PHD DISSERTATION

Development of Innovative Ingredients for Improved Microencapsulation

by Mia Falkeborg

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PREFACE

This Ph.D. thesis entitled "Development of Innovative Ingredients for Improved Microencapsulation" was submitted as a partial fulfillment of the requirements for obtaining a Ph.D. degree from Aarhus Graduate School of Science and Technology, Aarhus University. The research presented herein was conducted at Aarhus University from September 2011 through August 2014; and at Rensselaer Polytechnic Institute (RPI), Troy, NY, USA, from September 2013 to February 2014.

The work was supervised by Assoc. Prof. Zheng Guo (co-supervisor 2011-2012 and main supervisor 2012-2014), Prof. Xuebing Xu (main supervisor 2011-2012 and co-supervisor 2012-2014), Assoc. Prof. Marianne Glasius (co-supervisor), and Prof. Jonathan Dordick (supervisor at RPI).

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SUMMARY

Microencapsulation is a widely used tool for protection of sensitive ingredients against external stresses in food, medical, and technical applications. Generally, the encapsulation method and capsule materials need to be optimized for individual applications, and considerations such as matrix compatibility, stability, and release properties, need to be taken into account for each specific use. This Ph.D. study was aimed at developing innovative materials for use in microencapsulation systems. Through rational modification of natural materials, innovative encapsulation materials were synthesized, expanding the range of available materials for microencapsulation of sensitive ingredients.

The major focus was placed on development of ingredients with application potential in microencapsulation of marine oil, which requires the formation of marine oil-in-water emulsions. For preparation of stable marine oil-in-water emulsions, strong antioxidants and effective emulsifiers are needed. Starting from *alginate*, suitable emulsifiers and antioxidants were developed and applied in the stabilization of oil-in-water emulsions. Alginate is a polysaccharide composed of α-L-guluronate and β-D-mannuronate. Enzymatic depolymerization of alginate with an alginate lyase has been reported to increase the antioxidant activity of alginate, and the initial focus of this Ph.D. project was placed on characterization of this enzymatic reaction and its products: alginate oligosaccharides (AOs). The time-course of alginate depolymerization was followed to prepare AOs with the lowest possible degree of polymerization (DP). The structure and composition of the AOs were characterized, revealing that the AOs were composed of *4-deoxy-erythro-hex-4-enopyranosyluronate* in the non-reducing end, followed by 1, 2, or 3 units of mannuronate and/or guluronate in arbitrary order. The AOs were found to be good antioxidants as they were able to inhibit lipid oxidation in an emulsion to a higher extent than ascorbic acid under some conditions. Extensive analyses were performed to determine which functional group(s) of the AOs were responsible for the antioxidant activity. The studies revealed that AOs scavenged free radicals through hydrogen abstraction presumably from the carbon-bonded hydrogens, with contribution from radical

addition to the conjugated alkene acid structure, which formed during the enzymatic depolymerization.

According to the polar paradox theory, amphiphillic antioxidants provide optimal protection to emulsified oil. Thus, attempts were made to hydrophobically modify the AOs to introduce amphiphilicity. Two types of hydrophobic modifications were in focus: Type A; esterification of the carboxylic group(s) of AOs with alkyl alcohols, and type B; esterification of the hydroxyl group(s) of AOs with fatty acids. Though the primary effort of this Ph.D. study was placed on this topic, no successful results can be reported.

Focus was then placed on the modification of polymeric alginate for the purpose of introducing new properties and forming innovative materials with potential applications in microencapsulation. Polymeric alginate was modified with succinic anhydride (SAC0) and dodecenyl succinic anhydride (SAC12), in an aqueous reaction system. Process variables such as temperature, reaction time, and molar ratio of substrates, were optimized towards a high degree of succinylation. At optimum conditions, the degree of succinylation in alg-SAC0 and alg-SAC12 were 24.5% ± 2.7 and 33.9% ± 3.5, respectively. To evaluate the emulsion-stabilizing properties of the modified alginates, marine oil-in-water emulsions with 30% oil were formed, stabilized by native and modified alginate (alg-SAC12), and compared to similar emulsions stabilized by β-lactoglobulin (BLG) or phosphatidyl-choline (PC) representing emulsifiers commonly used in food products. The storage stability of emulsions stabilized by alg-SAC12 was significantly increased compared to emulsions stabilized by native alginate, BLG, or PC. By analysis of the emulsions' microstructures, alg-SAC12 was found to have good surface activity, and stabilized emulsions by adsorption to the oil-water interface, while native alginate stabilized emulsions by acting as a thickening agent. Emulsions stabilized by alg-SAC12 had a larger negative zeta potential, which provided repulsive forces between oil droplets, thus enhancing the emulsions' stability. Alg-SAC12 was furthermore found to possess a higher antioxidant activity compared to native alginate, in terms of ability to scavenge hydroxyl radicals. This dual-functionality, i.e. emulsification- and antioxidation properties, is particularly interesting considering the application in marine oil-in-water emulsions, and the development of this material represents one of the key findings of this Ph.D. research.

A second area of application of modified alginates was in microencapsulation of enzymes, for the purpose of shielding these sensitive catalysts against external stresses. Native alginate can

form strong hydrogels in which enzymes can be entrapped, thus protecting the enzyme and allowing it to be recycled. Serving as model enzymes, lipases were encapsulated in hydrogels formed from modified alginates. Alg-SAC12 in particular showed promising properties in this regard. Preliminary results on this topic are presented in this thesis.

In addition to the work described above, contributions were made to other research project, including the synthesis and application of phosphatidyl-saccharides. Phospholipids are known to form liposomes which have the potential to encapsulate sensitive ingredients; however, drying processes are usually required, which can disrupt the liposome structure. Cryoprotectants such as sugars are needed to stabilize the liposomes during dehydration processes. The idea of designing phospholipids conjugated with sugar structures was to develop compounds able to form liposomes with increased stability. Mono-, di-, and trisaccharides were enzymatically conjugated to the phosphatidyl group of phospholipids and the resulting phosphatidyl-saccharides were employed as the sole component for preparation of nanoliposomes. These nanoliposomes demonstrated significantly enhanced stability during storage and during dehydration / rehydration processes.

Finally, as an independent part of the Ph.D. study, novel antimicrobial enzymes were studied during a visiting stay at the Rensselaer Polytechnic Institute (RPI) in Troy, New York. Peptidoglycan hydrolases are a class of modular enzymes which degrade the peptidoglycan layer of specific bacteria species. The specificity of individual peptidoglycan hydrolases towards bacteria species allows their use as antibacterial agents, as they rapidly kill pathogenic bacteria while not affecting the commensal microflora. During the stay at RPI, novel enzymes with activity against *Staphylococcus aureus* and *Bacillus anthracis* were expressed and characterized. In both cases, promising observations were made, and the results are currently being further examined at RPI.

SAMMENFATNING

Mikroindkapsling anvendes bredt til beskyttelse mod udefrakommende påvirkninger på følsomme ingredienser i fødevarer og medicin og øvrige tekniske applikationer. Generelt skal både indkapslingsmetoden og indkapslingsmaterialer optimeres til hver enkelt applikation, og matrixkompatibilitet, stabilitet og release-egenskaber skal overvejes for hver enkelt anvendelsesform. Målet med dette Ph.D. studie var at udvikle innovative materialer til anvendelse i mikroindkapslingssystemer. Gennem rationel modificering af naturlige materialer er en række indkapslingsmaterialer blevet syntetiseret, hvilket har udvidet rækken af tilgængelige materialer til brug til i indkapsling af følsomme ingredienser.

Hovedfokus har været placeret på udvikling af ingredienser med applikationspotentialer inden for mikroindkapsling af fiskeolie i emulsion-basered former. Det kræver kraftige antioxidanter og effektive emulgatorer at fremstille og anvende stabile fiskeolie-i-vand emulsioner. Med udgangspunkt i alginat udvikledes anvendelige emulgatorer og antioxidanter som stabilisatorer af olie-i-vand emulsioner. Alginat er et polysakkarid, som er sammensat af α-L-guluronat og β-D-mannuronat. Enzymatisk depolymerisering af alginat med alginat lyase har vist sig at øge antioxidantaktiviteten af alginat. Fokus i dette Ph.D. projekt var at karakterisere denne enzymatiske reaktion og dens produkter, alginat-oligosakkarider (AOs). Forløbet af alginat depolymeriseringen blev evalueret for at fremstille alginat-oligosakkarider med lavest mulig grad af polymerisering (DP). Strukturen og sammensætningen af alginat-oligosakkariderne blev karakteriseret, hvilket viste at alginat-oligosakkariderne var sammensat af *4-deoxy-erythro-hex-4-enopyranosyluronat* i den ikke-reducerende ende, efterfulgt af henholdsvis en, to eller tre enheder af mannuronat og/eller guleronat i vilkårlig rækkefølge. Alginat-oligosakkariderne viste sig at være gode antioxidanter, eftersom de var i stand til at inhibere lipidoxidation i en emulsion i højere grad end askorbinsyre under givne omstændigheder. Der blev udført omfattende analyser for at klarlægge hvilke funktionelle grupper i alginat-oligosakkariderne der var årsag til antioxidantaktiviteten. Studierne afslørede at antioxidantaktiviteten hovedsagligt skyldes radikalneutralisering gennem hydrogenabstraktion,

antageligvis fra kulstofbundne hydrogen, med bidrag fra radikaladditionen til den konjugerede alkensyrestruktur, som opstår under den enzymatiske depolymerisering.

Ifølge "The Polar Paradox" yder amfibiske antioxidanter optimal beskyttelse til olie i emulsioner. De hydrofile alginat-oligosakkarider blev derfor modificeret med hydrofobiske enheder for at gøre dem overfladeaktive. Projektet har fokuseret på to typer af hydrofobiske modificeringer: Type A – esterifisering af carboxylgrupperne i alginat-oligosakkariderne med alkyl-alkoholer, og Type B – esterifisering af hydroxylgrupperne i alginat-oligosakkariderne med fedtsyrer. Ingen succesfulde resultater kan præsenteres fra denne del af projektet.

Fokus blev dernæst placeret på at fremstille modificeret polymerisk alginat med det formål at udvikle innovative materialer med potentielle anvendelsesmuligheder inden for mikroindkapsling. Polymerisk alginat blev modificeret i et vandigt reaktionssystem, med succinic anhydrider (succinat, SAC0) og dodecenyl succinic anhydrider (SAC12). Gennem optimering af procesvariable nåede graden af succinylering i alg-SAC0 24,5% ± 2,7, og i alg-SAC12 nåede graden af succinylering 33,9% ± 3,5. Fiskeolie-i-vand emulsioner med 30% olie, og med dodecenyl succinyleret alginat som stabilisator, blev sammenlignet med lignende emulsioner stabiliseret med umodificeret alginat, eller β-lactoglobulin eller phosphatidylcholin som repræsenterer bredt anvendte emulgatorer i fødevareprodukter. Opbevaringsstabiliteten af emulsionerne stabiliseret med modificeret alginat var markant forbedret sammenlignet med emulsioner stabiliseret med umodificeret alginat, β-lactoglobulin eller phosphatidylcholin. Konfokal lysscannings mikroskopi viste at den modificerede alginat havde god overfladeaktivitet og at den stabiliserede emulsioner ved adsorption til olie-vand grænsefladen, mens umodificeret alginat stabiliserede emulsioner ved at fungere som tykningsmiddel. Emulsioner stabiliseret med modificeret alginat havde et større negativt zeta-potentiale, hvilket gav frastødende kræfter mellem oliedråberne, og på den måde forbedrede emulsionens stabilitet. Den modificerede alginat viste sig endvidere at have en højere antioxidantaktivitet sammenlignet med umodificeret alginat, vurderet ud fra evnen til at neutralisere hydroxyl radikaler. Denne dobbelt-funktionalitet i form af emulgator og antioxidant aktivitet var særdeles interessant med henblik på stabilisering af fiskeolie emulsioner, og udviklingen af dette materiale repræsenterer et af nøgleresultaterne af denne Ph.D. forskning.

Et andet område for anvendelse af modificeret alginat var i mikroindkapsling af enzymer. Umodificeret alginat kan danne stærke hydrogeler hvori enzymer kan mikroindkapsles,

hvorved enzymerne bliver beskyttet. I dette studie blev lipaser anvendt som modelenzymer. Lipaser blev mikroindkapslet i hydrogeler dannet fra modificeret alginat og resultaterne var lovende. Indledende resultater herfra er præsenteret i denne afhandling.

Som tillæg til ovenstående arbejde er der bidraget til andre forskningsprojekter, inklusiv syntetiseringen og anvendelsen af phosphatidyl-sakkarider. Phospholipider er kendt for at danne liposomer, som har potentiale til at indkapsle følsomme bioaktive ingredienser. I denne sammenhæng kræves normalvis en tørringsproces, som kan ødelægge liposomstrukturen. Der er derfor brug for tørrebeskyttere som eksempelvis sukre under tørringsprocessen. Ideen med at designe phospholipider konjugeret med sukkerstrukturer var således at udvikle phospholipider der var i stand til at danne liposomer med forbedret stabilitet. Mono-, di- og trisakkarider blev enzymatisk konjugeret med phosphatidylgruppen af phospholipidet, og de resulterende phosphatidyl-sakkarider blev anvendt i dannelsen af liposomer. Nanoliposomerne viste sig at have signifikant forbedret stabilitet under opbevaring og under tørringsprocesser.

Endelig, som en uafhængig del af dette Ph.D. studie, blev antimikrobielle enzymer studeret under et forskningsophold ved Rensselaer Polytechnic Institute (RPI) i Troy, New York, USA. Peptidoglycan hydrolaser tilhører en klasse af modulærer enzymer som degraderer peptidoglycanlaget i specifikke bakterier. Specificiteten af peptidoglycanhydrolaser gør det muligt at anvende dem som antibiotiske midler, idet de kan dræbe patogene bakterier uden at påvirke den naturlige bakterieflora. Under opholdet ved RPI blev enzymer med virkning mod *Staphylococcus aureus* og *Bacillus anthracis* studeret. Begge studier førte til lovende observation som nu bliver studeret yderligere ved RPI.

LIST OF ABBREVIATIONS

Alg-SAC0:	Alginate modified with succinic anhydride
Alg-SAC12:	Alginate modified with dodecenyl succinic anhydride
AOs:	Alginate oligosaccharides
BCA:	Bicinchoninic acid (protein assay)
BLG:	*β*-lactoglobulin
CAL-B:	*Candida antartica* lipase B
CRL:	*Candida rugosa* lipase
DP:	Degree of polymerization
PC:	Phosphatidyl-choline
ROS:	Reactive oxygen species
TBARS:	Thiobarbituric acid reactive species

Reagents, solvents, and solutions

ABTS:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DMAP:	4-(N,N-dimethylamino)pyridine
EDTA:	Ethylenediaminetetraacetate dehydrate
PBS:	Phosphate buffered saline
SAC0:	Succinic anhydride
SAC12:	Dodecenyl succinic anhydride
TBA:	Thiobarbituric acid
TRIS:	Tris(hydroxymethyl)aminomethane

Techniques:

CLSM:	Confocal laser scanning microscopy
ESI-MS:	Electrospray ionization mass spectrometry
ELSD:	Evaporative light scattering detection
FTIR:	Fourier transformed infrared spectrometry
HPLC:	High performance liquid chromatography
PDA:	Photodiode array detection
SEC:	Size exclusion chromatography
TLC:	Thin layer chromatography

LIST OF PUBLICATIONS

The following publications and manuscripts are included as part of this thesis:

I: Mia Falkeborg, Ling-Zhi Cheong, Carlo Gianfico, Katarzyna Sztukiel, Kasper Kristensen, Marianne Glasius, Xuebing Xu, Zheng Guo, 2014. "Alginate Oligosaccharides: Enzymatic Preparation and Antioxidant Properties Evaluation". Food Chemistry; 164, pp 184-195.

II. Mia Falkeborg, Zheng Guo. "Dodecenyl Succinylated Alginate as a Novel Dual-function Emulsifier for Improved Fish Oil-in-water Emulsions". Manuscript, submitted to Food Hydrocolloids in 2014.

III. Shuang Song, Ling-Zhi Cheong, Mia Falkeborg, Lei Liu, Mingdong Dong, Henrik Max Jensen, Kresten Bertelsen, Michael Thorsen, Tianwei Tan, Xuebing Xu, Zheng Guo, 2013: "Facile Synthesis of Phosphatidyl Saccharides for Preparation of Anionic Nanoliposomes with Enhanced Stability". PLoS ONE; 8(9), pp. e73891.

The following manuscript is in preparation and is partly included in this thesis:

IV. Mia Falkeborg, Allen Ndonwi Shu, Zheng Guo. "Alkyl-succinylations of Alginate as Biocompatible Materials for Encapsulation of Lipases".

Contributions were made to the following publications which are not part of this thesis:

V. Derya Kahveci, Zheng Guo, Ling-Zhi Cheong, Mia Falkeborg, Worawan Panpipat, Xuebing Xu, 2013: "Oxidative Stability of Enzymatically Processed Oils and Fats". In: A. Logan, U. Nienaber, X. Pan (Eds.), *Lipid oxidation: Challenges in Food Systems*. AOCS Press, Urbana, IL. pp. 211-242.

VI: Renjun Gao, Mia Falkeborg, Xuebing Xu, Zheng Guo, 2013: "Production of Sophorolipids with Enhanced Volumetric Productivity by means of High Cell Density Fermentation". Applied Microbiology and Biotechnology; 97, side 1103-1111.

VII: Mia Falkeborg, Claire Berton-Carabin, Ling-Zhi Cheong, 2015: "Ionic Liquids in the Synthesis of Antioxidants Targeted Compounds". In: X. Xuebing, Z. Guo, L. Z. Cheong (Eds.), *Ionic Liquids for Lipid Processing and Analysis: Opportunities and Challenges*. AOCS Press, Urbana, IL.

1 BACKGROUND AND OBJECTIVES

Marine oils have a high content of long chain omega-3 (n-3) polyunsaturated fatty acids (PUFAs). Extensive research has shown that increased consumption of n-3 PUFAs has a number of beneficial health effects, and the importance of these fatty acids in human nutrition and disease prevention is scientifically recognized (Shahidi et al. 2004; 2005; Narayan et al. 2006; Ruxton et al. 2007). It has been suggested that the typical western diet, which is high in n-6 fatty acids but low in n-3 fatty acids, may not supply the appropriate balance of PUFAs for proper biological function of the body. This imbalance is believed to cause a variety of diseases, including cardiovascular diseases and inflammatory disorders (Din et al. 2004; Wall et al. 2010). For this reason, there is an interest in producing regularly consumed food products enriched with marine oils (Kolanowski et al. 1999; Jacobsen et al. 2007; Taneja et al. 2012). Problems with enrichment of food with marine oils occur, as n-3 PUFAs are highly sensitive and prone to oxidation due to their high degree of unsaturation. It is hence important to stabilize the n-3 PUFAs when producing food products enriched with marine oil. This can be done by the use of antioxidants, which prevent or delay lipid oxidation (Wanasundara et al. 2005; Jacobsen et al. 2008; Shahidi et al. 2010). Additionally, microencapsulation of the marine oil has been shown to be effective in protecting the marine oil, while also providing increased compatibility with the food product of interest (Taneja et al. 2012). Microencapsulation of marine oil can be done through several approaches, all based on the requirement of a stable marine oil-in-water emulsion. Oil-in-water emulsions are thermodynamically unfavorable systems and stabilizers such as emulsifiers are required to provide kinetic stability to the emulsions (McClements et al. 2000). Special focus needs to be placed on the oil-water interface, and on the location of antioxidants in emulsions. Antioxidants present at the oil-water interface provide better protection, than antioxidants present in the aqueous phase (Shahidi et al. 2011). When designing marine oil-in-water emulsions, optimal physical and oxidative stability is thus obtained when antioxidants are incorporated into the oil-water interface, e.g. as a part of the emulsifier.

Through rational molecular design, this Ph.D. study aimed at developing innovative ingredients with application potentials in the physical and oxidative stabilization of marine oil-in-water

emulsions. Starting from the natural polysaccharide *alginate*, the main objective was to design and synthesize innovative ingredients, and to evaluate their application potential as physical and oxidative stabilizers of marine oil-in-water emulsions.

The potential of enzymatic depolymerization of alginate for the purpose of preparing strong antioxidants was evaluated. The products of alginate depolymerization, alginate oligosaccharides (AOs), have antioxidant activity and the main objective was to propose a mechanism for this antioxidant activity, in order to identify which functional group(s) of the AOs were responsible for the activity.

Attempts were made to introduce amphiphilicity to the AOs by modifying them with hydrophobic moieties, for the purpose of tailoring them for use in oil-in-water emulsions. Two types of hydrophobic modifications were in focus, (1) esterification of the carboxyl group(s) of AOs with alkyl alcohols, and (2) esterification of the hydroxyl group(s) of AOs with fatty acids. Attempts were made to find optimal reaction conditions for these hydrophobic modifications by evaluating various chemical and enzymatic reaction systems.

