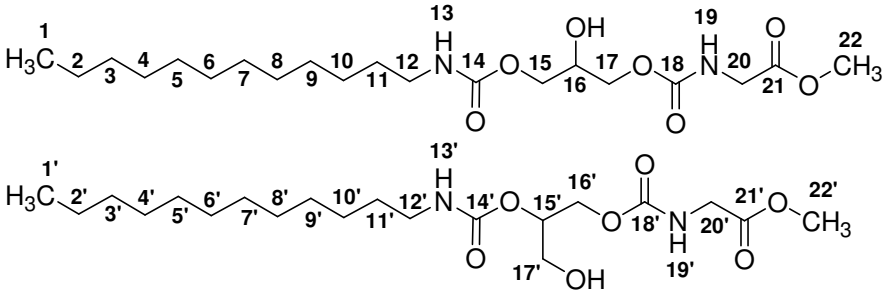
4.29 (m, 9 H, H-9, H-10, H-11, H-11´, H-14), 4.92 (t, 1 H, H-10´), 5.09 (s, 1 H, H-7_Z, H-7_Z´), 5.35 (s, 1 H, H-7_E, H-7_E´), 5.68 (s, 1 H, H-13_Z, H-13_Z´), 5.96 (s, 1 H, H-13_E, H-13_E´) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 14.0 (C-1, C-1´), 22.6 (C-2, C-2´), 26.4 (C-4, C-4´), 29.8 (C-5, C-5´), 31.5 (C-3, C-3´), 41.2 (C-6, C-6´), 42.6 (C-14, C-14´), 52.4 (C-16, C-16´), 61.4 (C-9´), 63.5 (C-11´), 65.7 (C-9), 66.3 (C-11), 68.6 (C-10), 73.0 (C-10´), 156.2 (C-8, C-8´), 156.8 (C-12, C-12´), 170.8 (C-15, C-15´) ppm.

(3-Dodecylcarbamoyloxy-2-hydroxy-propoxycarbonylamino)-acetic acid methyl ester 10:

(2-Oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-glycine methyl ester (**6**, 1.04 g, 4.45 mmol) and 1-dodecylamine (824.3 mg, 4.45 mmol) were dissolved in acetonitrile (10 mL) and stirred at 50 °C for 6.5 h and for 3 d at r.t. The solvent was evaporated and the product crystallised from $CHCl_3$ after addition of Et_2O. The product was filtrated and dried in vacuo. Yield: 1.64 g (88.0 %) white solid.



^{1}H-NMR ($CDCl_3$, 300 MHz):

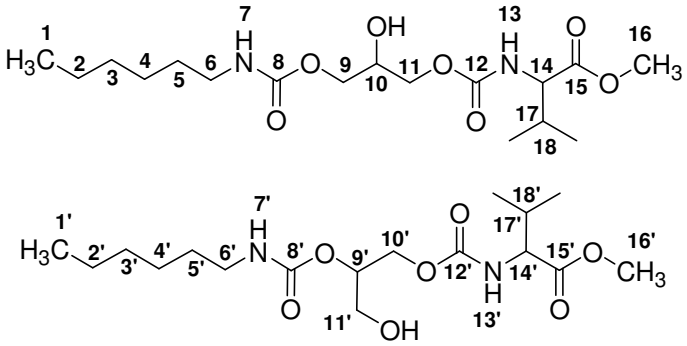
δ = 0.88 (t, 6 H, H-1, H-1´), 1.26 (s, br, 36 H, H-2 – H-10, H-2´ - H-10´), 1.49 (m, 4 H, H-11, H-11´), 3.15 (q, 4 H, H-12, H-12´), 3.71 (d, 2 H, H-17´), 3.76 (s, 6 H, H-22, H-22'), 3.97 (d, 4 H, H-20, H-20´), 4.02 – 4,30 (kB, 7 H, H-15, H-16, H-17, H-16´), 4.88 – 4.94 (m, 1 H, H-15´), 5.12 – 5.19 (m, 1 H, N*H*), 5.67 – 5.72 (m, 1H, N*H*) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 14.1 (C-1, C-1´), 22.7 (C-2, C-2´), 26.8 (C-10, C-10´), 29.3 – 29.8 (C-4 - C-9, C-11, C-4' - C-9', C-11'), 31.5 (C-3, C-3´), 41.2 (C-12, C-12´), 42.6 (C-20, C-20´), 52.4 (C-22, C-22´), 61.5 (C-17´), 63.4 (C-16´), 65.7-66.3 (C-15,-17), 68.7 (C-16), 72.6 (C-15'), 156.6-156.8 (C-14, C-18, C-14', C-18'), 170.6 (C-21, C-21´) ppm.