An additional objective of this Ph.D. study was to modify the alginate polymer with hydrophobic moieties for the purpose of introducing strong emulsification properties. This was approached through modifications with succinic anhydrides, with and without an alkyl chain. The objective was to optimize the process variables to achieve a high degree of succinylation, and to evaluate the properties of the resulting materials. The modified alginates were applied as stabilizers of marine oil-in-water emulsions, and characteristics such as emulsion microstructure and storage stability were evaluated, and compared to emulsions formed with common emulsifiers. Furthermore, the antioxidant properties of the modified alginates were evaluated.

In addition to microencapsulation of marine oils, other types of microencapsulation systems were evaluated in this Ph.D. study.

Polymeric alginate finds a range of applications in the area of microencapsulation, in particular due to its ability to form strong hydrogels. The ability of the modified alginates to form

hydrogels was evaluated. Serving as model compounds for sensitive ingredients, the objective was to encapsulate lipases in hydrogels formed from modified alginates, and to evaluate the encapsulation efficiency and the activity of the encapsulated lipases.

Contributions were additionally made to research concerning nanoliposomes, which are well known microencapsulating agents. Rational modifications were made to natural phospholipids, and the modified phospholipids were applied in the formation of nanoliposomes. The characteristics and stability of the nanoliposomes were evaluated and compared to nanoliposomes formed from native phospholipids.

Chapter 2 of this thesis presents the basic knowledge related to the research, including a review of microencapsulation techniques, oil-in-water emulsion science, and lipid oxidation reactions and antioxidants. A review of relevant literature is given on the topics of alginate and alginate modification including enzymatic depolymerization. An introduction to the structure and formation of hydrogels is also given.

The following chapters of the thesis present the experimental and analytical methods (Chapter 3), a summary and discussion of the main results (Chapter 4), and a conclusion of the work and future outlooks (Chapter 5). At the end of the thesis, two research papers/manuscripts are presented, followed by a conference poster, and three appendices presenting (1) information on the properties of the alginate lyase S employed in the study, (2) a summary of the work regarding nanoliposomes, including the related research paper, and (3) a summary of the work performed during the visiting stay at RPI, concerning expression and characterization of antimicrobial enzymes.

2 INTRODUCTION

2.1 MICROENCAPSULATION OF MARINE OILS

The simplest system for encapsulation and delivery of marine oils into food products is in the form of oil-in-water emulsions. The structure and basic science regarding oil-in-water emulsions is presented in section 2.2. Marine oil emulsions have been applied in a range of products, especially milk-based products, where emulsification is a key step in the production (Chee et al. 2005; Let et al. 2007; Ye et al. 2009; Kralovec et al. 2012). Formation of emulsions requires an input of energy to homogenize the phases. A variety of different homogenization methods are available, including high shear mixers, high-pressure homogenizers, colloid mills, and ultrasonic homogenizers (McClements et al. 2007).

Simple oil-in-water emulsions can be modified through the layer-by-layer electrostatic deposition technique (Guzey et al. 2006). Layer-by-layer electrostatic deposition involves coating oil droplets in a primary emulsion, which is stabilized by ionic emulsifiers, by adsorbing oppositely charged emulsifiers, thus forming a secondary emulsion with a two-layered interface (Guzey et al. 2006; Zuidam et al. 2010).

The most widely used method for microencapsulation of marine oils is spray-drying (Desai et al. 2005; Barrow et al. 2007; Kralovec et al. 2012). In this process, a marine oil-in-water emulsion is spray-dried in the presence of a suitable matrix material, in which the oil will be embedded in the final dry product. The process is economically favorable and provides great flexibility, however, the oil loading capacity is generally low (normally less than 30% marine oil per gram final microencapsulate) and the dried particles have a high amount of surface oil (Barrow et al. 2007; Kralovec et al. 2012). Spray-dried marine oil encapsulates have been applied in food products such as yoghurt (Estrada et al. 2011) and in short shelf-life products such as bread (Barrow et al. 2007). The spray-drying technique is less suitable for liquid food products, as the carrier materials usually are water-soluble (Sagalowicz et al. 2010).

An additional commonly used technique for microencapsulation of marine oils is complex coacervation. Coacervation is the separation of a colloidal system into two liquid phases, and complex coacervation is coacervation caused by the interaction of two oppositely charged colloids (de Kruif et al. 2004). The phase more concentrated in colloid component is termed the coacervate. Microencapsulation of marine oils using complex coacervation involves emulsification of the marine oil in the initial hydrocolloid solution; during coacervation, the coacervate will deposit around the oil droplets. The use of complex coacervation results in high oil loadings (normally more than 60%), and the amount of surface oil is very low (normally less than 0.02%, compared to 0.2-1% for spray-dried emulsions) (Barrow et al. 2007). Marine oil encapsulated using complex coacervation has been applied in food products such as yogurt (Tamjidi et al. 2012), and the use of complex coacervation for microencapsulation and delivery of n-3 PUFAs into a variety of food products has been commercially successful (Barrow et al. 2007).

2.2 EMULSIONS

In oil-in-water emulsions, oil droplets are surrounded by an aqueous continuous phase and the region between the oil droplets and the continuous phase is the interface (McClements et al. 2000). Because of the incompatibility of the phases, the interface area tends to minimize over time, making emulsions thermodynamically unstable. Hence, emulsions need to be physically stabilized to avoid spontaneous separation of the phases.

One type of stabilizer is thickening agents, which increase the viscosity of the emulsion's aqueous phase and slow the creaming of oil droplets. A second type is emulsifiers, which are amphiphillic molecules that can adsorb at the oil-water interface, forming a protective coating around the oil droplets and preventing aggregation (McClements 2007). In food applications, a few main categories of emulsifiers are generally described, such as low molecular weight emulsifiers, also referred to as surfactants, phospholipids, and amphiphillic biopolymers such as proteins (Hasenhuettl et al. 2008; Dickinson 2012).

2.3 LIPID OXIDATION AND ANTIOXIDANTS

A basic introduction to lipid autooxidation and the effect of antioxidants is given in the following section, written with inspiration from Falkeborg et al. (2015). Alternative lipid oxidation pathways, e.g. photooxidation, are not considered.

The reaction pathways of lipid oxidation are well established, and the subject has been extensively reviewed (Frankel 1985; 2005; Gardner 1989; Schaich 2005; Cheng et al. 2007). Generally, lipid oxidation follows three main steps: initiation, propagation, and termination, as illustrated in Figure 1.

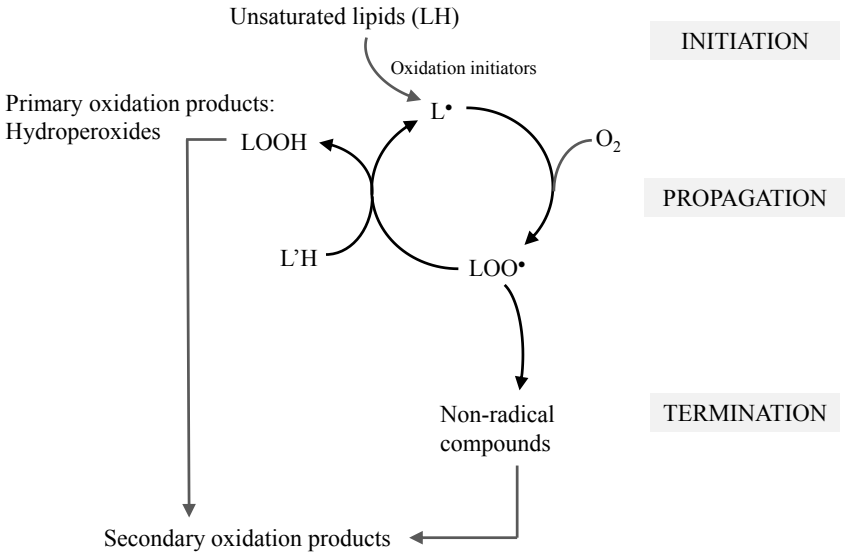


Figure 1: General reaction scheme of lipid oxidation.

The direct reaction between an unsaturated lipid (LH) and triplet oxygen ($^3O_2$) cannot occur spontaneously because of the high activation energy induced by the spin barrier between lipids and $^3O_2$ (Belitz et al. 2009b). This spin barrier can be overcome under certain environmental conditions such as heat or radiations, and/or in the presence of chemical initiators such as

transition metals. Such initiators can produce lipid radicals by abstracting a hydrogen atom from an unsaturated fatty acid, forming an alkyl radical ($L^{\bullet}$) (reaction 1).

$$LH \rightarrow H + L^{\bullet} \qquad \text{(reaction 1)}$$

During the propagation step, alkyl radicals react with $^3O_2$, forming peroxyl radicals ($LOO^{\bullet}$, reaction 2).

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \qquad \text{(reaction 2)}$$

Peroxyl radicals are unstable, and can abstract hydrogen atoms from other unsaturated fatty acids (L'H), forming hydroperoxides (LOOH) and alkyl radicals (reaction 3).

$$LOO^{\bullet} + L'H \rightarrow LOOH + L'^{\bullet} \qquad \text{(reaction 3)}$$

The newly formed alkyl radicals can in turn react with $^3O_2$, hence propagating the chain reaction. Eventually, radical molecules may combine, forming stable, non-radical compounds (termination).

Hydroperoxides are referred to as the primary oxidation products. They can undergo oxidation, reduction, or decomposition, which, as for the termination reactions, lead to formation of secondary oxidation products. A broad range of secondary oxidation products can be produced throughout the reactions, including aldehydes, alcohols, and ketones. These compounds are largely responsible for the flavor defects that characterize oxidized lipids (Coupland et al. 1996; Jacobsen et al. 2007).

A common way to counteract lipid oxidation is to use antioxidants, which can intervene at different stages of the lipid oxidation reaction (Frankel 1996; Wanasundara et al. 2005;

Laguerre et al. 2007; Jacobsen et al. 2008; Shahidi et al. 2010). A distinction is made between preventive antioxidants that prevent or delay the initiation step, and chain-breaking antioxidants that limit the propagation of the radical chain reaction. Preventive antioxidants act by suppressing the effect of oxidation initiators (Laguerre et al. 2007; Shahidi et al. 2010). Hence, this category of antioxidants includes metal chelators such as ethylenediaminetetraacetic acid (EDTA) (Laguerre et al. 2007; Shahidi et al. 2010). Chain-breaking antioxidants act by reacting with radicals and converting them to stable, non-radical products (radical scavenging). The resulting antioxidant radicals are more stable in that the unpaired electron is delocalized, e.g. around conjugated double bonds, forming stable resonance structures. The stabilized antioxidant radicals are hence less likely to participate in lipid oxidation reactions and the chain reaction is interrupted.

When considering lipid oxidation in emulsions, special focus needs to be placed on the oil-water interface. The interfacial area can promote contacts between oxidizable lipids and prooxidant compounds, such as metal ions dissolved in the aqueous phase, and as a consequence, the susceptibility of lipid oxidation in an oil-in-water emulsion is increased compared to bulk oil (Coupland et al. 1996; Frankel et al. 2002; Jacobsen et al. 2007; Shahidi et al. 2011).

Antioxidants present at the oil-water interface in an oil-in-water emulsion provide better protection, than antioxidants present in the aqueous phase (Coupland et al. 1996; Yuji et al. 2007; Shahidi et al. 2011). An early theory describing this phenomenon is the polar paradox (Porter 1980; Frankel et al. 1994). It states that polar, hydrophilic antioxidants are more effective in non-polar media such as bulk oils, compared to non-polar, hydrophobic antioxidants. Conversely, in multiphase systems such as oil-in-water emulsions, hydrophobic antioxidants tend to be more active than hydrophilic antioxidants. In bulk oil, hydrophilic antioxidants locate preferentially at the air-oil interface, i.e. where the lipids get into contact with atmospheric oxygen, whereas hydrophobic antioxidants remain buried in the bulk phase. In oil-in-water emulsions, hydrophilic antioxidants are located in the aqueous phase, whereas hydrophobic antioxidants are located in the oil droplets, where they can effectively counteract lipid oxidation.

It has been shown that the antioxidant activity of “lipophilized” antioxidants (i.e., hydrophilic antioxidants modified with an alkyl chain) does not increase linearly with the alkyl chain length, but that optimal antioxidant activity is achieved for a medium alkyl chain length (the cutoff effect) (Laguerre et al. 2009; 2010; 2013; Shahidi et al 2011; Liu et al. 201). For this reason,

lipophilization of antioxidants needs to be optimized separately for different antioxidants and for individual applications.

2.4 MATERIALS FOR MICROENCAPSULATION OF LIPIDS

Dairy proteins are widely used as stabilizers of marine oil-in-water emulsions. Caseins and the globular whey proteins (especially *β*-lactoglobulin) represent the two distinct classes of dairy proteins (Dickinson 1998). They are capable of providing physical stability to emulsions, and furthermore, they have been found to have antioxidant activity and can hence inhibit lipid oxidation (Djordjevic et al. 2004; Day et al. 2007). Marine oil emulsions stabilized by dairy proteins have been applied in food products such as processed cheese, yoghurt, and milk (Chee et al. 2005; Let et al. 2007; Ye et al. 2009), and dairy proteins have been found capable of assisting in formation of multilayered marine oil emulsions (Taherian et al. 2011).

For microencapsulation of marine oils using spray-drying, commonly used matrix materials include gum arabic, and other gums such as locust bean gum or guar gum, modified starch, modified cellulose, glucose syrups and maltodextrins, polysaccharides such as alginate, as well as dairy and soy proteins (Drusch, Sefert, et al. 2006; Drusch et al. 2007; 2008; 2009; Hogan et al. 2003; Kolanowski et al. 2004; Desai et al. 2005; Jafari et al. 2008).

For complex coacervation of marine oils, the most studied encapsulation system is the combination of gelatin and gum arabic (Desai et al. 2005). These materials have been used for microencapsulation of marine oil for use in yoghurt (Tamjidi et al. 2012). Other materials used in complex coacervation include proteins (bovine serum albumin, soy proteins, and whey proteins) combined with polysaccharides (gum arabic, carrageenan, chitosan, and pectin) (Desai et al. 2005; Kralovec et al. 2012).

To improve the microencapsulation properties of natural biopolymers, specific modifications are being made, especially in the case of polysaccharides. Modification of the natural starch biopolymer with octenylsuccinate forms an ingredient with high emulsifying properties that is

commonly used in the food industry (Sweedman et al. 2013). Octenylsuccinate-derivatized starch has been applied as matrix material for microencapsulation of marine oil by spray-drying (Drusch et al. 2006; Anwar et al. 2011). Other lipid-based ingredients that have been successfully encapsulated in octenylsuccinate-derivatized starch include orange oil (Verdalet-Guzman et al. 2013), *β*-carotene (de Paz et al. 2013), and vitamin A (Xie et al. 2010).

Other polysaccharides, too, have been modified with hydrophobic moieties in order to increase their emulsion-stabilizing effect. Reports have shown that hydrophobically modified cellulose showed increased ability to stabilize oil-in-water emulsions compared to native cellulose (Karlberg et al. 2005; Sun et al. 2007), and modified cellulose has been applied in spray-drying based microencapsulation of marine oils (Kolanowski et al. 2004).

Alginate has found applications in microencapsulation of oil-based products. Tan et al. (Tan et al. 2009) encapsulated marine oil by spray-drying in matrix materials composed of alginate/starch blends. They found that inclusion of alginate in the matrix resulted in rounder microspheres with higher oil encapsulation efficiencies. Li et al. (2010) showed that alginate, being an anionic biopolymer, functioned well as the outer layer in multi-layered 10% oil-in-water emulsions formed by layer-by-layer electrostatic deposition of *β*-lactoglobulin (inner layer), chitosan (intermediate layer), and pectin or alginate (outer layer). As detailed in section 2.8, alginate hydrogels can be formed by divalent cations such as Ca^{2+}; a feature that has been exploited in the microencapsulation of wheat germ oil and evening primrose oil (Chan et al. 2000) and olive oil (Sun-Waterhouse et al. 2011).

2.5 ALGINATE – AN ABUNDANT SUSTAINABLE RESOURCE

Alginate is a linear polysaccharide composed of *α*-L-guluronate (G) and its C5 epimer *β*-D-mannuronate (M), arranged as homopolymeric blocks (polymannuronate (MM) and polyguluronate (GG)), and heteropolymeric blocks (strictly alternating GM blocks and random G/M blocks) (Lee et al. 2012; Pawar et al. 2012; Hay et al. 2013). Guluronate and mannuronate

are uronates with carboxylate groups at the C5 positions, the configuration of which represents the difference between the two pyranoses. The structure of alginate is illustrated in Figure 2.

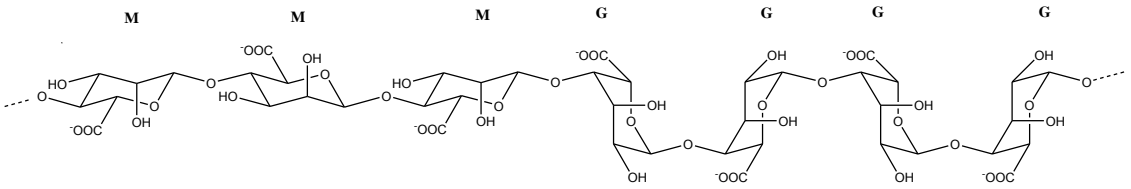


Figure 2: Structure of alginate – three mannuronate (M) units and four guluronate (G) units.

Alginate can be obtained from various genera of brown algae as well as from a few bacterial species. The commercial available alginate is currently extracted from farmed brown seaweed, where the alginate forms a structural part of the cell wall and constitutes up to 40% of the dry matter (Hay et al. 2013). Approximately 30,000 tons of alginate is produced annually, which is estimated to be less than 10% of the total amount of biosynthesized alginate (Pawar et al. 2012; Hay et al. 2013). Alginate can hence be considered an abundant, renewable resource of biomaterial.

Alginate in its sodium carboxylate form is soluble in water, where is forms viscous solutions. Pawar et al. (2011) performed a detailed solubility study of alginates, showing the Na-alginate is soluble in water only, and insoluble in all organic solvents under consideration. Protonated alginate (i.e. alginic acid) is not fully soluble in any solvent, including water (Pawar et al. 2011).

Currently, alginate has a broad range of applications owing to its relative low cost, low toxicity, and biocompatibility. In biomedicine, alginate is typically used in the form of hydrogels for e.g. wound dressings and tissue engineering, and as material for encapsulation and controlled release of drugs, enzymes, and cells (Lee et al. 2012; Hay et al. 2013). In the food industry, alginate is applied as a stabilizer, gelling agent, and viscosity regulator (Wong et al. 2000). Based on the properties and abundance of alginate, there is a potential for further expanding its use as a sustainable material for the production of a range of bioproducts.

2.6 ALGINATE DEPOLYMERIZATION

Alginate lyases (E.C. 4.2.2.3/11) catalyze the depolymerization of alginate by breaking the glycosidic linkages between the mannuronate and/or guluronate units. With few exceptions, alginate lyases work by an endo-active *β*-elimination mechanism leading to the formation of a double bond in the resulting AOs (Gacesa 1992; Wong et al. 2000; Kim et al. 2011). Alginate lyases exert their function through a three-stage mechanism, where the carboxylate of the substrate (alginate) is first neutralized by the formation of a salt bridge to an amino acid in the active site of the enzyme. Then, a base-catalyzed abstraction of the proton at C5 occurs with formation of a resonance-stabilized enolate anion intermediate. Finally, electrons are transferred from the carboxyl group, forming a double bond between C4 and C5, and eliminating the 4-0-glycosidic bond (Gacesa 1992). The non-reducing terminal residue of AOs is *4-deoxy-erythro-hex-4-enopyranosyluronate*; this residue is followed by 1, 2, or more units of mannuronate and/or guluronate in arbitrary order, depending on the nature of the lyase. Figure 3 depicts AOs with a degree of polymerization (DP) of 2-4.

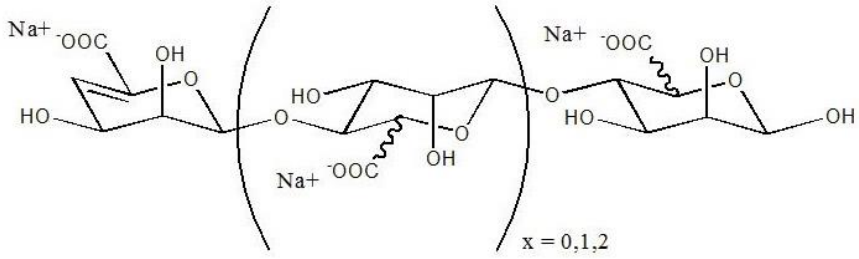


Figure 3: Alginate oligosaccharides with DP = 2-4.

Alginate lyases can vary in their specificity for mannuronate or guluronate, and/or in their specificity for the orientations of glycosidic bonds between mannuronate and guluronate units (M-G versus G-M) (Heyraud et al. 1996). Most reported alginate lyases have some activity against all glycosidic bonds in alginate, but can favor one type over others.

When enzymatically depolymerizing alginate, reaction variables such as temperature, buffer type, pH, and ionic strength, should be optimized for individual types of alginates and alginate

lyases. Generally, the reactions reported in literature take place in TRIS-HCl buffer close to neutral pH and at temperatures around 25-40°C. A review of alginate depolymerizations and the resulting DPs are given in Table 1.

The majority of alginate lyases are endo-active, releasing AOs with DP ≤ 2. One example of an exo-active alginate lyase was reported by Kim and Chung, et al. (2012). Treatment of sodium alginate with this alginate lyase released unsaturated monomeric alginate units.

A few reports have indicated that AOs have antioxidative properties. Zhao et al. (2012) showed that enzymatically prepared AOs could inhibit ferrous-induced lipid oxidation in an egg yolk emulsion to a greater extent than observed for ascorbic acid at the same concentrations. They further showed, that AOs were able to scavenge both superoxide (O_2^{*-}) and hydroxyl ($^{*}OH$) radicals. However, contradicting results were shown by Wang et al. (2007), who in a similar assay showed that AOs did not influence ferrous-induced lipid oxidation in egg yolk emulsions, and that AOs only scavenge hydroxyl radicals and not superoxide radicals. Trommer et al. (2005) reported, that alginic acid has a pro-oxidative effect on lipids in emulsion.

Some reports have shown that the molecular weight of alginate influences its antioxidant activities. Lower-molecular weight fractions produced by enzymatic depolymerization (Zhao et al. 2012) or by radiation-induced degradation (Sen 2011) were shown to have higher antioxidant activity, compared to higher-molecular weight fractions.

Further studies were obviously required to clarify the antioxidant activity of AOs and the molecular basis for this activity. The first main objective of this Ph.D. study was thus to prepare AOs by enzymatic depolymerization of alginate, and to investigate the antioxidant properties of the resulting AOs. Specifically, the aim was to identify the functional group(s) of AOs responsible for the antioxidant activity. The increased understanding of the antioxidant activity of AOs, and which functional group(s) in the structure was responsible for this activity, should aid in the design of hydrophobic modifications of AOs.

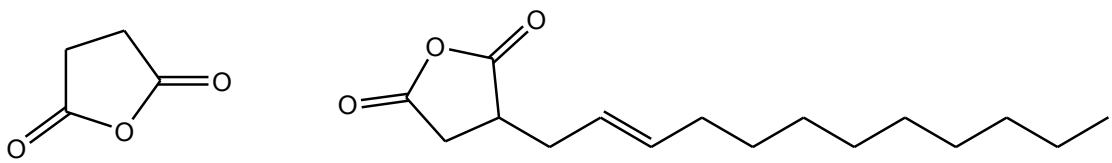
Table 1: Alginate depolymerization. DP: Degree of polymerization.

Starting material	Alginate lyase	Buffer / pH / temperature	DP	Reference
Sodium alginate from *L. Japonica*	Alginate lyase from *Vibro* sp.510	50 mM TRIS-HCl / pH 7.5 / 28˚C	2, 3, 4	(Zhang et al. 2004)
Sodium alginate (commercial, unspecified)	Recombinant exo-alginate lyase Alg17C from *Saccharophagus degradans* 2-40	20 mM TRIS-HCl / pH 6.0 / 40˚C	1	(Kim, Chung, et al. 2012)
Sodium alginate (commercial, unspecified)	Recombinant endo-alginate lyase Alg7D from *Saccharophagus degradans*	20 mM TRIS-HCl / pH 7.0 / 50˚C	2, 3, 4, 5	(Kim, Ko, et al. 2012)
Sodium alginate from brown algae (commercial)	Alginate lyase aly-SJ02, from *Pseudoalteromonas* sp.SM0524	50 mM TRIS-HCl 0.25 mM NaCl / pH 8.5 / 50˚C	2, 3	(Li et al. 2011)
Homopolymeric guluronic acid	Alginate lyase (unspecified)	50 mM TRIS-HCl / pH 7.5 / 30˚C	2, 3	(Liu et al. 2002)
Homopolymeric polymannuronate	Haliotis tuberculata alginate lyase	50 mM TRIS-HCl / pH 7.5 / 25˚C	2, 3, 4, 5	(Heyraud et al. 1996)
Homopolymeric polyguluronate	Klebsiella pneumoniae alginate lyase	50 mM TRIS-HCl / pH 7.5 / 20˚C	2, 3, 4	(Heyraud et al. 1996)

2.7 MODIFICATION OF POLYMERIC ALGINATE

Numerous derivatizations of polymeric alginate have been performed in order to tailor this biopolymer towards specific applications in the area of microencapsulation, especially in biomedicine (Pawar et al. 2012). Amphiphillic derivatives of alginate have been prepared by chemically esterifying the carboxyl groups with alkyl alcohols (Broderick et al. 2006; Yang et al. 2012; 2013), alkyl halides (Pelletier et al. 2000; De Boisseson et al. 2004), cholesterol (Yang et al. 2007), and alkylamines (Vallee et al. 2009). Generally, the reported methods involve the use of toxic organic solvents, such as formamide/dimethyl formamide (Yang et al. 2007; Yang et al. 2012; 2013) or dimethyl sulfoxide (Pelletier et al. 2000), and/or toxic coupling reagents and catalysts, such as 4-(N,N-dimethylamino) pyridine (DMAP) and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (Yang et al. 2012; 2013) or DMAP and N,N'-dicyclohexylcarbodiimide (Yang et al. 2007).

A few reports have shown the possibility of modifying alginate with succinic anhydrides for the purpose of tailoring the alginate towards microencapsulation of bacteria (Le-Tien et al. 2004; Rao et al. 2008). With inspiration from this work, and from the octenylsuccinate-derivatized starch commonly used in the food industry, this Ph.D. project aimed at modifying alginate with succinic anhydrides (SAC0) and dodecenyl succinic anhydrides (SAC12) for the purpose of introducing new properties to the alginate. The structures of SAC0 and SAC12 are illustrated in Figure 4. The high reactivity of anhydrides towards hydroxyl groups eliminates the need for (toxic) catalysts, and the modification of alginate with SAC0 and SAC12 can take place in an aqueous reaction system.



SAC0 SAC12

Figure 4: Structures of succinic anhydride (SAC0) and dodecenyl succinic anhydride (SAC12).

2.8 HYDROGELS

Hydrogels are networks of polymers that have a high affinity for water, but are prevented from dissolving due to their cross-linked structure (Liu et al. 2012). The most common way to prepare hydrogels from alginate is to combine an aqueous solution of alginate with ionic cross-linking agents, such as Ca^{2+}. The divalent cations coordinate to guluronate moieties on adjacent alginate chains, forming a cross-linked network generally termed the egg-box model of network (Lee et al. 2012; Pawar et al. 2012). An illustration of the alginate hydrogel network is given in Figure 5. In Figure 5, the smooth regions represent polymannuronate; while the sawtooth-like regions represent polyguluronate regions (*cf*. Figure 2). Ca^{2+} coordinates primarily to polyguluronate, and the insert illustrates the suggested orientation of this coordination.

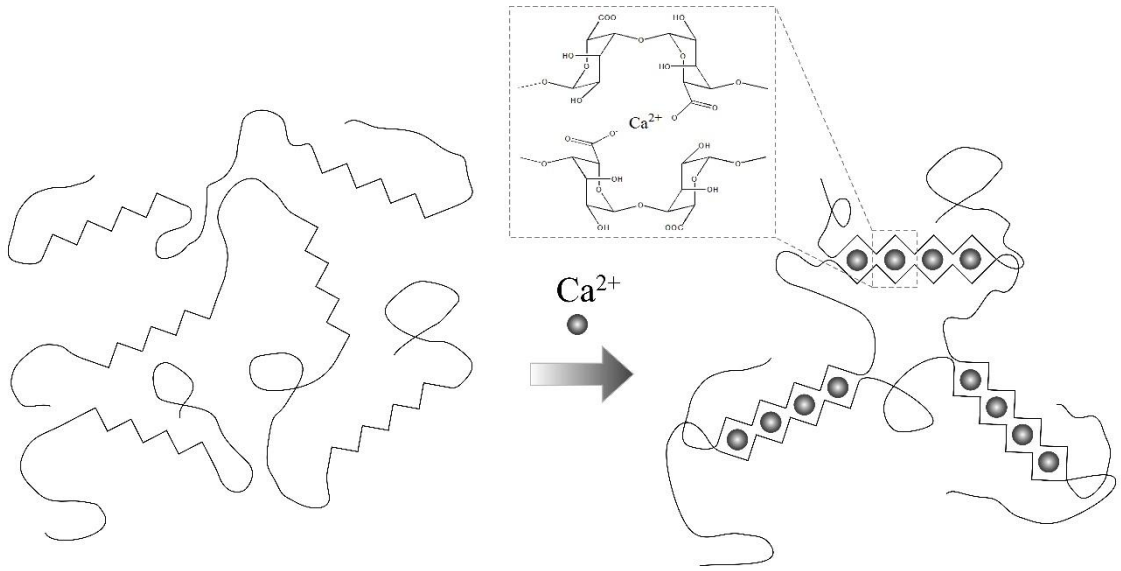


Figure 5: Alginate hydrogel formed with Ca^{2+}.

Alginate hydrogels are widely used to encapsulate sensitive ingredients, in particular, probiotics (Dong et al. 2013), live cells for use in biomedicine (Lim et al. 1980), as well as enzymes for various technical applications (Won et al. 2005; Zhang et al. 2014). Encapsulation of enzymes is generally used to increase the operational stability of these catalysts in the various areas they are applied (Hwang et al. 2013). Encapsulation of enzymes in alginate hydrogels is carried out

by mixing the enzyme with an aqueous solution of alginate, and then adding the alginate-enzyme solution drop-wise to an aqueous solution of calcium chloride. Alginate hydrogel beads form upon contact with the calcium ions, and the enzyme is entrapped within the alginate hydrogel network (Won et al. 2005; Zhang et al. 2014).

Encapsulation of enzymes in alginate hydrogels shield the enzymes and protect them from direct contact with external factors, but the method has drawbacks related to mass transfer limitations and low enzyme loading (Hwang et al. 2013). A particular disadvantage with alginate hydrogel beads is that they swell in buffer solutions, when Ca^{2+} is exchanged with Na^{2+}, resulting in enzyme leakage and inhibition of the recycling efficiency of the enzyme. To overcome this, coating of the alginate beads with e.g. chitosan or silica has been shown to be useful in avoiding leakage of the enzyme from the alginate beads (Won et al. 2005). Thus, Toscano et al. (2014) showed that chitosan-coated alginate beads were superior in lipase encapsulation efficiency compared to simple alginate beads or chitosan beads.

In this study, lipases were applied as model enzymes to evaluate the encapsulation efficiency of hydrogels prepared from modified alginates. Lipases (E.C. 3.1.1.3) catalyze cleavage of ester bonds between organic alcohols and fatty acids, and the reverse reaction (Reis et al. 2009). The loading capacity and the activity of the encapsulated lipases were evaluated. The results obtained to date are preliminary and additionally studies are required to fully characterize the potential of the modified alginates in this area.

3 EXPERIMENTAL METHODS

The experimental methods of this research are presented in four main sections. Section 3.1 summarizes the methods applied for alginate depolymerization and characterization of the resulting AOs, with reference to Paper I, in which the detailed experimental details are given. In section 3.2, the methods for the hydrophobic modifications of AOs are presented. The results of this section are unpublished due to the less successful outcomes, and all experimental details are presented herein. In section 3.3, the experimental details for modification of alginate with SAC0 and SAC12 are presented. This section is expected to form part of Manuscript IV (*Mia Falkeborg, Allen Ndonwi Shu, Zheng Guo: Alkyl-succinylations of alginate as biocompatible materials for encapsulation of lipases*). In section 3.4, the details for the application of modified alginates (1) in marine oil-in-water emulsions, and (2) in hydrogels for lipase encapsulation, are presented. The application of alg-SAC12 in marine oil-in-water emulsions is presented in detail Paper II. The application of modified alginates in lipase encapsulation in hydrogels is expected to form part of Manuscript IV, and all experimental details are presented in section 3.4.

3.1 PREPARATION OF ALGINATE OLIGOSACCHARIDES

In the first part of the research, alginate was enzymatically depolymerized, and the resulting AOs were characterized. The sodium alginate used in this study was provided by DuPont, previously Danisco, and originated from brown algae. The alginate lyase was donated by Nagase Enzymes, Japan, and was derived from *sphingobacterium*. Information about the optimal reaction conditions for this enzyme was provided by the producer and is presented in Appendix 1 of this thesis.

For optimization of the depolymerization reaction, process variables such as reaction medium (0.05 M ammonium acetate (NH_4Ac), 0.05 M TRIS-HCl at pH 7), and reaction time, were evaluated. The structure and composition of the resulting AOs were determined by Fourier transformed infrared spectrometry (FTIR), thin layer chromatography (TLC), and electrospray ionization mass spectrometry (ESI-MS), as described in Paper I. Additionally, size-exclusion chromatography (SEC) was performed, in a 2.6 x 55 cm glass column with Superdex^{TM} 30 prep grade from GE Healthcare. Elution was performed with 0.1 M NH_4Ac adjusted to pH 4.5 with HCl at 0.4 mL/min, and the eluting compounds were visualized using UV detection at 235 nm (Finnigan Surveyor PDA Plus Detector, Thermo Scientific).

To evaluate the antioxidant activity of AOs, emulsions were prepared with linoleic acid in phosphate buffered saline (PBS) pH 7.2 using Tween® 20 as stabilizer. AOs were added in varying amounts, and lipid oxidation was initiated by the addition of ferrous-ions. The formation of oxidation products was determined using thiobarbituric acid (TBA), which reacts with malondialdehyde and other secondary oxidation products (generally, termed TBA reactive species, TBARS) to form colored complexes, which can be quantified in a spectrophotometer.

In addition to evaluating the alginates' influence on lipid oxidation in emulsions, their ability to scavenge free radicals (hydroxyl, superoxide, and the stable ABTS) was also determined, according to the methods described in Paper I. To elucidate the antioxidant mechanism of the AOs, comparative studies were made on various alginate preparations such as, mannuronate- and guluronate rich fractions, acid- and salt forms, and polymeric, oligomeric, and monomeric forms of alginate, prepared as described in Paper I.

3.2 MODIFICATIONS OF ALGINATE OLIGOSACCHARIDES

According to the polar paradox and cut-off theories, hydrophobic modifications of the AOs were attempted in order to tailor the AOs for use in oil-in-water emulsions. Strategies for the hydrophobic modifications of AOs included enzymatic or chemical esterification of (1) the carboxyl groups of AOs with alkyl alcohols, and (2) the secondary hydroxyl groups of AOs

with fatty acids. For each type of modification, the general approach included development of a suitable synthesis method using commercial glucuronic acid as a model compound for AOs. The structure of glucuronic acid is presented in Figure 6, alongside the structures of the monomers of alginate in their acid forms (guluronic acid and mannuronic acid).

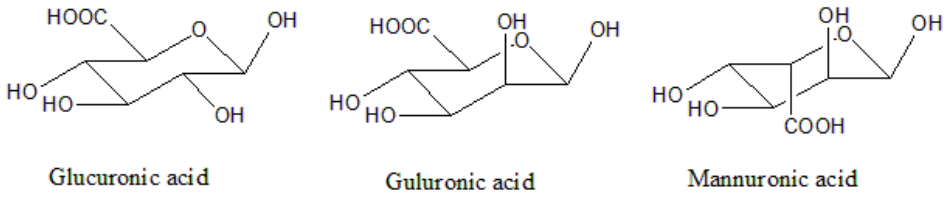


Figure 6: Structures of glucuronic acid, guluronic acid, and mannuronic acid.

Glucuronic acid differs from guluronic acid only in the orientation of the C2 hydroxyl, and from mannuronic acid additionally in the orientation of the C5 carboxyl group, and as such, glucuronic acid represents a suitable model compound for AOs.

Several synthesis strategies were evaluated in each of the two main categories of modification, alkyl alcohol esterification, and fatty acid esterification, as summarized in section 3.2.1 and section 3.2.2, respectively.

3.2.1 Alkyl Alcohol Esters of Alginate Oligosaccharides

The reaction between the model compound, glucuronic acid, and an alkyl alcohol is illustrated in Figure 7.

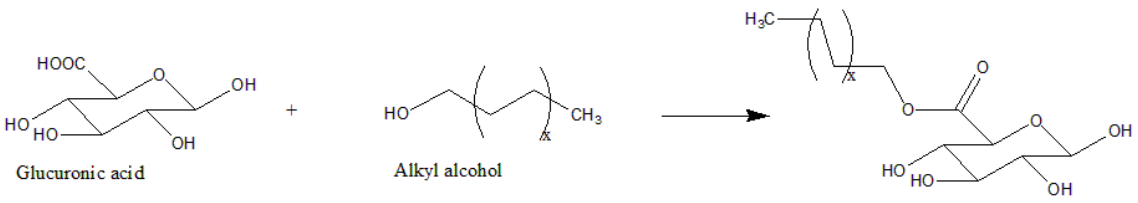


Figure 7: Esterification of glucuronic acid with an alkyl alcohol.

3.2.1.1 Enzymatic synthesis

The enzymatic esterification of glucuronic acid with octanol was performed according to the method of Moreau et al. (2004), as follows. Glucuronic acid (2.5 mmol) was combined with octanol (2.5 mmol) and 500 mg freshly activated molecular sieves (3Å, 8-12 mesh) in 10 mL *tert*-butanol at 60°C. The reaction was initiated by the addition of 100 mg lipase (Novozym 435 from Novozymes) and continued for 48 hours at 150 rpm and 60°C. The lipase and molecular sieves were removed by centrifugation and the reaction medium was diluted in 20 mL pure H_2O. The ester product and unreacted octanol were then extracted in 3 x 20 mL dichloromethane, which were pooled and evaporated to dryness under vacuum at 35°C and re-dissolved in chloroform/methanol 1/1. The ester product was then purified using TLC.

Glucuronic acid was replaced in the above-described reaction with AOs in their sodium carboxylate form, and AOs in their acid form; the latter was prepared by ion exchange as described in Paper I.

3.2.1.2 Solvent-free system – screening of chemical catalysts

A chemical synthesis method in solvent-free systems was attempted. Octanol was applied in excess and served as both acyl donor and solvent. Glucuronic acid (1.0 mmol) was combined with octanol (6.0 mmol) and 0.01 mmol catalyst (sulfuric acid, toluene sulfonic acid, camphor sulfonic acid, lithium chloride, ferric chloride, or cupper chloride) in 15 mL screw-capped vials. The vials were incubated at 65°C under magnetic stirring at 200 rpm for 24 hours. Aliquots (10 µL) were withdrawn periodically and analyzed by TLC and high-performance liquid chromatography (HPLC).

3.2.1.3 Equimolar reaction in organic solvent – solvent screening

A second type of chemical synthesis method was attempted, in which the substrates, glucuronic acid and octanol, were applied in equimolar amounts, in an organic solvent. Glucuronic acid (1.0 mmol) was combined with octanol (1.0 mmol) and 0.01 mmol sulfuric acid or ferric chloride, in 2 mL solvent (dimethyl formamide, *tert*-butanol, acetonitrile, hexane, diethyl ether, pyridine, 2-butanone, toluene, dimethyl sulfoxide, acetone, tetrahydrofuran) in 15 mL screw-

capped vials. The vials were incubated at 65°C under magnetic stirring at 200 rpm for 24 hours. Aliquots (10 µL) were withdrawn periodically and analyzed by TLC and HPLC.

3.2.1.4 Analytical methods for alkyl alcohol esterification

TLC analyses were performed on silica plates (Merck Silica Gel 60, glass plates, 20 x 20 cm) using chloroform/methanol/water 64/15/2 v/v/v as developing solvent. The compounds were visualized by spraying with dichlorofluorescein 10% in ethanol followed by irradiation with UV light; followed by spraying with 10% v/v sulphuric acid in ethanol and heating at 110°C for 10 min.

For purification of compounds using TLC, the above development system was used, but TLC plates were only stained at the leftmost 2 cm with dichlorofluorescein only. The glucuronate esters were scraped off and extracted from the silica gel three times in chloroform/methanol 1/1 v/v. The extracts were pooled and checked for purity by HPLC before evaporation of solvent to dryness at 35°C under reduced pressure.

HPLC analyses were performed on a Thermo Fisher Scientific system equipped with an online degasser, quaternary pump, and thermostated column compartment. Separation was performed on a reversed phase column (C-18, 25 cm x 4.6, 5 µm) at 30°C using a methanol-water gradient as mobile phase at 1 mL/min, as follows; 75 % methanol for 5 minutes, increased to 90% methanol over 5 minutes, held at 90% methanol for 5 minutes, decreased to 75% methanol over 4 minutes, and finally recalibrated at 75% methanol for 6 minutes. The elute was analyzed by photodiode array (PDA) detection (scanning wavelengths) and by evaporative light scattering detection (ELSD) (40°C, 3.5 mbar nebulizer pressure), serially connected. ELSD was used to quantify the amounts of glucuronic acid and glucuronate esters, while PDA detection was used to monitor the elution of solvents and octanol.

ESI-TOF-MS analyses were performed using a Bruker micro TOF-Q mass spectrometer in positive ESI mode. Samples were infused into the ESI source using the following settings: capillary voltage 4.00 kV, nebulizer pressure 3.4 bar, gas flow rate 10.0 L/min, and temperature 180°C. The resulting mass spectra were analyzed using Bruker Daltonics Data Analysis 3.4 software.

3.2.2 Fatty Acid Esters of Alginate Oligosaccharides

The second type of hydrophobic modification of AOs of interest was the esterification of the hydroxyl groups with fatty acids. The reaction scheme is shown in Figure 8. The position of esterification is chosen arbitrarily and the position can vary, including the possibility of multiple ester-formations in one glucuronic acid molecule.