2-(3-Hexylcarbamoyloxy-2-hydroxy-propoxycarbonylamino)-3-methyl-butyric acid methyl ester 11:

(2-Oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-valine methyl ester (**7**, 730 mg, 2.65 mmol) and 1-hexylamine (351 µL, 2.72 mmol) were dissolved in $CHCl_3$ (12.5 mL) and stirred at 40 °C for 9 h: The solution was washed thrice with 5% HCl and 5% NaOH, the organic phase was dried over $MgSO_4$, filtrated and the product was dried in vacuo. Yield: 507.7 mg (50.9 %) yellow oil.



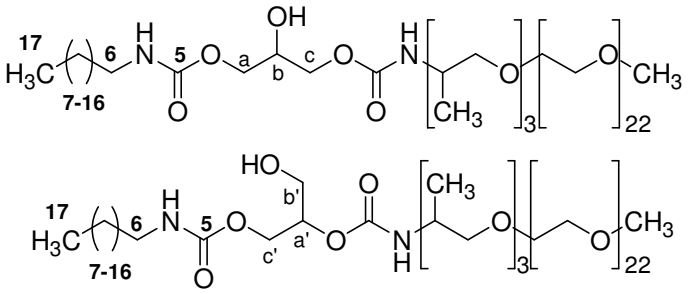
^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 0.86–0.98 (m, 18 H, H-1, H-1´, H-18, H-18´), 1.29 (s, 12 H, H-2, H-2´, H-3, H-3´, H-4, H-4´), 1.44–1.49 (m, 4 H, H-5, H-5´), 2.15-2.17 (m, 2 H, H-17, H-17´), 3.16 (q, 4 H, H-6, H-6´), 3.70 (m, 2 H, H-9´), 3.75 (s, 6 H, H-16, H-16´), 3.99–4.28 (m, 8 H, H-9, H-10, H-11, H-11´, H-14), 4.88–4.95 (m, 1 H, H-10´), 5.02 (s, 1 H, H-7_Z, H-7_Z´), 5.21 (s, 1 H, H-7_E, H-7_E´), 5.36 (s, 2 H, H-13_Z, H-13_Z´), 5.64 (d, 2 H, H-13_E, H-13_E´) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 14.0 (C-1, C-1´), 17.6 (C-18, C-18´), 19.0 (C-18, C-18´), 22.6 (C-2, C-2´), 26.4 (C-4, C-4´), 29.8 (C-5, C-5´), 31.2 (C-3, C-3´), 31.5 (C-17, C-17´), 41.2 (C-6, C-6´), 52.2 (C-16, C-16´), 59.2 (C-14, C-14´), 61.3 (C-10´), 63.4 (C-11´), 65.8 (C-9), 66.2 (C-11), 68.6 (C-10), 73.1 (C-9´), 156.5 (C-8, C-8´), 156.7 (C-12, C-12´), 172.7 (C-15, C-15´) ppm.

Reaction of Jeffamine XTJ-506 with (2-oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-1-dodecylamine 12



(2-Oxo-1,3-dioxolan-4-ylmethoxycarbonyl)-1-dodecylamine (**5**, 165.0 mg, 0.50 mmol) and Jeffamine XTJ-506 (507.5 mg, 0.51 mmol) were stirred at 80 °C for 36.5 h. Samples were taken after 7, 12.5, 16 and 27 h and analysed by NMR spectroscopy.

^{1}H-NMR ($CDCl_3$, 300 MHz):

δ = 0.86-0.90 (m, 3H, H-17), 1.02-1.15 (m, 9H, *CH₃*, XTJ-506), 1.26 (s, 18H, H-8,-9,-10,-11,-12,-13,-14,-15,-16), 1.48 (s, 2H, H-7), 3.13-3.17 (m, 2H, H-6), 3.42 (s, 3H, OMe), 3.54-3.64 (m, 97H, PO, EO), 3.88-4.36 (m, 8H, H-a, -b, -c, -a', -b'), 5.10-5.50 (m, 2H, H-c', N*H*) ppm.

^{13}C-NMR ($CDCl_3$, 75 MHz):

δ = 14.0 (C-1), 17.2 (CH_3), 19.5 (CH_3), 22.7 (C-16), 26.7 (C-8), 29.3-29.9 (C-7,C-9, -10, -11,-12,-13,-14), 31.9 (C-15), 41.2 (C-6), 47.4 (NH*C*H(CH_3)), 59.0 (OCH_3), 61.3 (C-b'), 63.3 (C-c´), 65.9 (C-c,-a), 68.8 (C-b), 70.5 – 75.3 (EO, PO) ppm.

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Aachen, 05.01.2010