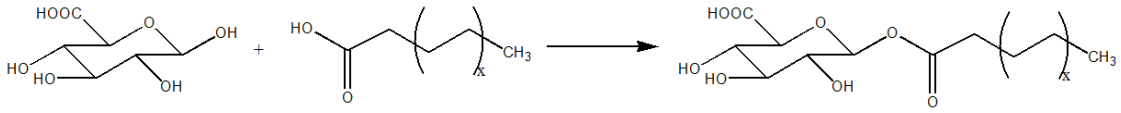


Figure 8: Esterification of glucuronic acid with a fatty acid.

Focus was placed entirely on enzymatic catalysis, and varying types of reaction systems were evaluated, as described in the following.

3.2.2.1 Reactions in organic solvents

The modified method of Kou and Xu et al. (1998) was used, as follows: Specified amounts of oleic acid and glucuronic acid were combined with 100 mg freshly activated molecular sieves in 3 mL 2-butanone at 65°C. One hundred mg Novozym 435 was added and the reactions were continued under magnetic stirring at 300 rpm for 7 days. The ratios of glucuronic acid to oleic acid were varied according to Table 2 (reactions 1-4). A reaction with no enzyme was included as negative control (reaction 5). An additional reaction using lauric acid as fatty acid part was performed (reaction 6). Samples were withdrawn daily and diluted 1-1 in chloroform/methanol 1/1 v/v and centrifuged. The supernatants were analyzed by TLC and ESI-MS.

Table 2: Molar ratios of substrates. Conditions: 3 mL 2-butanone, 100 mg lipase, 65°C, molecular sieves, 300 rpm, 7 days.

Reaction	Glucuronic acid [mmol]	Oleic acid [mmol]
1	1.0	1.0
2	2.0	1.0
3	5.0	1.0
4	1.0	5.0
5* no lipase	1.0	1.0
6	5.0	1.0* lauric acid

3.2.2.2 Solvent-free reactions with/without organic adjuvants

The modified method of Ye et al. (2010) was used, as follows: Twenty mmol molten lauric acid at 80°C was combined with 5 mmol glucuronic acid. Two hundred mg Novozym 435 was added, and the reaction was continued for 7 days at 80°C under magnetic stirring at 500 rpm (reaction 7 in Table 3). Additional reactions were performed, in which 5 g acetone or 5 g 2-butanone (reactions 8 and 9, respectively) was included as adjuvants. The containers were open to atmosphere allowing for free solvent evaporation, which according to literature was completed within 24 hours (Ye et al. 2010). Samples were withdrawn daily and diluted 1-1 in chloroform/methanol 1/1 v/v and centrifuged. The supernatants were analyzed by TLC.

Table 3: Solvent-free reactions. Conditions: 20 mmol lauric acid, 5 mmol glucuronic acid, 200 mg lipase, 80°C, 500 rpm, 7 days.

Reaction	Adjuvant
7	None
8	5 g acetone
9	5 g 2-butanone

3.2.2.3 Substrate immobilization

With inspiration from the methods of Castillo et al. (1997) and Torres et al. (2001), the influence of immobilization of the glucuronic acid onto a solid carrier was analyzed. Oleic acid (1.40 mmol) and glucuronic acid (0.35 mmol) were combined in 2 mL hexane. One hundred mg molecular sieves and/or 136 mg silica gel, corresponding to 2-1 w/w of glucuronic acid (Silica gel 60, from Fluka) was added (reactions 10-12 in Table 4), and the reactions were initiated by the addition of 100 mg Novozym 435, and continued for 7 days at 60°C under magnetic stirring at 200 rpm. An additional reaction with no addition of either molecular sieve or silica gel was performed (reaction 13). Samples were withdrawn daily and diluted 1-1 in chloroform/methanol 1/1 v/v and centrifuged. The supernatants were analyzed by TLC.

Table 4: Use of molecular sieves and/or silica gel. Conditions: 1.40 mmol oleic acid, 0.35 mmol glucuronic acid, 2 mL hexane, 100 mg lipase, 60°C, 200 rpm, 7 days

Reaction	Molecular sieves (100 mg)	Silica gel (136 mg)
10	+	+
11	+	-
12	-	+
13	-	-

3.2.2.4 Reaction in ionic liquid (reaction 14)

Reaction in ionic liquids was performed with inspiration from the methods of Ha et al. (2010). One hundred mg glucuronic acid was combined with 2 mL ionic liquid (methyl trioctyl ammonium trifluoro acetate, >98%, from IoLiTec Ionic Liquids Technologies, Germany) and stirred at >1000 rpm at 80°C for one hour. Undissolved glucuronic acid was removed, and 4 mL molten lauric acid was added under high-speed stirring (>1000 rpm) at 80°C. The temperature was adjusted to 60°C and the stirring speed to 600 rpm. The reaction was initiated by the addition of 250 mg Novozym 435 and continued for 5 days. TLC analysis of this reaction mixture was not applicable due to its complexity; instead, the reaction mixture was diluted and injected into the ESI-MS without purification of individual products.

3.2.2.5 Analytical methods for fatty acid esterification

TLC analyses were performed according to the method by Kou and Xu et al. (1998), as follows: 2 μL diluted sample was spotted onto a silica-gel plate (10 cm x 10 cm) and the plate was developed for 2.2 cm in chloroform/methanol/water 64/10/1 v/v/v. The slightly dried plate was then developed on 7.5 cm with hexane/diethyl ether/acetic acid 70/30/1 v/v/v. The products were visualized by spraying with 10% v/v sulphuric acid in ethanol followed by heating at 110°C for 10 min. The retention factors of the reaction mixture constituents were compared to the retention factors of pure glucuronic acid and fatty acid, and to retention factors of saccharide monoesters reported in literature. According to Kou and Xu et al. (1998), the retention factor of saccharide monoesters is approximately one third of the retention factor of fatty acid, while for diesters the retention factor is approximately two thirds of the fatty acid.

ESI-TOF-MS analyses were performed using a Bruker micro TOF-Q mass spectrometer in negative ESI mode. Samples were infused into the ESI source using the following settings: capillary voltage 4.00 kV, nebulizer pressure 3.4 bar, gas flow rate 10.0 L/min, and temperature 180°C. The resulting mass spectra were analyzed using Bruker Daltonics Data Analysis 3.4 software.

3.3 MODIFICATIONS OF ALGINATE

In this part, polymeric alginate was modified with succinic anhydride (SAC0) and dodecenyl succinic anhydride (SAC12). The reaction scheme between alginate and SAC0, and SAC12, and the final succinylated products, are presented in Figure 9.

The reactions were performed with inspiration from the methods reported by Rao et al. (2008) and Le-Tien et al. (2004). Small-scale reactions were performed to optimize process variables to achieve a high degree of succinylation (section 3.3.1). Optimum process variables were then applied to larger-scale succinylation as described in section 3.3.2.

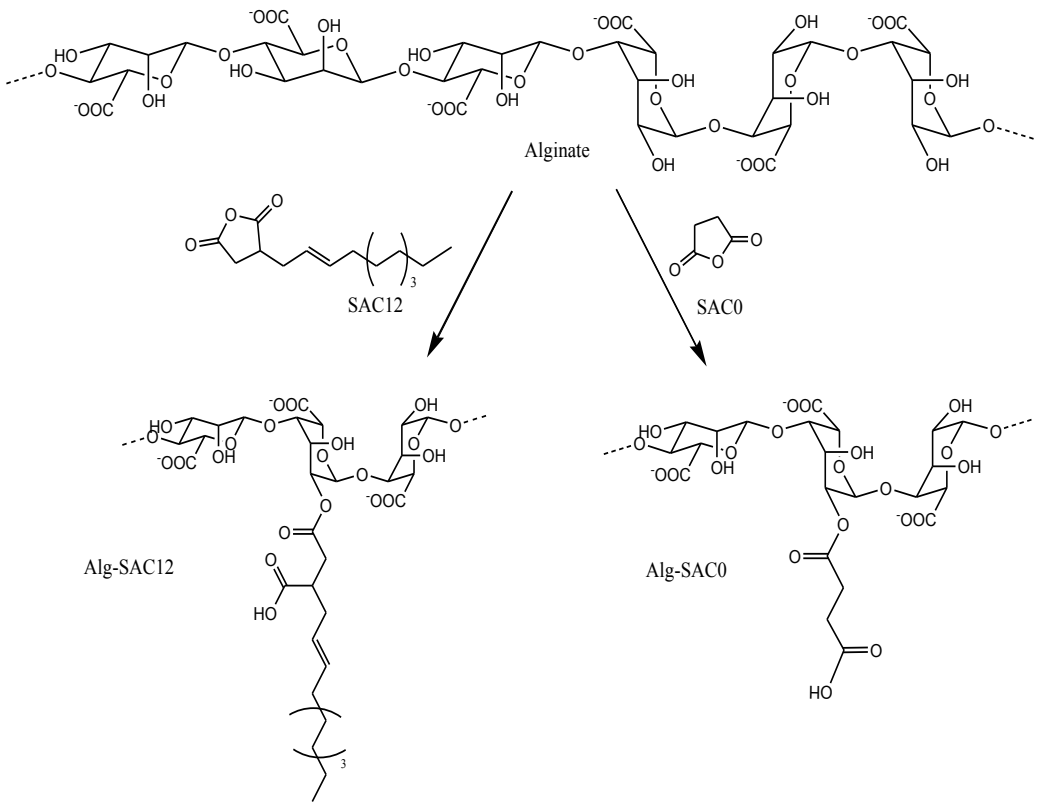


Figure 9: Modification of alginate with dodecenyl succinic anhydride (SAC12) and succinic anhydride (SAC0).

3.3.1 Small-scale Alginate Succinylations

Small-scale alginate succinylations for process optimization were performed in 20 mL capped vials, thermostated using circulating water, and stirred with magnetic bars at 300 rpm. Sodium alginate (100 mg) was dissolved in 10 mL pure water, and the pH was adjusted to 7.0 ± 0.3 using 1% sodium hydroxide (NaOH) and an automated pH-meter (inoLab pH 7110, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). Generally, reactions were performed for 4 hours at 30°C with 202.2 mg SAC0 or 538.1 mg SAC12, corresponding to a 4-times molar excess of succinic anhydrides with respect to alginate monomeric units (i.e. sodium guluronate and mannuronate units with molecular weights 198.01 g/mole). The succinylated products (alg-SAC0 and alg-SAC12) were obtained by precipitation with 35 mL ethanol and centrifugation at 4,000 rpm for 10 min at 20°C. Unreacted succinic anhydrides and water was removed by washing four times with 50 mL acetone. The degree of succinylation

(DS) was determined by titration (section 2.3). The kinetics of the reaction was followed by determining the degree of succinylation after varying reaction times (1-8 hours). Optimization towards a high degree of succinylation was performed by varying the reaction temperature (25, 30, 40, and 50°C) and by varying the molar excess of succinic anhydrides (1, 2, 3, 4, and 5-times molar excess with respect to alginate monomers).

3.3.2 Larger-scale Alginate Succinylations

Larger scale preparations of succinylated alginates were performed at optimum conditions in 250 mL screw-capped flasks thermostated at 30°C with circulating water. Alginate (750 mg) was dissolved in 75 mL H_2O, followed by the addition of 1516 mg SAC0 or 4036 mg SAC12, corresponding to a 4-times molar excess of SAC0 and SAC12. The succinylations were performed during 4 hours with magnetic stirring at 400 rpm. The pH was adjusted hourly to 7.0 ± 0.3 using 3% NaOH. The succinylated alginates were isolated by precipitation in 300 mL ethanol and centrifuged at 8,000 rpm for 20 min at 20°C, and washed four times with 200 mL acetone. The succinylated alginates were finally dried at room temperature for 48 hours.

3.3.3 Characterization of Modified Alginates

The degree of succinylation was determined with inspiration from the acidification-based method reported by Plate et al. (2012). The method is described in Paper II for alginate and alg-SAC12. A similar method was applied to alg-SAC0.

The structures of the modified alginates were confirmed with FTIR (Paper II).

3.4 APPLICATION OF MODIFIED ALGINATE

The modified alginates prepared in this study were evaluated for their ability to (1) form stable marine oil-in-water emulsions and (2) to form hydrogels with the ability to encapsulate lipases.

Initial experiments showed that alg-SAC12, and only to a lesser extent alg-SAC0, had improved emulsification properties, and for that reason, only alg-SAC12 was evaluated further for its ability to stabilize marine oil-in-water emulsions. The formation and characterization of marine oil-in-water emulsions was performed as detailed in Paper II.

Using methods inspired by Won et al. (2005) and Zhang et al. (2014), lipases (*Candida rugosa* lipase, CRL, and *Candida antartica* lipase B, CAL-B) were encapsulated in alginate hydrogel beads. CRL was provided as a solid preparation containing 15% enzyme in lactose (determined in present study by the BCA protein assay), which was dissolved in water to 15 mg solids / mL. CAL-B was provided as a liquid preparation with 4% enzyme solids in an aqueous sorbitol/glycerol formulation, which was used as received.

Alginate, alg-SAC0, or alg-SAC12, was dissolved in water to 10 g/L. For encapsulation of CAL-B, 1.5 mL alginate solution was combined with 0.1 mL CAL-B and mixed at room temperature for 10 min. For encapsulation of CRL, 0.4 mL alginate solution was combined with 0.1 mL CRL solution, and mixed at room temperature for 10 min. Portions of 0.2 mL of these alginate-lipase solutions were added drop-wise (approximately 0.5 mL/min) from a plastic syringe through a 27-gauge needle (0.4 x 20 mm) into 3 mL 1 M $CaCl_2$ at 3-5°C under stirring with a magnetic bar. The alginate beads were left in the $CaCl_2$ solution for 30-40 min to cure. The $CaCl_2$ solution was then carefully drained using a thin-tipped pipette, and the alginate beads were washed 3 times with 3 mL H_2O.

To determine the encapsulation efficiency and enzyme loading, the protein content was determined in the recovered $CaCl_2$ fraction and in the wash fractions, using the Thermo Scientific Pierce™ BCA Protein Assay Kit *enhanced protocol*, suitable for detecting protein concentrations in the range 5-250 µg/mL. Standard curves, prepared from solutions of bovine serum albumin in H_2O ($R^2 = 0.9975$) and in 1 M $CaCl_2$ solution ($R^2 = 0.9918$), were used to determine the lipase concentrations in the residual $CaCl_2$ solutions and in the wash fractions.

The activities of the lipases in free and immobilized forms were determined spectrometrically using *para*-nitrophenolbutyrate (p-NPB) (50 mM in acetonitrile) as substrate. Beads formed from 0.2 mL alginate-lipase solution were transferred to polystyrene cuvettes and dispersed in 0.975 mL 50 mM TRIS-HCl buffer at pH 7.3. Immediately after addition of 0.025 mL p-NPB solution, the absorbance at 405 nm was determined every 1 second in a UV-visible spectrophotometer (Cary 50Bio, Varian, Australia). The occurrence of *para*-nitrophenol over time was determined using the extinction coefficient, 18,000 M^{-1} cm^{-1} (Zhang et al. 1991). The enzyme activity was determined as the rate of increase in *para*-nitrophenol concentration determined from the slope of the linearization of at least 10 data points. Activity was expressed in units of μM *para*-nitrophenol / sec, and specific enzymatic activity was expressed as enzymatic activity per mg protein entrapped in the beads, as determined from the protein concentration determinations.

All encapsulation experiments and associated analyses were performed in triplicates, and the results are reported as means ± the standard deviations.

4 SUMMARY OF MAIN RESULTS

The major findings of this research are summarized in this chapter. As for the experimental section, this chapter is divided into four main parts. Section 4.1 summarizes the results from alginate depolymerization and characterization of the antioxidant activities of the resulting AOs, with reference to Paper I. Section 4.2 presents the observations regarding the attempts to hydrophobically modify AOs. As will be presented, no successful outcomes can be reported from this part, and the results are not intended for publishing. Section 4.3 summarizes the results regarding modification of polymeric alginate with succinic anhydrides and the optimization of reaction variables. These results are expected to be presented in Manuscript IV. In section 4.4, the main results regarding application of the modified alginates in marine oil-in-water emulsions, as well as in hydrogel formation for lipase entrapment, are presented. The results regarding marine oil-in-water emulsions are presented in detail in Paper II, where intensive discussions of the results also are given.

4.1 PREPARATION OF ALGINATE OLIGOSACCHARIDES

With inspiration from existing literature, summarized in Table 1, alginate was enzymatically depolymerized for preparation of AOs. In most reports on the subject of enzymatic depolymerization of sodium alginate, TRIS-HCl buffer is used as the reaction buffer (*cf*. Table 1); and SEC is applied to separate the AOs from the TRIS salt and to fractionate the AOs. Accordingly, TRIS-HCl buffer was originally used as the reaction medium for the depolymerization of alginate in this study. At the end of reaction, the enzymatic catalyst was removed by heat denaturation coupled with centrifugation, and the resulting reaction mixture was fractionated by SEC (Figure 10).

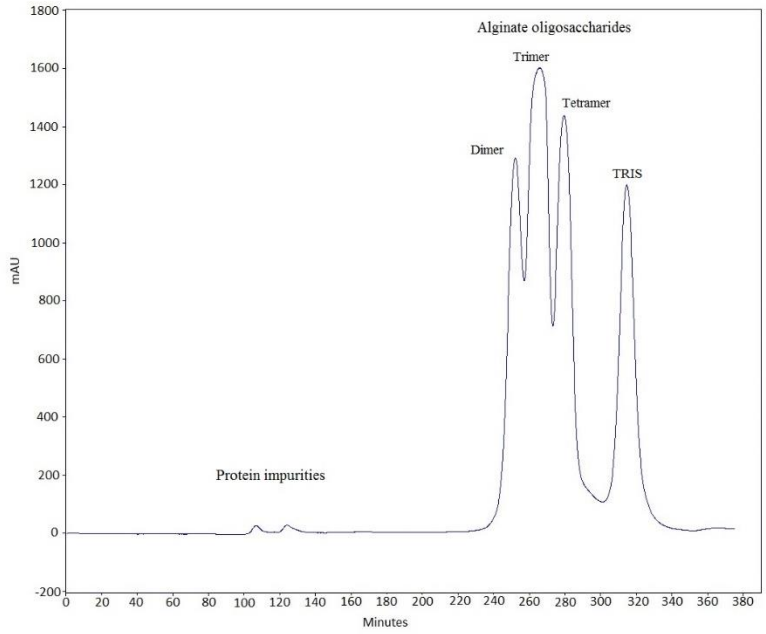


Figure 10: Size-exclusion chromatogram of alginate depolymerization reaction mixture.

As indicated in Figure 10, the AOs were composed of three oligomers eluting from 240 min to 290 min. The TRIS salt and catalyst impurities eluted at 315-325 min and 105-135 min, respectively, as confirmed by analysis of pure standards of these compounds. The fractions with AOs were collected and analyzed with TLC, confirming that the three oligomers were dimers, trimers, and tetramers, of mannuronate and/or guluronate.

In this study, AOs were to be applied as the starting material in the synthesis of amphiphillic antioxidants for use in microencapsulation of marine oil in oil-in-water emulsions. As such, substantial amounts of AOs were required. The trough-put of SEC was inadequate to obtain AOs on the required scale, and alternatives were sought to simplify the process and avoid the use of SEC.

Depolymerization of alginate proceeds through a β-elimination reaction, which does not change the pH of the medium during course of reaction. As a consequence, no actual buffer was required to maintain a constant pH; the only requirement was the presence of salts to provide a certain ionic strength in the reaction medium. Ammonium acetate was an interesting alternative to TRIS, as this salt is volatile and hence can be removed from the reaction mixture by evaporation. Accordingly, the depolymerization of alginate was performed in 0.05 M

ammonium acetate, and the resulting AOs were isolated by evaporation of the reaction medium under vacuum. The structure and composition of the AOs were characterized by FTIR, ESI-MS, and TLC, confirming that the AOs had a DP of 2-4 (Figure 3), as presented in Paper I.

The antioxidant activity of AOs was evaluated in model emulsions of linoleic acid. AOs were found to inhibit the formation of secondary oxidation products in a concentration-dependent manner, and at some concentrations, the AOs inhibited lipid oxidation to a higher extent than ascorbic acid, in accordance with the findings of Zhao et al. (2012). Polymeric alginate, too, was found to inhibit lipid oxidation, but it also led to a very high viscosity of the linoleic acid emulsions. The antioxidant activity of alginate was independent of the relative contents of mannuronate and guluronate. These findings are presented and discussed in detail in Paper I.

AOs were found to possess hydroxyl and superoxide scavenging activities, and were able to scavenge the stable free radical ABTS. When comparing the scavenging activities of AOs to other structures of alginate, a mechanism for the radical scavenging activity could be elucidated. Thus, it was proposed that the radical scavenging activity of AOs originated mainly from the presence of the conjugated alkene acid structure, which formed during enzymatic depolymerization. AOs can act as chain-breaking antioxidants, and the AO-radicals are stabilized through allylic rearrangement according to the theory of resonance hybrids (Pauling 1960). An illustration of the proposed model for radical scavenging is presented in Paper I.

AOs thus represent potential new ingredients for use in lipid-based foods to prevent lipid oxidation. The focus of present study was specifically placed on the application of AOs in protection of emulsified marine oils. For the purpose of tailoring the AOs for this application, attempts were made to hydrophobically modify the AOs. The results from this part are summarized in the following section.

4.2 MODIFICATION OF ALGINATE OLIGOSACCHARIDES

After obtaining detailed information about the antioxidant activity of AOs, attempts were made to modify the AOs with hydrophobic moieties to tailor them for use in oil-in-water emulsions. Preservation of the conjugated alkene acid structure in the AOs was desired, as this functional group was found to be main responsible for the antioxidant activity. The results obtained from the two main categories of hydrophobic moieties, (1) esterification of the carboxyl groups in AOs with alkyl alcohols, and (2) esterification of the hydroxyl groups in AOs with fatty acids, are summarized in sections 4.2.1 and 4.2.2, respectively. AOs were replaced with the model compound, glucuronic acid, for reaction simplicity.

4.2.1 Alkyl Alcohol Esterification of Alginate Oligosaccharides

Blecker and Moreau et al. (Blecker et al. 2002; 2008; Moreau et al. 2004) showed that glucuronic acid can be esterified with an alkyl alcohol using enzymatic catalysis in an amphiphillic solvent. The method presented by Moreau et al. (2004) was successfully employed in present study for synthesis of the octanol ester of glucuronic acid. The product was isolated by TLC and formation of the expected octanyl glucuronate was confirmed with ESI-MS (exact mass 306.17 u). This purified ester served as a standard in TLC and HPLC analyses.

The enzymatic reaction was successful with the carboxylic acid in glucuronic acid, yet, the sodium carboxylate groups in AOs were less readily esterified. As a consequence, simply replacing glucuronic acid with AOs in this reaction system did not result in esterification of the AOs.

Attempts were made to perform the reaction with acidified AOs, prepared by ion exchange as described in Paper I. However, applying the enzymatic catalyst to the acidified AOs resulted in enzymatic deactivation, presumably due to the low pH of the reaction medium. To avoid inactivation by low pH, the use of chemical catalysis was examined.

Various Lewis and Brøndsted acids were examined for their ability to catalyze the Fischer esterification of glucuronic acid with octanol. For the first attempts, octanol was used in molar excess to function as the reaction solvent. The acid catalyst was applied as 1% mol/mol with

respect to glucuronic acid. After 24 hours, the reaction mixture was analyzed by TLC and HPLC to identify reaction products. Selected results from TLC analysis are presented in Figure 11. The positions of glucuronic acid and octanol, and the product, octanyl glucuronate, are indicated.

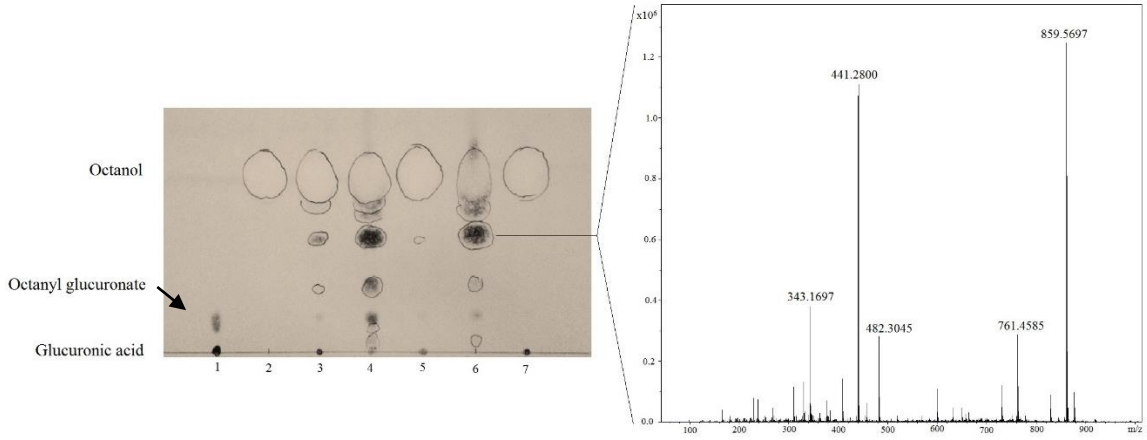


Figure 11: Left, TLC. Lane 1: Glucuronic acid. Arrow indicates lactonized form of glucuronic acid (cf. *Figure 12*). *Lane 2: Octanol. Lanes 3-7: Reaction mixture after 24 hours with catalysts $FeCl_3$ (3), H_2SO_4 (4), LiCl (5), toluene sulfonic acid (6), or no catalyst (7).*
Right: ESI-MS spectrum of reaction product isolated from TLC.

When using Lewis acids as catalysts (specifically, ferric chloride (lane 3), lithium chloride (lane 5), or cupper chloride (not presented)), conversion of glucuronic acid was very low. When using Brøndsted acids (specifically, sulfuric acid (lane 4), toluene sulfonic acid (lane 6) or camphor sulfonic acid (not presented)) several by-products formed in addition to the octanyl glucuronate ester. The majority of the by-products had retention factors closer to octanol.

The individual bands were purified from the TLC plates and analyzed by ESI-MS. The compound indicated by an arrow in Figure 11 was found to be the lactonized form of glucuronic acid (exact mass 176.03 u, ESI-MS spectrum is not presented). The ESI-MS spectrum of the major reaction product is presented right in Figure 11. The product was identified to be the di-substituted glucuronic lactone, illustrated in Figure 12. The exact mass of the di-substituted lactone is 418.29 u. The ESI-MS signal at 441.28 m/z in Figure 11 represents the compound (418.29 u + $1Na^+$), and the signal at 859.57 m/z represents the compound in a dimeric constitution (418.29 u x 2 + $1Na^+$).

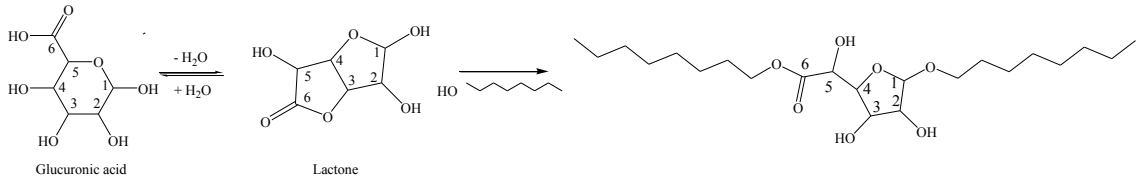


Figure 12: Glucuronic acid lactonization and reaction of lactone with octanol.

Alternative reaction systems were then evaluated, in which various organic solvents were applied to dissolve the reactants in equimolar amounts. A range of organic solvents with varying polarities (dielectric constants ranging from 1.9-46.7) were screened for their ability to properly dissolve the reactants and lead to formation of products. The reaction systems were composed of 1.0 mmol of each of the substrates (glucuronic acid and octanol) and 0.01 mmol catalyst, H_2SO_4 or $FeCl_3$, in 2 mL solvent.

For the organic solvents in consideration, glucuronic acid was only fully soluble in dimethyl sulfoxide, and only partly soluble in the remaining solvents. For several of the solvents, caramelization of the glucuronic acid was observed by the formation of a brown to black color. Generally, caramelization of saccharides involves a range of complex reactions occurring when the saccharide is heated in presence of an acidic or alkaline catalyst (Belitz et al. 2009a). The observed relative color intensity in each solvent, in presence of H_2SO_4, is presented in Figure 13. Generally, no correlation between the solvent polarity and the degree of browning was observed. Similar results were obtained using $FeCl_3$ as catalyst (not presented).

Compositions of the reaction mixtures in these solvents after 24 hours were analyzed by TLC and HPLC (plates and chromatograms are not presented herein). The reaction outcomes were found to be complex mixtures of products, primarily the glucuronic lactone and the di-substituted lactone illustrated in Figure 12, as well as several other, unidentified products. No effective method for controlling these side reactions was found in this study.

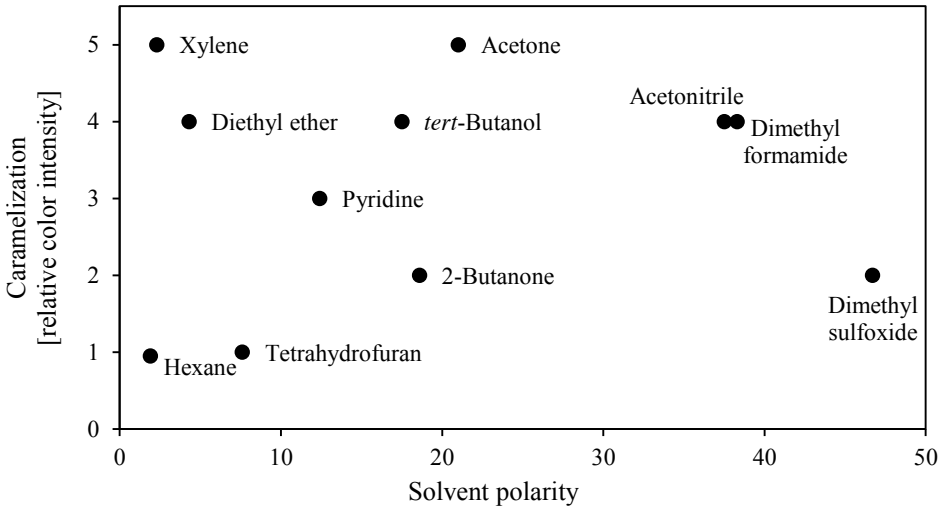


Figure 13: Formation of brown-colored products in the reaction system with equimolar amounts of glucuronic acid and octanol, and H_2SO_4.

The side-reactions involving lactonization are not expected to occur with AOs, as it requires a degree of flexibility in the hexose ring, which is limited in the oligomeric structure of AOs. Nonetheless, replacing glucuronic acid with AOs in any of the described reaction systems did not lead to esterification of the AOs.

4.2.2 Fatty Acid Esterification of Alginate Oligosaccharides

The second type of hydrophobic modification of AOs of interest was the esterification of the hydroxyl groups with fatty acids. Several reports exist regarding enzymatic synthesis of saccharide fatty acid esters, and the subject is extensively reviewed (Kennedy et al. 2006; Chang et al. 2009; Gumel et al. 2011; Kobayashi 2011). With inspiration from existing literature, a range of esterification methods using lipase as catalysts were examined. The enzymatic methods included reaction in organic solvent; reaction in solvent free systems; reaction in organic solvent with immobilization of substrate; and reaction in ionic liquids. For all methods, oleic acid and/or lauric acid was used as the fatty acid part. Lipase B from *Candida antartica*, commercialized as Novozym 435, was used as enzymatic catalyst. TLC was used to detect the formation of products, and ESI-MS was used to identify the products.

A central challenge in designing a suitable reaction system was to find a reaction medium, in which the saccharide and the fatty acid both were soluble. The initial strategy was to use an amphiphillic organic solvent. Kou and Xu et al. (1998) successfully synthesized a range of saccharide esters by combining the saccharide moiety and fatty acid moiety in an amphiphillic solvent (*tert*-butanol) with an enzymatic catalyst, at 40˚C, using molecular sieves to absorb the water formed during esterification. A modified version of this method was examined in this study for the synthesis of glucuronic acid oleate. The results from TLC analysis are presented in Figure 14. The reaction mixture were analyzed at initiation (day 0) and after 7 days of reaction.

TLC analysis of the reaction mixture with equimolar amounts of reactants (reaction 1 defined in Table 2) showed that one new product formed, however, the product yield was too low to allow for purification (substrate conversion estimated by TLC analysis: ~5-15%, lane 2 in Figure 14).

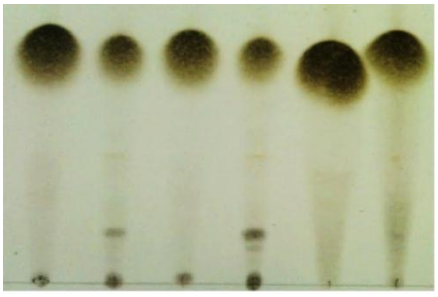


Figure 14: Synthesis of glucuronic acid oleate. Influence of substrate ratio (glucuronic acid to oleic acid). Lanes 1 and 2: Ratio 1-1 after 0 and 7 days. Lanes 3 and 4: Ratio 5-1 after 0 and 7 days. Lanes 5 and 6: Ratio 1-5 after 0 and 7 days.

Using an excess of one substrate is known to shift the equilibrium towards formation of products. Thus, the molar ratio of glucuronic acid to oleic acid was varied from 1-5 to 5-1 to evaluate the influence on the product yield. The outcomes are presented in lanes 4-7 in Figure 14. Excess oleic acid was found to inhibit product formation, probably due to enzymatic inactivation (lane 6 in Figure 14). Excess glucuronic acid, on the other hand, was found to lead to an increased yield of glucuronic acid oleate (estimated conversion of limiting substrate (oleic acid): ~15-25%), lane 4 in Figure 14).

ESI-MS-MS analysis confirmed that the product was glucuronic acid oleate with exact mass 458.26 u (457.26 m/z + $1H^+$, spectrum not presented). The glycoester was fragmented in MS-MS, and the fragments were found to be glucuronic acid and oleic acid. Similar estimated degree of conversion was observed using lauric acid as the fatty acid moiety (conversion of lauric acid: ~15-25%, TLC plate not presented); and the formation of glucuronic acid laureate with exact mass 376.21 u was confirmed with ESI-MS-MS (375.18 m/z + $1H^+$, spectrum not presented).

Excess of glucuronic acid was hence concluded to lead to an increased product yield, yet the conversion of saccharide remained very low. As the main objective of this study was to develop an esterification method which could be applied to AOs, a high conversion of the saccharide moiety was required to make the reaction feasible. To use an excess of AOs was not applicable. With inspiration from existing literature, alternative synthesis strategies were therefore examined.

Ye et al. (2010) synthesized fructose oleate in a solvent-free system, performing the reactions at elevated temperature at which the fatty acid could function as solvent. They included acetone in their reactions, which was allowed to evaporate from the reaction mixture. After evaporation, the reaction continued as a solvent-free. A similar method was examined in this project, studying the use of both acetone and 2-butanone as the solvent adjuvant, as well as a system which was completely solvent free (reactions 7-9 defined in Table 3). However, no formation of glucuronic acid laureate could be detected by TLC analysis.

The next synthesis strategy was based on the hypothesis, that inclusion of an apolar solvent which could completely dissolve the fatty acid, could overcome the inhibiting effect of excess fatty acid. In apolar solvents, however, polar substrates such as glucuronic acid can adsorb to the enzymatic catalyst, leading to a decrease in reaction rate. This can be overcome by including silica gel in the reaction mixture, to which the polar substrate will adsorb. As the concentration of free substrate decreases due to the progress of reaction, the polar substrate will be released from the silica (Castillo et al. 1997; Torres et al. 2001). This approach was applied to the synthesis of glucuronic acid esters in this study, yet no product formation could be detected by TLC analysis.

Ha et al. (2010) have successfully used ionic liquids as reaction medium for the synthesis of saccharide fatty acid esters. With inspiration from their results, ionic liquid was employed as a reaction solvent for the synthesis of glucuronic acid oleate. Glucuronic acid was found to have a very low solubility in the ionic liquid, and the reaction did not lead to formation of product. The complicated reaction mixture could not be applicably analyzed by TLC, but direct inject of the reaction mixture into the ESI-MS confirmed that no product had formed.

A summary of the results from TLC and ESI-MS analyses for reactions 1-14 is given Table 5.

Table 5: The presence or absence of glucuronic acid oleate in each of the reaction systems after 7 days, symbolized by "+" or "-", respectively. Double + ("++") indicates seemingly higher product formation as evaluated by visual inspection of the intensity of the TLC spot. No diesters were observed in any of the reaction systems. NA: not analyzed. (*Reaction 14 only 5 days).*

Reaction	TLC	ESI-MS
1	+	NA
2	+	NA
3	++	+
4	-	NA
5	-	NA
6	++	+
7	-	NA
8	-	NA
9	-	NA
10	-	NA
11	-	NA
12	-	NA
13	-	NA
14	NA	-

In summary, none of the examined methods were found capable of synthesizing glucuronic acid oleate in adequate yields. Several reasons for this can be proposed. Firstly, all hydroxyl groups

in glucuronic acid are secondary, which significantly lowers their reactivity, compared to the primary hydroxyl group in e.g. glucose and sucrose. Secondly, glucuronic acid has very low solubility in organic solvents due to its ability to form strong intermolecular hydrogen bonds, which lowers the reactivity of the hydroxyl groups.

In this study, glucuronic acid only served as model compound for AOs. The solubility of AOs in organic solvents was generally observed to be even lower than the corresponding solubility of glucuronic acid, and even in reaction systems where low amounts of product could be detected using glucuronic acid, no esters formed when using AOs.

Further attempts to find solutions to this challenge were terminated in the summer of 2013, prior to initiating the work at Rensselaer Polytechnic Institute in Troy, NY, USA. Following, focus was placed on the modification of native, polymeric alginate for preparation of an encapsulation material for use in preparation of stable marine oil-in-water emulsions.

4.3 MODIFICATION OF ALGINATE

Alginate was modified with SAC0 and SAC12, in an aqueous reaction medium at neutral pH and 30°C, with no use of catalysts or coupling reagents. The aqueous synthesis procedure and neutral conditions were beneficial, as one of the target applications of the resulting material was in food products.

The synthesis variables such as temperature, reaction time, and molar ratio of substrates, were optimized towards a high degree of succinylation. The degree of succinylation was determined by alkaline titration of the acidified product to determine the number of carboxyl groups, which increased with reaction with SAC0 and SAC12. Figure 15 shows the time-course of the succinylations using SAC0 and SAC12 in 4-times molar excess at 30°C.

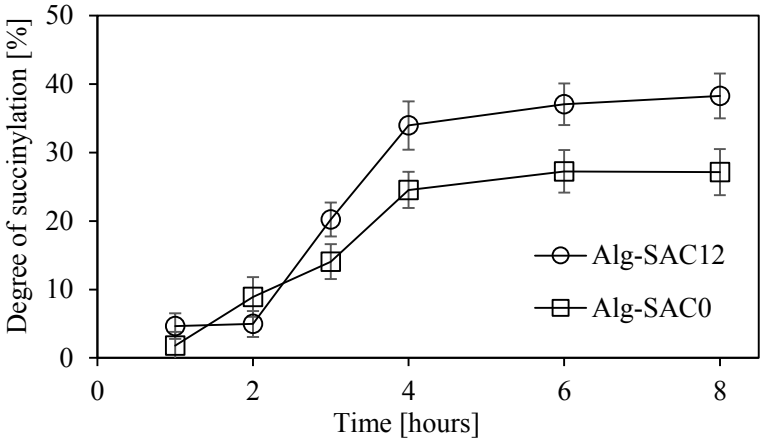


Figure 15: Time-course of alginate succinylation, using 4-times molar excess of SAC0 or SAC12, at 30°C.

For both reactions, no significant increase in the degree of succinylation was observed after 4 hours of reaction. The degree of succinylation after 4 hours in alg-SAC0 and alg-SAC12 were 24.5% ± 2.7 and 33.9% ± 3.5, respectively, which interprets to that, on average, every second monomeric units of alginate were modified with one SAC0 moiety, and that two out of three monomeric units of alginate were modified with one SAC12 moiety. Hence, the degree of succinylation increased when succinic anhydride had an alkyl substitution. This observation could be related to the water solubility of the substrates and their stability in water. Anhydrides are instable in water, and once an anhydride has reacted with water (ring opening), its reactivity decreases. Succinic anhydride with no alkyl substitution dissolved readily in water, and the effect of water on the anhydride moiety could have caused the lower degree of succinylation, compared to SAC12, which had a much lower solubility in water.

The influence of temperature was examined for the reaction between alginate and SAC12 using 4-times molar excess of SAC12 (Figure 16). Thirty degrees C was found to be the optimum temperature, leading to the highest degree of succinylation after four hours. Lower reaction temperature slowed the reaction rate and decreased the substrate's (SAC12) solubility in water. At higher temperatures, the anhydride moiety degraded faster, hence inactivating the substrate and leading to a lower degree of succinylation in the alginate.

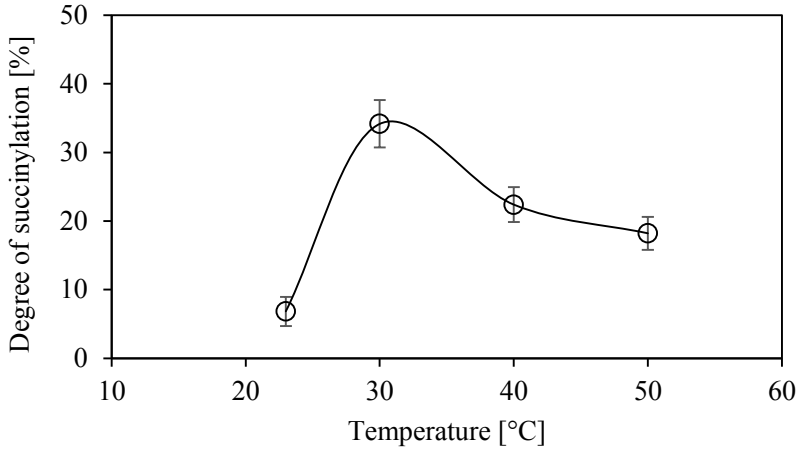


Figure 16: Influence of temperature on degree of succinylation, using SAC12 in 4-times molar excess, after 4 hours.

The effect of increasing the excess of SAC12 was studied and the results are presented in Figure 17. As expected, the degree of succinylation increased with increasing amounts of SAC12. The degree of succinylation levels off at four-time molar excess, and no significant increase in degree of succinylation was observed by increasing the molar ratio further. This phenomenon could be related to an effect of steric hindrance, in that, due to the bulky structure of the dodecenyl moieties, every monomeric unit in alginate cannot be succinylated.

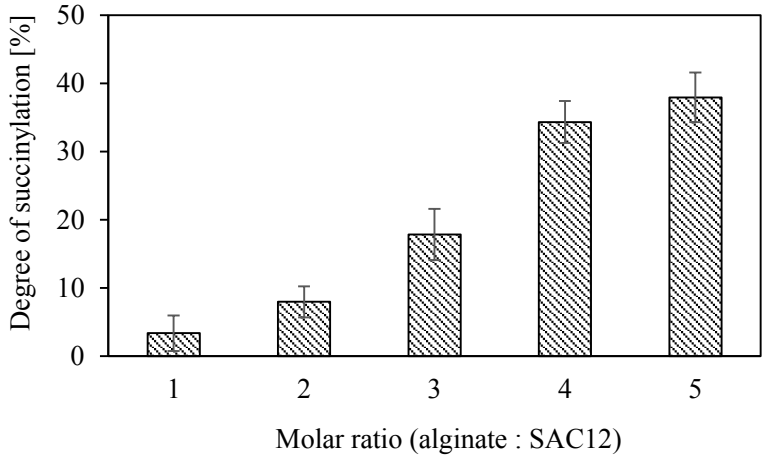


Figure 17: Influence of varying the molar excess of SAC12 with respect to alginate, after 4 hours at 30°C.

The successful succinylations of alginate were confirmed with FTIR (Figure 18). The FTIR spectra of alginate and alg-SAC12 are presented and interpreted in Paper II, but a coherent presentation of the FTIR spectra of alginate, alg-SAC12, and alg-SAC0, is given in the following.

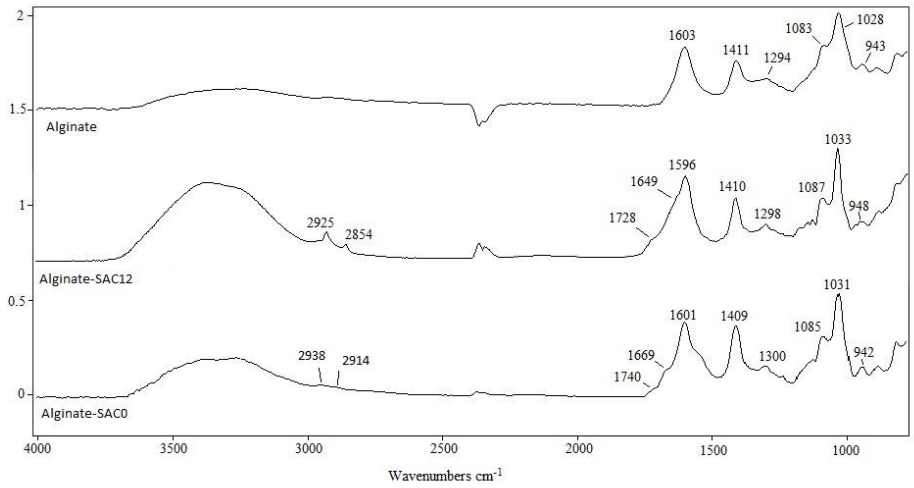


Figure 18: FTIR spectra of alginate, alg-SAC12, and alg-SAC0.

The succinylated alginates showed bands assignable to the sodium alginate backbone, as well as bands assignable to substitutions. Distinct bands assignable to the alkane moieties of the substitutions were detected at 2925 cm^{-1} and 2854 cm^{-1} in alg-SAC12, and at 2938 cm^{-1} and 2914 cm^{-1} in alg-SAC0 (weak bands). In the spectrum of succinylated alginates, the C=O asymmetric stretching vibrations of the carboxylates in the alginate backbone, the free acids in the substitutions, and the ester bonds between alginate and succinic anhydride, are not well separated, but can be identified at 1596 cm^{-1}, 1649 cm^{-1}, and 1728 cm^{-1} for alg-SAC12; and at 1601 cm^{-1}, 1669 cm^{-1}, and 1740 cm^{-1} for alg-SAC0. The band of the carboxylate asymmetric stretching vibrations (1603 cm^{-1}) is expectedly also present in the unmodified alginate. In all spectra, two bands at ~1080 cm^{-1} and ~1030 cm^{-1} occur. These are assigned to the C–O and C–C stretching vibrations of the pyranose rings in alginate. The bands at ~945 cm^{-1} in each spectra are assigned to the 1→4 glycosidic linkages in alginate (Chandia et al. 2001). The alginates all have the characteristic broad absorbance band in the region 3200–3500 cm^{-1}, assignable to the

hydroxyl stretching vibrations. The intensity of this band is higher in the succinylated alginates, as it is contributed from the O–H stretching vibration of the free carboxylic acids (Yadav 2005).

4.4 APPLICATION OF MODIFIED ALGINATES

The properties of the modified alginates were evaluated with focus on two distinct applications. In section 4.4.1, the application of modified alginate (specifically, alg-SAC12) in marine oil-in-water emulsions is presented. The detailed results from this part are presented in Manuscript II. In section 4.4.2, the application of modified alginate in the formation of hydrogels for lipase encapsulation is presented.

4.4.1 Marine Oil-in-water Emulsions

Emulsions with 30% marine oil were formed using the modified alginates as stabilizers. Alg-SAC0 did not show significantly modified properties in terms of emulsification stabilization compared to native alginate, and was not considered further for this purpose.

No high-energy methods, such as homogenization, were employed for the formation of emulsions; only simple magnetic stirring was used to mix the ingredients and form the emulsions, which naturally led to formation of emulsions with larger oil droplets compared to values generally reported in literature. Emulsions stabilized by alg-SAC12 and by native alginate, as well as by β-lactoglobulin (BLG) and phosphatidyl-choline (PC), were prepared similarly for comparison.

Characteristics such as pH, average droplet size, and zeta potential, of each emulsion were analyzed. The results are presented in detail in Paper II. Most importantly, all emulsions had a negative surface charge; however, emulsions stabilized by alg-SAC12 had a significantly larger negative surface charge, which provided strong repulsive forces between the oil droplets, stabilizing the emulsions. Additionally, emulsions stabilized by alg-SAC12 had smaller oil

droplets (7.64 ± 1.06 μm) compared to emulsions stabilized by native alginate (22.02 ± 10.16 μm) as determined by dynamic light scattering analyses.

Confocal laser scanning microscopy (CLSM) analyses of the emulsions provided interesting insights into the stabilizing mechanisms of native and modified alginate. Selected CLSM images are presented in Figure 20 and Figure 19. As was also observed by dynamic light scattering, CLSM confirmed that the average oil droplet size was significantly smaller in emulsions stabilized by alg-SAC12 compared to emulsions stabilized by native alginate. As shown in Figure 20, native alginate distributed almost homogeneously in the aqueous phase and did not pack at the oil-water interface. Native alginate hence stabilized the emulsions mainly through its action as a thickening agent, increasing the viscosity of the emulsions' aqueous phase and slowing the creaming of oil droplets. Alg-SAC12, on the other hand, showed significant surface activity and was present almost exclusively in the interfacial region of the emulsions, coating the surface of the oil droplets (Figure 19). Only low amounts of alg-SAC12 could be observed in the aqueous phase. Oil droplets in both emulsions appeared well separated without indication of droplet flocculation.

The increased amphiphilicity of alg-SAC12 provided increased storage stability to the marine oil-in-water emulsion. The smaller oil droplets and the large negative surface charge, led to a lower creaming index after 11 days of storage at 5°C, compared to the corresponding emulsions stabilized by native alginate. Good storage stability of oil-in-water emulsions is a prerequisite for their application in food products, and alg-SAC12 hence showed good potential for application in food emulsions.

Marine oil in oil-in-water emulsions is particularly sensitive towards oxidation, *cf*. Chapter 1, and antioxidants are hence required to protect the emulsified oil against oxidation. As presented in Paper I, native alginate scavenges hydroxyl radicals and ABTS radicals to some extent. Preservation, and even enhancement, of hydroxyl radical scavenging activity was observed for alg-SAC12, as presented in Paper II, whereas the opposite was observed for ABTS radical scavenging. As detailed in Paper II, the reason for this is found in the radical scavenging mechanism of native and succinylated alginates; alg-SAC12 possessed lower hydrogen-donation activity compared to native alginate, but an increased ability to act as an acceptor in radical addition reactions.

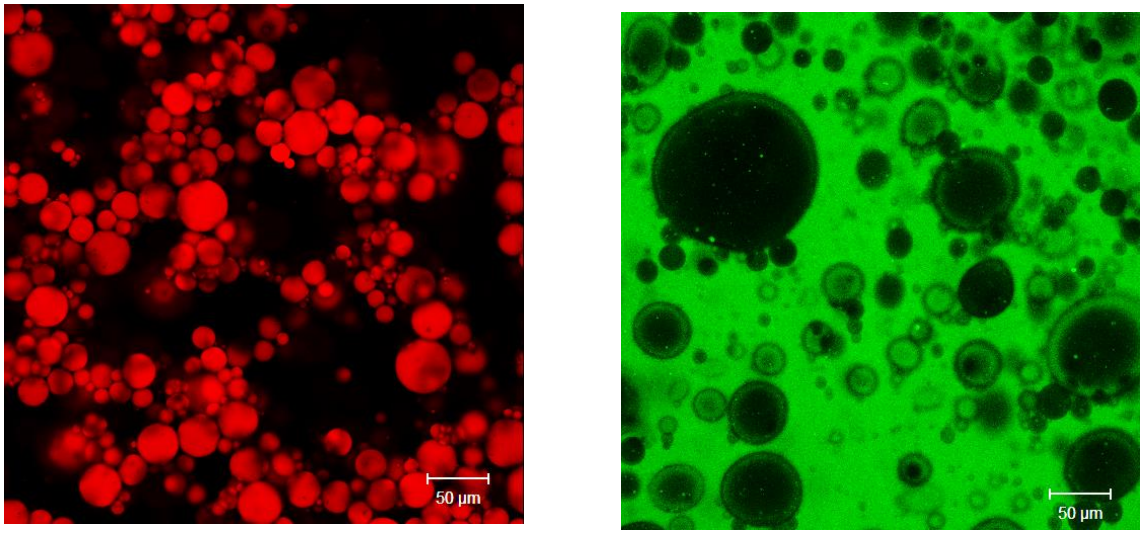


Figure 20: CLSM images of 30% marine oil-in-water emulsions stabilized by native alginate. Left: Nile Red fluorescence probe for lipid phase imaging. Right: Rhodamine 6G fluorescence probe for alginate imaging.

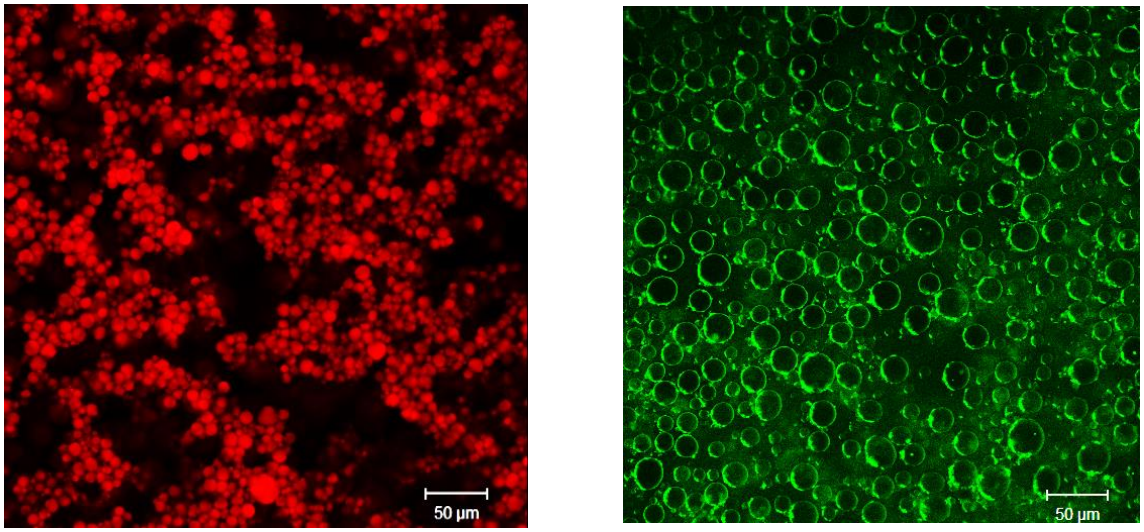


Figure 19: CLSM images of 30% marine oil-in-water emulsions stabilized by alg-SAC12. Left: Nile Red fluorescence probe for lipid phase imaging. Right: Rhodamine 6G fluorescence probe for alginate imaging.

Other mechanisms besides free radical scavenging influence the rate and extent of lipid oxidation in emulsions. Depending on their surface charge, oil droplets can attract oppositely charged compounds, such as prooxidants or antioxidants, depending on the presence and charge of such compounds (Mei et al. 1999; McClements et al. 2000). The large negative surface charge in emulsions stabilized by alg-SAC12 could attract prooxidant transition metal ions present in a food matrix, thus increasing the rate of lipid oxidation (Mei et al. 1999). To overcome this it is suggested to apply this emulsifier in combination with other biomaterials, e.g. by coating the stabilized droplets by positively charged biopolymers through layer-by-layer electrostatic deposition (Guzey et al. 2006).

4.4.2 Hydrogels for Lipase Encapsulation

The native and succinylated alginates were used as matrix materials for the immobilization of lipases (CAL-B and CRL). The aqueous solutions of alginates (native alginate, alg-SAC0, or alg-SAC12) formed beads upon contact with the $CaCl_2$ solution. The beads appeared transparent for unloaded alginate hydrogels and turbid/white for lipase-loaded alginate hydrogels. The beads were approximately 1-4 mm in diameter and retained their shape throughout the analyses.

The results presented in the following should be regarded as preliminary only. The methods were generally associated with variability, and additional repeats should be performed before final conclusions can be made.

For encapsulation of CAL-B in alginate hydrogels, the enzyme was mixed with alginate (native or modified) in an initial ratio of 0.26 mg enzyme per 1 mg alginate. This corresponds to a high excess of lipase compared to the amount which expectedly can be encapsulated in alginate hydrogel beads according to existing reports (Won et al. 2005; Zhang et al. 2014). When in contact with the calcium chloride solution, the alginate formed hydrogel beads, in which part of the lipase was entrapped. The final amount of entrapped lipase was determined by measuring the amount of free protein in the residual calcium chloride fraction, and in the wash fractions. The results are presented in Figure 21, which shows that no significant difference between the three polymers could be detected. For all alginates, approximately 80-90 µg CAL-B could be

encapsulated in 1 mg polymer. For comparison, Won et al. (2005) encapsulated approximately 16 μg lipase in 1 mg alginate.

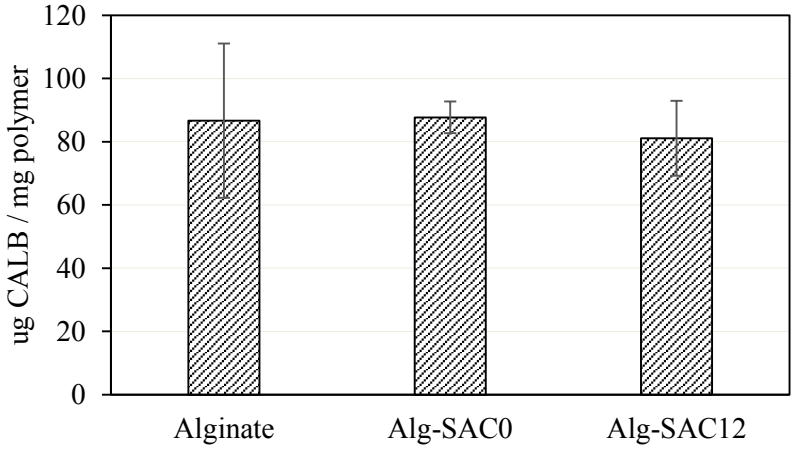


Figure 21: Enzyme (CAL-B) load in alginate, alg-SAC0, and alg-SAC12 hydrogel beads.

The activity of the lipase encapsulated in the polymers was determined and is presented in Figure 22. As for the enzyme loading efficiency, no significant difference between the three polymers could be detected. The specific activity of free CAL-B was 20 μM/sec/mg, and the specific activities of the encapsulated lipases were thus significantly lower. The major reason is expected to be hindrance of the lipases in the innermost parts of the hydrogel beads to reach the p-NPB substrate.

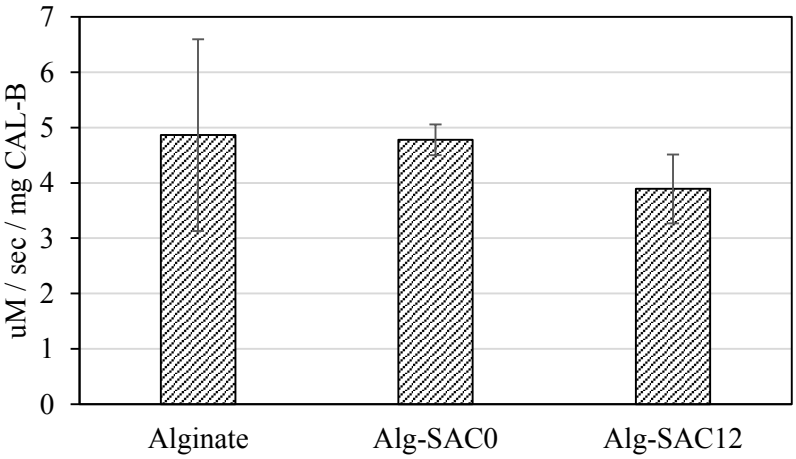


Figure 22: Specific activity of CAL-B encapsulated in alginate, alg-SAC0, and alg-SAC12.

For encapsulation of CRL, a significant lower initial ratio of enzyme to polymer was applied (56 µg CRL per 1 mg polymer). The final encapsulation efficiency has yet to be determined accurately; in the following an encapsulation efficiency of 50% is assumed – corresponding to a final enzyme load of 28 µg CRL per mg alginate.

The specific activity of the encapsulated CRL is presented in Figure 23. The results represent data from three, individual repeats of enzyme encapsulation and specific activity determinations; the error bars remain narrow, however, due to the lack of naturally varying data regarding the actual enzyme load in the hydrogel beads.

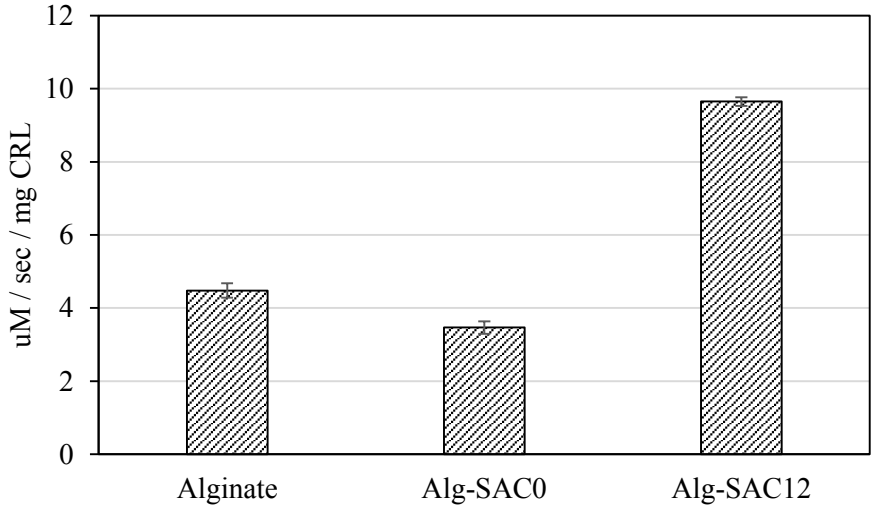


Figure 23: Specific activity of CRL encapsulated in alginate, alg-SAC0, and alg-SAC12.

As can be seen in Figure 23, the specific activity of CRL encapsulated in alg-SAC12 hydrogel was more than double the specific activity of CRL encapsulated in alginate or alg-SAC0 hydrogels. Due to the lacking data for enzyme load, this observation could be due to an actual higher enzyme load in alg-SAC12 hydrogel beads, compared to alginate or alg-SAC0 hydrogel beads, opposed to the assumed 50% encapsulation efficiency assumed for all polymers. Alternatively, it could be due to an activating effect of the modified polymer on the CRL. In complex lipases such as CRL, a lid covers the substrate-binding site. Upon contact with the interface, the lip opens to activate the lipase (Rehm et al. 2010). CAL-B has no activating lid in

its structure (Salis et al. 2003). One explanation for the observations in this study is based on the hypothesis, that alg-SAC12, with its hydrophobic moieties, can encapsulate CRL in an active form in which the lid is kept open. This would have no effect on CAL-B, as it does not contain a lid in its structure. However, additional research is required to elucidate the encapsulation mechanisms of the alginates further.

5 CONCLUSIONS AND PERSPECTIVES

With the work presented in this Ph.D. thesis, the range of available microencapsulation materials has been expanded. Starting from alginate, innovative materials have been prepared, which can be applied in microencapsulation of marine oil in oil-in-water emulsions, and potentially in microencapsulation of lipases in hydrogel beads. The versatile nature of alginate allows its modification towards various applications, and the hydrophobic modification of alginate, in particular, have been shown to endow alginate with new properties, which have been illustrated for various applications.

The growing consumer preference for natural ingredients has motivated the use of green and non-toxic synthesis methods throughout the study. Alginate oligosaccharides (AOs) were prepared by enzymatic depolymerization of alginate in an all-aqueous system at moderate temperature and pH. By replacing the commonly used TRIS-HCl buffer with an ammonium acetate solution as reaction medium, the need for isolation using chromatography was omitted, allowing for a larger throughput in the preparation of AOs. The AOs were found to be good antioxidants, and represent as such potential new ingredients for use in food products. A mechanism for the radical scavenging was elucidated, which proposes that the radical scavenging activity of AOs originated mainly from the presence of the conjugated alkene acid structure formed during enzymatic depolymerization. AOs act as chain-breaking antioxidants, and the AO-radicals are stabilized through allylic rearrangement according to the theory of resonance hybrids.

The specific aim of this Ph.D. study was to apply these oligosaccharides in the protection of microencapsulated marine oil. For that purpose, attempts were made to hydrophobically modify the AOs to tailor them for use in oil-in-water emulsions. Two types of hydrophobic modifications were examined in this study. The first included the esterification of the carboxyl group of AOs, represented by glucuronic acid, with alkyl alcohols. An enzymatic method based on the use of lipase was successfully applied in the synthesis of octanyl glucuronate; however, this synthesis method did not yield the corresponding esters of AOs. A range of synthesis methods based on chemical catalysis in organic solvents were investigated, yet none of them

were found capable of synthesizing octanyl glucuronate, primarily due to the formation of by-products, in particular, the lactone form of glucuronic acid and the lactone di-substituted with octanol. The second type of hydrophobic modification of AOs in focus was the fatty acid esterification of the hydroxyl groups in AOs, represented by glucuronic acid. Reactions performed in amphiphillic organic solvent (2-butanone) led to the formation of glucuronic acid oleate and glucuronic acid laureate, the structures of which were confirmed by ESI-MS (positional isomers were not confirmed). However, the conversion of saccharide was very low, and the method was not applicable in the synthesis of AOs fatty acid esters. Alternative strategies were not successful in improving the product yield, and it was concluded, that fatty acid esterification of glucuronic acid, representing AOs, was not a feasible strategy to produce the desired AO-based amphiphillic antioxidants.

Polymeric alginate was modified with succinic anhydrides in a reaction system resembling that of the commercially accepted system for preparation of modified starch. Alg-SAC12 was able to efficiently stabilize marine oil-in-water emulsions, which have the potential to be applied in food products for the purpose of fortifying the food with n-3 PUFAs. The viscosity of emulsions stabilized by alg-SAC12 was reduced compared to emulsions stabilized by native alginate, thus increasing its application potential for further processes, such as spray drying. Alg-SAC12 was found to possess an interesting dual-functionality in terms of good emulsification properties combined with antioxidant properties. Lipid oxidation in an emulsion takes place primarily at the oil-water interface, i.e. where the modified alginate is located in the emulsion, and the modified alginate hence provides good protection against lipid oxidation. Though the antioxidant activity of alg-SAC12 was only determined *in vitro*, i.e. by measuring its ability to scavenge free radicals, it can reasonably be assumed that this emulsifier is capable of stabilizing marine oil-in-water emulsions both physically and oxidatively. It is suggested that future research with this emulsifier is carried out in a food product of interest, as it is well known that multiple factors in a food system influences the physical and oxidative stability of emulsified marine oil.

Generally, systems for microencapsulation of sensitive ingredients should be optimized for individual applications, and only general guidelines can be drawn for e.g. marine oil-in-water emulsions in food. The microencapsulation materials presented in this work are not optimized towards a specific application or a specific food product, but have been developed to represent

new tools for use in the design of microencapsulation systems. Thus, the research was focused on illustrating the emulsion-stabilizing properties of alg-SAC12, and the antioxidant properties of alginate oligosaccharides; but leaving the design of specific areas of application to later research. Emulsions stabilized by alg-SAC12 could e.g. be spray dried to convert the emulsified oil to a dry form. Alternatively, as the emulsion stabilized by alg-SAC12 had a large negative zeta potential, it could form a suitable "primary" emulsions, which could be stabilized by positively charged polymers.

The succinylated alginates were found capable of forming hydrogels, in which lipases can be encapsulated. Additional research on this topic is required before final conclusions can be drawn, but the results presented herein indicate a potential for alg-SAC12 to encapsulate lipases with a lid-structure in an open, active form. Such properties could be exploited in the design of suitable carrier systems for lipases for use in various technical applications.

It is concluded, that the materials presented herein represent promising new materials, in particular, the dual-functionality of dodecenyl succinylated alginate in terms of emulsifying properties and antioxidant properties. Future research on this topic is suggested to focus on encapsulation of marine oil in this material using e.g. spray-drying or similar techniques, and evaluation of its properties in a food product of interest. Also the effect of AOs in actual foods should be evaluated, as current study only has shown their potential antioxidant effects.

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7 PAPERS AND APPENDICES

Paper I: Mia Falkeborg, Ling-Zhi Cheong, Carlo Gianfico, Katarzyna Sztukiel, Kasper Kristensen, Marianne Glasius, Xuebing Xu, Zheng Guo, 2014. "Alginate Oligosaccharides: Enzymatic Preparation and Antioxidant Properties Evaluation". Food Chemistry 164, pp 184-195.

Manuscript II: Mia Falkeborg, Zheng Guo. "Dodecenyl Succinylated Alginate as a Novel Dual-function Emulsifier for Improved Fish Oil-in-water Emulsions". Submitted to Food Hydrocolloids, under review (2014).

Conference poster: Mia Falkeborg, Ling-Zhi Cheong, Carlo Gianfico, Katarzyna Sztukiel, Kasper Kristensen, Marianne Glasius, Xuebing Xu, Zheng Guo, 2014. "Alginate Oligosaccharides: Enzymatic Preparation and Evaluation of their Antioxidant Properties". Recipient of "Best Poster Award" in the Lipid Oxidation and Quality Section.

Appendix 1. Properties of Alginate Lyase S. Provided by Nagase Enzymes, Kyoto, Japan.

Appendix 2: Enzymatic Synthesis of Phosphatidyl-saccharides and their Application in the Formation of Stable Liposomes. Including **Paper III**: Shuang Song, Ling-Zhi Cheong, Mia Falkeborg, Lei Liu, Mingdong Dong, Henrik Max Jensen, Kresten Bertelsen, Michael Thorsen, Tianwei Tan, Xuebing Xu, Zheng Guo, 2013: "Facile Synthesis of Phosphatidyl Saccharides for Preparation of Anionic Nanoliposomes with Enhanced Stability". PLoS ONE; 8(9), pp. e73891.

Appendix 3: Expression and Characterization of Antimicrobial Enzymes. A summary of the research from Rensselaer Polytechnic Institute, September 2014 – February 2014.

Paper I

Alginate Oligosaccharides: Enzymatic Preparation and Antioxidant Property Evaluation

Mia Falkeborg, Ling-Zhi Cheong, Carlo Gianfico, Katarzyna Sztukiel, Kasper Kristensen, Marianne Glasius, Xuebing Xu, Zheng Guo, 2014.

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Alginate oligosaccharides: Enzymatic preparation and antioxidant property evaluation

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ABSTRACT

Alginate oligosaccharides (AOs) prepared from alginate, by alginate lyase-mediated depolymerization, were structurally characterized by mass spectrometry, infrared spectrometry and thin layer chromatography. Studies of their antioxidant activities revealed that AOs were able to completely (100%) inhibit lipid oxidation in emulsions, superiorly to ascorbic acid (89% inhibition). AOs showed radical scavenging activity towards ABTS, hydroxyl, and superoxide radicals, which might explain their excellent antioxidant activity. The radical scavenging activity is suggested to originate mainly from the presence of the conjugated alkene acid structure formed during enzymatic depolymerization. According to the resonance hybrid theory, the parent radicals of AOs are delocalized through allylic rearrangement, and as a consequence, the reactive intermediates are stabilized. AOs were weak ferrous ion chelators. This work demonstrated that AOs obtained from a facile enzymatic treatment of abundant alginate is an excellent natural antioxidant, which may find applications in the food industry.

1. Introduction

Compounds with antioxidant activity are attracting considerable attention due to their functions in food preservation and in human health (Shahidi & Zhong, 2010). Currently, both synthetic and natural antioxidants have widespread applications; however, due to the growing trend in consumer preference for natural food ingredients, the interest in using antioxidants from natural sources is increasing (Wanasundara & Shahidi, 2005). This study presents the use of marine alginate as a source of biomaterial in the preparation of natural antioxidants. Alginate is a polysaccharide originating from marine algae, composed of α-L-guluronate and β-D-mannuronate arranged as linear homopolymeric and heteropolymeric blocks (Benvegnu & Sassi, 2010; Pawar & Edgar, 2012). Guluronate and mannuronate are both uronates with carboxylate groups at their C5 positions. The configuration of the carboxylate groups represents the difference between the two (Fig. 1). Approximately 30,000 tons of alginate is produced annually, which is estimated to be less than 10% of the total amount of biosynthesized alginate (Pawar & Edgar, 2012). Hence, alginate can be considered an abundant resource of biomaterials.

During the enzymatic depolymerization of alginate by alginate lyases, the glycosidic linkages are cleaved through *endo*-active β-elimination, and double bonds between the C4 and C5 carbons in the non-reducing terminal residues of the resulting alginate oligosaccharides (AOs) are formed (Fig. 1) (Kim, Lee, & Lee, 2011; Wong, Preston, & Schiller, 2000). Complete depolymerization of alginate by β-eliminating lyases leads to the formation of unsaturated dimers, trimers, and possibly higher oligosaccharides depending on the nature of the lyase (Wong et al., 2000; Zhang et al., 2004).

Zhao et al. (Zhao, Li, Xue, & Sun, 2012) reported that AOs prepared by enzymatic depolymerization of alginate prevent lipid oxidation in emulsions, and they showed that AOs can scavenge hydroxyl (·OH) and superoxide ($O_2^{\cdot-}$) radicals. However, Wang et al. (Wang et al., 2007) reported that AOs have no influence on lipid oxidation in emulsions, and that AOs scavenge only hydroxyl radicals and not superoxide radicals. Trommer and Neubert (2005) reported, that alginic acid has a pro-oxidative effect on lipids in emulsion. Further studies are obviously required to clarify the antioxidant activity of AOs and the molecular basis for this activity. This study aims to clarify the molecular mechanism of the

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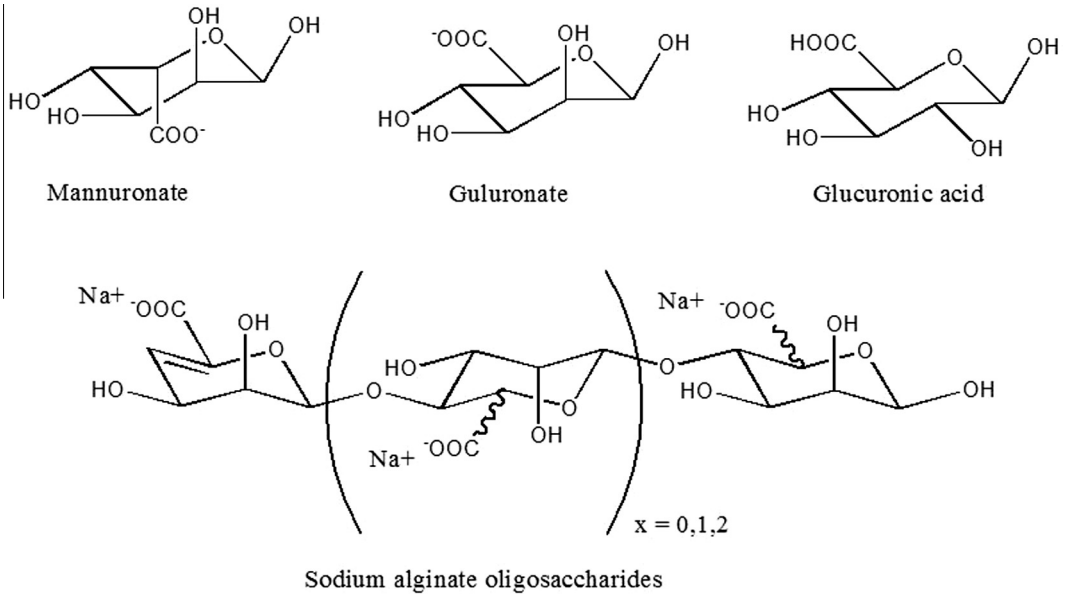

Fig. 1. Saccharide structures.

antioxidant activity of AOs through comparative studies with other polymeric and monomeric forms of alginate.

Several antioxidant mechanisms have been proposed generally for carbohydrates, including the ability to scavenge reactive oxygen species such as superoxide radicals and hydroxyl radicals. Three main mechanisms exist by which compounds can directly scavenge free radicals, namely single electron transfer (SET), hydrogen atom transfer (HAT), and radical addition to e.g. double bonds (Hernandez-Marin & Martinez, 2012). Through their theoretical studies of the radical scavenging capability of carbohydrates, Hernandez-Marin et al. (Hernandez-Marin & Martinez, 2012) concluded that SET is less likely to occur, and that HAT mainly occurs from carbon-bonded hydrogens. Radical addition is generally not considered a possible antioxidant mechanism of carbohydrates, as they do not commonly contain double bonds or aromatic rings. In their study of a range of antioxidants, Peshev et al. (Peshev, Vergauwen, Moglia, Hideg, & Van den Ende, 2013) observed that compounds with a carbon–carbon double bond were superior antioxidants. The double bond provides an opportunity for radical addition, which becomes the preferred radical reaction over SET and HAT. As enzymatically prepared AOs have double bonds in their structure, we hypothesized that AOs are endowed with increased antioxidant activity in terms of increased radical scavenging capability through both hydrogen abstraction and radical addition.

The present study aimed to investigate the antioxidant properties of AOs prepared by complete depolymerization of sodium alginate by a β-eliminating lyase. The release of unsaturated saccharides during depolymerization was followed by spectrophotometric analyses. The AOs were recovered when the lowest possible degree of polymerization of alginate was obtained, i.e. when additional enzymatic treatment did not lead to additional depolymerization. The composition and antioxidant properties (lipid oxidation inhibition, radical scavenging activity ($^{\cdot}OH$, $O_2^{\cdot-}$, and $ABTS^{\cdot}$), and ferrous ion chelating activity) of the AOs were investigated. Comparative studies were made on polymeric, oligomeric, and monomeric forms of alginate; on mannuronate- and guluronate-rich fractions; and on acid and salt forms of the AOs, in order to determine which functional group(s) of AOs are responsible for their antioxidant activity.

2. Materials and methods

2.1. Materials

Sodium alginate (Grindsted® Alginate FD 170) was provided by DuPont, previously Danisco, Brabrand, Denmark. This alginate originated from brown algae, and the ratio of α-L-guluronate units to β-D-mannuronate units was 40–60. Alginate lyase S from *Sphingobacterium* was provided by Nagase Enzymes, Kyoto, Japan. Amberlite® 200 Na^+ strong cation exchanger resin, Tween® 20, ammonium acetate, phosphate buffered saline (PBS) 0.01 M pH 7.4, D-glucuronic acid, ascorbic acid, ferrous sulfate ($FeSO_4$), trichloroacetic acid, thiobarbituric acid (TBA), potassium hydroxide (KOH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$), sodium salicylate, hydrogen peroxide (H_2O_2), xanthine oxidase (from bovine milk, 0.11 U/mg solid, XOD), hypoxanthine (minimum 99%, HPX), nitrotetrazolium blue chloride (NBT), ferrous chloride ($FeCl_2$), 3-(2-pyridyl)-5, 6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt (ferrozine), and disodium ethylenediaminetetraacetate dehydrate (EDTA–$Na_2{\cdot}2H_2O$), were purchased from Sigma–Aldrich. Linoleic acid 96% was obtained from Zhongchuan Biotechnology Co Ltd. Anqing, China.

2.2. Enzymatic depolymerization of sodium alginate and purification of AOs

Sodium alginate (3.0 g) was depolymerized in 150 ml 0.05 M ammonium acetate using alginate lyase S at a concentration of 5% w/w of sodium alginate, at 35 °C and 200 rpm. Aliquots of 200 μl of the reaction mixture were withdrawn at time: 0, 0.5, 1, 2, 4, 8, 12, 24, 27, 30, 33, 48, 51, 53, and 72 h. The lyase in the samples was denatured at 110 °C for 15 min and removed by centrifugation for 5 min at 10,000 rpm at room temperature. The release of unsaturated saccharides was determined by measuring the absorbance of the supernatant at 234 nm in a UV–visible spectrophotometer (Cary 50Bio, Varian, Australia) using a quartz cuvette. The supernatant was diluted in pure water to obtain a value of absorbance within the accurate range of the spectrophotometer. The course of depolymerization was additionally followed

by thin layer chromatography (TLC). After 72 h, the lyase was denatured by incubation at 110 °C for 15 min and removed by centrifugation. The AOs were purified and dried by evaporation of water and ammonium acetate at 20 mbar and 65 °C, after which the AOs were obtained in their sodium salt form as free-flowing powder. The structure and composition of the AOs was confirmed by TLC, electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS), and Fourier-transformed infrared spectrometry (FTIR) analyses.

2.3. Acidification of AOs

To produce AOs on acid form, dried sodium AOs were dissolved in deionized water to a concentration of 200 mg/ml and passed 3 times over Amberlite® 200 resin, which had been activated by 1 M HCl. The acidified AOs were purified and dried by evaporation of water and excess ions (Na^+, H^+ and Cl^-) at 20 mbar and 65 °C. The acidification process was confirmed by FTIR, and the composition of the acidified AOs was determined by TLC.

2.4. Characterization of AOs by TLC, ESI-TOF-MS, and FTIR

TLC analyses were performed on silica-coated glass plates (Merck Silica Gel 60, 20 × 20 cm) using 1-butanol/formic acid/water 4/6/1 v/v/v as developing solvent. The saccharides were visualized by spraying with 10% v/v sulfuric acid in ethanol followed by heating at 110 °C for 10 min (Li et al., 2011).

ESI-TOF-MS analyses were performed using a Bruker micro TOF-Q mass spectrometer in negative ESI mode. Sodium AOs were dissolved in deionized water and infused into the ESI source using the following settings: capillary voltage 4.00 kV, nebulizer pressure 3.4 bar, gas flow rate 10.0 L/min, and temperature 180 °C. The resulting mass spectra were analyzed using Bruker Daltonics Data Analysis 3.4 software.

FTIR spectra were recorded in absorbance mode in the 4000–650 cm^{-1} region at a resolution of 4 cm^{-1}, using a Qinterline QFA-flex spectrometer equipped with a deuterium triglycine sulfate detector. The samples were placed in their pure solid form in a Pike attenuated total reflectance (ATR) device thermostated at 25 °C. The spectra were ratioed against a single-beam spectrum of the clean ATR crystal. The resulting spectra were analyzed using GRAMS/AI software.

2.5. Preparation of mannuronate- and guluronate-rich fractions of alginate

To prepare mannuronate- and guluronate-rich fractions of alginate, a method based on partial acid hydrolysis of alginate followed by pH adjustment was used. The method was originally reported by Haug et al. (Haug, Larsen, & Smidsrod, 1966) and is frequently used for the preparation of mannuronate- and guluronate-rich fractions of alginate (Chandia, Matsuhiro, & Vasquez, 2001; Fenoradosoa et al., 2010; Leal, Matsuhiro, Rossi, & Caruso, 2008; Sakugawa, Ikeda, Takemura, & Ono, 2004). Three grams sodium alginate in 300 ml deionized water was heated under reflux at 100 °C for 20 min, with 9 ml of 3 M HCl to hydrolyze the strictly heteropolymeric fractions (alternating guluronate and mannuronate), which are most easily hydrolyzed. The cooled suspension was then centrifuged at 10,000*g* for 20 min, and the precipitate containing the homopolymeric and the random heteropolymeric fractions was suspended in 300 ml of 0.3 M HCl and heated under reflux at 100 °C for 2 h to hydrolyze the remaining heteropolymeric fractions. The cooled suspension was centrifuged at 10,000*g* for 20 min, and the precipitate containing only the homopolymeric fraction was neutralized with 1 M NaOH. The homopolymeric fraction was then separated into mannuronate- and guluronate-rich fractions by adjusting the pH to 2.85 ± 0.05 with 1 M HCl, at which polymannuronate is soluble and polyguluronate is insoluble. After centrifugation at 10,000*g* for 15 min, the soluble mannuronate-rich fraction and the insoluble guluronate-rich fraction were collected and neutralized with 1 M NaOH. To ensure a high degree of purity, the pH of both fractions was re-adjusted to 2.85 ± 0.05, and the fractions were collected by centrifugation and neutralized with 1 M NaOH. The saccharides were finally precipitated in ethanol, washed with ethanol, and dried under nitrogen.

2.6. Antioxidant properties

2.6.1. Inhibition of iron-induced lipid oxidation

The ability of AOs, acidified AOs, sodium alginate, glucuronic acid, polymannuronate and polyguluronate, to inhibit iron-induced lipid oxidation in an emulsion was evaluated by the TBA reactive species (TBARS) assay (Guillen-Sans & Guzman-Chozas, 1998). Emulsions of linoleic acid in PBS buffer were prepared using Tween 20 as emulsifier (2% oil w/w and 1% emulsifier w/w). Varying concentrations of saccharides (0–175 mg/ml) were included in the emulsions (total volume 1.5 ml) to evaluate their effect on lipid oxidation. The pH of all emulsions was determined using a pH-meter (inoLab (pH 7110), Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). Oxidation was accelerated by adding 250 µl of 25 mM $FeSO_4$ followed by incubation under magnetic stirring at 400 rpm for 15 min at room temperature. The formation of secondary oxidation products was detected by the modified procedure of Zhao et al. (2012), as follows. Trichloroacetic acid (0.5 ml) and 0.7% TBA in 0.05 M KOH (1 ml) was added to each sample which were then incubated at 100 °C for 15 min. Following that, the samples were cooled at room temperature for 10 min and centrifuged at 4000*g* for 1 min. TBA formed coloured complexes with the secondary oxidation products (Guillen-Sans & Guzman-Chozas, 1998), which were detected in a UV–visible spectrophotometer (Cary 50Bio, Varian, Australia) at 534 nm. To account for the lack of specificity of the TBARS assay, as described by Guillen-Sans et al. (Guillen-Sans & Guzman-Chozas, 1998), the absorbance of compounds and TBA-complexes, which did not originate from lipid oxidation, was taken into account, by including negative controls prepared by replacing the linoleic acid emulsion with pure PBS. The absorbances of the negative controls were withdrawn from the absorbances of the respective samples with emulsion. This difference in absorbance at a given saccharide concentration is notated A_S, and the percentage inhibition at each concentration of each saccharide was calculated according to Eq. 1, in which A_0 refers to the absorbance of a sample with no saccharides or antioxidants.

$$Inhibition\ [\%] = \frac{A_0 - A_S}{A_0} * 100 \quad (1)$$

The initial oxidative status of the linoleic acid was tested by measuring the TBARS content of the emulsion without addition of $FeSO_4$, and it was found that it did not contain measurable TBARS. Samples were analyzed in triplicate, and duplicate spectrophotometric measurements were made for each sample. Ascorbic acid was analyzed correspondingly for comparison. The results are presented as inhibition percentage *versus* concentration of saccharide.

2.6.2. ABTS radical scavenging

ABTS radical scavenging activity was examined using the modified procedure of Re et al. (Re et al., 1999). ABTS radicals ($ABTS^{\cdot}$) were generated by incomplete oxidation of ABTS with $K_2S_2O_8$ by combining equal volumes of 7 mM ABTS in deionized water and 2.45 mM $K_2S_2O_8$, followed by incubation in the dark for 24 h. The resulting $ABTS^{\cdot}$ solution was diluted in pure water to give the final

ABTS· solution an absorbance of A_{734nm} = 0.700 ± 0.020. The saccharides were dissolved in deionized water in the concentration range 0–100 mg/ml, and 10 μl saccharide solution was added to 1 ml ABTS· solution and carefully mixed, and the decrease in absorbance over time at 734 nm was measured at room temperature. The percentage of ABTS· scavenged at each time point was calculated according to Eq. 2, in which $A_{INITIAL}$ refers to the initial absorbance (A_{734nm} = 0.700 ± 0.020) and A_S refers to the absorbance after a given time of reaction with a given concentration of saccharide.

$$ABTS\ scavenging\ [\%] = \frac{A_{INITIAL} - A_S}{A_{INITIAL}} * 100 \quad (2)$$

Samples were analyzed in duplicate, and triplicate spectrophotometric measurements were made for each sample.

2.6.3. Hydroxyl radical scavenging

The capacity of the saccharides to scavenge hydroxyl radicals was examined by detecting their competition with salicylic acid for the reaction with hydroxyl radicals, as described by Smirnoff et al. (Smirnoff & Cumbes, 1989). Hydroxylation of salicylic acid leads to formation of dihydroxy benzoic acid, which absorbs at 510 nm. This reaction is suppressed by the presence of hydroxyl radical scavengers, which compete with the salicylic acid for the hydroxyl radicals. In this assay, the hydroxyl radicals were generated from H_2O_2 through the Fenton reaction in a reaction mixture containing 0.6 mM H_2O_2, 0.6 mM $FeSO_4$ and 1 mM sodium salicylate, and varying concentrations (0–15 mg/ml) of saccharides (total volume 1.5 ml). The reaction mixture was incubated at 37 °C for 20 min after which the absorbance at 510 nm was determined. The absorbance of the saccharides themselves at 510 nm was taken into account by withdrawing the absorbance of negative controls, which were prepared at each saccharide concentration by replacing salicylic acid with pure water. This difference in absorbance, at each saccharide concentration, was notated A_S, and the hydroxyl scavenging activity was calculated according to Eq. 3, in which A_0 refers to the absorbance of a control with no scavenger. All samples were analyzed in triplicate using freshly prepared stock solutions for each repeat. Ascorbic acid was analyzed correspondingly for comparison.

$$Hydroxyl\ scavenging\ [\%] = \frac{A_0 - A_S}{A_0} * 100 \quad (3)$$

2.6.4. Superoxide radical scavenging

The capacity of the saccharides to scavenge superoxide radicals was examined by detecting their competition with NBT for the reaction with superoxide, according to the method of Mora-Pale et al. (Mora-Pale, Kwon, Linhardt, & Dordick, 2012). Upon reduction by superoxide, NBT forms formazan, which absorbs at 560 nm. This reaction is suppressed by the presence of superoxide scavengers, which compete with NBT for the superoxide. In this assay, the superoxide radicals were generated from the hypoxanthine–xanthine oxidase system. The reaction mixture contained 0.113 μM NBT, 0.208 μM HPX, 0.014 U XOD, and varying concentrations (0–5 mg/ml) of saccharides (total volume 120 μl) in PBS buffer. The assay was performed using 96-well plates and a SpetraMax M5 plate reader. The reaction was initiated by the addition of XOD and the absorbance at 560 nm was read after 15 min at 25 °C after when the absorbance had reached a stable plateau. The absorbance of the saccharides themselves at 560 nm was taken into account by withdrawing the absorbance of negative controls, which were prepared at each saccharide concentration by replacing XOD with pure PBS buffer. This difference in absorbance, at each saccharide concentration, was notated A_S, and the superoxide scavenging activity was calculated according to Eq. 4, in which A_0 refers to the absorbance of a control with no scavenger. All samples were analyzed in triplicate using a freshly prepared XOD solution for each repeat. Ascorbic acid was analyzed correspondingly for comparison.

$$Superoxide\ scavenging\ [\%] = \frac{A_0 - A_S}{A_0} * 100 \quad (4)$$

2.6.5. Ferrous ion chelating activity

The ability of the saccharides to chelate ferrous ions was assayed using the modified method of Le et al. (Le, Chiu, & Ng, 2007). The saccharides were dissolved in pure water in the concentration range 0–100 mg/ml, and 1400 μl of this saccharide solution was combined with 0.06 μmol $FeCl_2$ in 100 μl pure water (total volume 1.5 ml) and mixed under slow magnetic stirring (100 rpm) for 10 min. The ferrous ions remaining free in solution after 10 min were quantified by adding 100 μl ferrozine (5 mM in pure water) and measuring the absorbance of the ferrous–ferrozine complex at 562 nm after an additional 10 min. The absorbance of the saccharides themselves at 562 nm was taken into account by withdrawing the absorbance of negative controls, which were prepared at each saccharide concentration by replacing ferrozine with pure water. This difference in absorbance at a given saccharide concentration, was notated A_S, and the chelation activity was calculated according to Eq. 5, in which A_0 refers to the absorbance of a sample with no chelating agents included.

$$Chelating\ activity\ [\%] = \frac{A_0 - A_S}{A_0} * 100 \quad (5)$$

All samples were analyzed in triplicate using freshly prepared stock solutions for each repeat. EDTA was analyzed correspondingly as positive control.

2.7. Data analysis

Data processing including regression analyses was performed in Microsoft Excel 2010. One-way analysis of variance (one-way ANOVA) was performed using Microsoft Excel Analysis Toolpak (2010). When applicable, the double standard deviations are shown as error bars around the average of the obtained data.

3. Results and discussion

3.1. Preparation and structural identification of AOs

Sodium alginate was enzymatically depolymerized using alginate lyase S with inspiration from the methods by Liu et al. (Liu, Jiang, Liao, & Guan, 2002) and Zhang et al. (Zhang et al., 2004). In their work, Tris–HCl was used to buffer the enzymatic reaction at pH 7.5. However, as the depolymerization of alginate does not change the pH of the medium, no actual buffer was required to maintain the pH of the reaction medium; the only requirement was the presence of salts to provide the required ionic strength of the reaction medium. Ammonium acetate was an interesting alternative to Tris, as this salt is volatile and can be removed from the reaction mixture by evaporation. The reaction temperature (35 °C) was chosen based on information of activity and stability from the enzyme producer. Reaction factors, such as enzyme load (5% w/w of alginate), concentration of ammonium acetate (0.05 M), and pH (neutral) were determined after optimization studies (results not presented herein).

The formation of AOs over time was followed by spectrophotometric analyses at 234 nm and by TLC analysis and the time course of the depolymerization is shown in Fig. 2A. Fig. 2A1 shows, that the rate of depolymerization was highest during the first 2 h of reaction and 37% of the final absorbance was reached within this

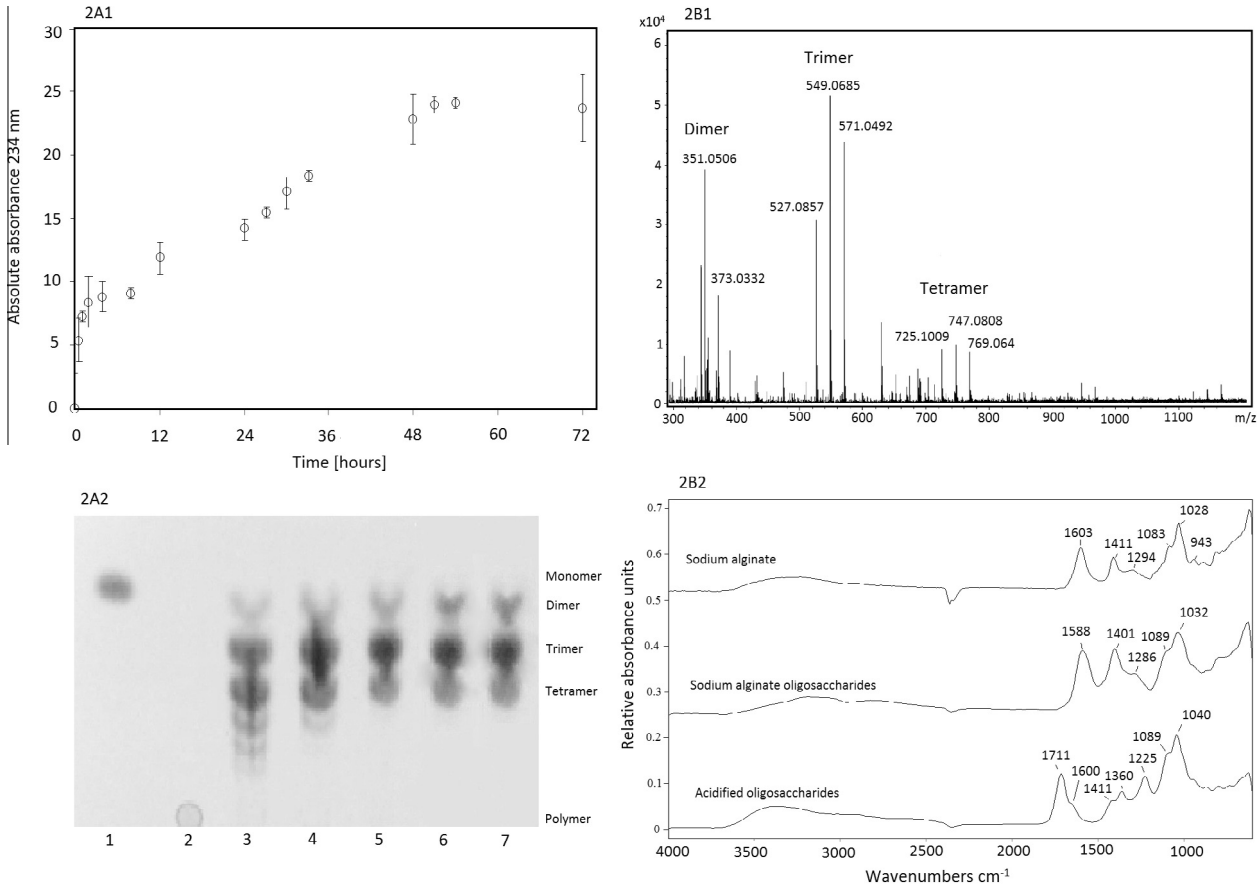

Fig. 2. Time course of alginate depolymerization by A1: spectrophotometric analyses (absorbance at 234 nm × dilution factor) and A2: TLC (Lane 1: glucuronic acid. Lanes 2–6: sodium alginate after 0, 1, 5, 23, and 72 h of depolymerization, respectively. Lane 7: Acidified AOs). Structural determinations of AOs by B1: ESI-TOF-MS and B2: FTIR.

time. After 2 h, the absorbance increased linearly (R^2 = 0.9869) at a rate of 0.32 absorbance units per hour until 48 h of depolymerization. These observations indicate that the lyase had a higher activity towards the starting material (sodium alginate) than towards the polysaccharide intermediates. After 48 h, no significant ($p < 0.05$) increase in absorbance was observed, indicating that the equilibrium oligosaccharide mixture had been reached. To ensure that this observation was not due to lyase inactivation, fresh lyase was added after 48 h of depolymerization, and no increase in absorbance was observed (data not presented). Thus, AOs with the lowest possible degree of depolymerization could be obtained from this reaction system after 48 h of depolymerization. To ensure completely similar compositions of AOs throughout the study, all samples for antioxidant analyses and structural characterizations were collected after 72 h of depolymerization.

TLC analysis of the reaction mixture at 0, 1, 5, 23 and 72 h showed that the large polymers were internally cleaved into smaller units, evidenced by the increasing TLC retention factors of the alginate during depolymerization (Fig. 2A2). The formation of polymeric intermediates with larger retention factors compared to alginate confirms that the depolymerization reaction progressed through *endo*-cleaving activity of the lyase. At the end, the mixture contained only dimers, trimers, and tetramers.

ESI-TOF-MS analysis verified that AOs obtained after 72 h of depolymerization were composed of dimers, trimers, and tetramers (Fig. 2B1). No monomers could be detected. Each oligomer gave multiple signals in the MS-spectra, depending on the counter-ion of the carboxylate group. Dimers gave signals at 351.05 m/z ($M+H^+-2Na^+$) and 373.03 m/z ($M-1Na^+$); trimers gave signals at 527.09 m/z ($M+2H^+-3Na^+$), 549.07 m/z ($M+H^+-2Na^+$), and 571.05 m/z ($M-1Na^+$); tetramers gave signals at 725.10 m/z ($M+2H^+-3Na^+$), 747.08 m/z ($M+H^+-2Na^+$), and 769.06 m/z ($M-1Na^+$); where M corresponds to the respective sodium alginate oligomer. The exact masses determined by ESI-TOF-MS confirmed the expected formation of a double bond in the non-reducing ends of the AOs. The dimers, trimers, and tetramers were hence composed of 4-deoxy-erythro-hex-4-enopyranosyluronate in the non-reducing end, followed by 1, 2, or 3 units, respectively, of mannuronate and/or guluronate in an arbitrary order (Fig. 1).

AOs obtained after 72 h of depolymerization were acidified by ion exchange. TLC analysis showed that acidification did not change the composition of the AOs (comparison of lanes 6 and 7 in Fig. 2A2), thus confirming that the low pH of the ion exchange did not hydrolyze the glycosidic linkages in the AOs.

Fig. 2B2 shows the FTIR spectra of sodium alginate, sodium AOs, and acidified AOs. In carboxylates as the sodium alginate and sodium AOs, the asymmetric stretching vibration of C=O occurs at ~1600 cm^{-1}, meanwhile in carboxylic acids as the acidified AOs, this vibration occurs at ~1700 cm^{-1} (Gomez-Ordonez & Ruperez, 2011; Yadav, 2005). This shifting is caused by the higher decrease in double bond character of the C=O by resonance in carboxylates, compared to carboxylic acids (Yadav, 2005). The positions of the C=O asymmetric stretching vibrations in the three spectra confirmed the successful acidification of the AOs (1603 cm^{-1} and 1588 cm^{-1} for sodium alginate and sodium AOs, respectively, and 1711 cm^{-1} for acidified AOs). Some absorbance in the ~1600 cm^{-1} region can be observed in the spectrum of the acidified AOs, indicating that some of the carboxyls remained on carboxylate form even after repeated ion exchange. The band in the ~1401–1411 cm^{-1} region of each spectrum is assigned to the

C—OH deformation vibrations. In the top two spectra, this band is contributed from the symmetric stretching vibrations of the C=O carboxylates (Leal et al., 2008). The symmetric C=O stretching vibration of carboxylic acids, as in the acidified AOs, do not absorb in the infrared (Yadav, 2005) and as expected, this band has lower intensity in the spectrum of acidified AOs. The band at 1225 cm^{-1} in the acidified AOs spectrum is assigned to the C—O stretching vibration of the carboxylic acid (Yadav, 2005) and the band is expectedly not present in the top two spectra. In each spectrum, two bands at ~1080 cm^{-1} and ~1030 cm^{-1} occur. These are assigned to the C—O and C—C stretching vibrations of the pyranose rings (Fenoradosoa et al., 2010; Gomez-Ordonez & Ruperez, 2011; Leal et al., 2008). The band at 943 cm^{-1} in the sodium alginate spectrum is assigned to the 1→4 glycosidic linkages (Chandia et al., 2001). In the depolymerized alginate samples, the majority of these bands have been broken, and the band is expectedly nearly undetectable in the bottom two spectra. All the saccharides have the characteristic broad absorbance band in the region 3200–3500 cm^{-1}, assignable to the hydroxyl stretching vibrations. The acidified AOs additionally show a very broad band at 2500–3300 cm^{-1}, assignable to the O—H stretching vibration of the carboxylic acid (Yadav, 2005).

To prepare mannuronate- and guluronate-rich fractions of alginate a method consisting of partial acid hydrolysis of alginate followed by pH adjustment was used. It was found that 200–300 mg polyguluronate and 270–370 mg polymannuronate could be isolated for each 1000 mg alginate hydrolyzed. According to Haug et al. (Haug et al., 1966) the homopolymeric fractions prepared this way are 80–90% pure. The isolated mannuronate- and guluronate-rich fractions, along with polymeric sodium alginate, AOs, acidified AOs, and glucuronic acid, were subjected to various colorimetric assays for the characterization of their antioxidative properties, as presented in the following.

3.2. Antioxidant properties

3.2.1. Inhibition of iron-induced lipid oxidation

The TBARS assay was used to determine the overall antioxidant activity of sodium AOs, acidified AOs, sodium alginate, glucuronic acid, and the mannuronate- and guluronate-rich fractions, by determining their ability to inhibit iron-induced lipid oxidation of emulsified linoleic acid. AOs (>50 mg/ml) were able to completely inhibit formation of TBARS in the linoleic acid emulsion (100% inhibition); in contrast to ascorbic acid, which was able to inhibit only up to 89% (Fig. 3A). A similar concentration-dependent inhibition of lipid oxidation was shown by Zhao et al. (Zhao et al., 2012). Zhao et al. showed, that AOs inhibited the formation of TBARS in an egg yolk emulsion by a factor of 1.2–3.3 compared to ascorbic acid. AOs are hence strong antioxidants and can effectively protect emulsified unsaturated fatty acids from iron-induced oxidation. To determine which functional group(s) of AOs were responsible for this antioxidant activity, comparative studies were made on polymeric and monomeric forms of alginate; on mannuronate- and guluronate-rich fractions; and on acid- and salt forms of the AOs.

Due to the high viscosity of the lipid emulsions with polymeric alginate, data points could only be collected with large deviations at lower saccharide concentrations (0–50 mg/ml) (Fig. 3B). At low concentrations (<25 mg/ml), polymeric alginate and AOs inhibited the formation of TBARS to a similar extent. At higher concentrations (25–50 mg/ml) the inhibition activity of polymeric alginate decreased. It should be noted that the data point collected at high alginate concentration was less repeatable due to the high sample viscosity. Alginate was able to inhibit the formation of up to 79% of the TBARS (at 15 mg/ml). The antioxidant mechanism of alginate is possibly radical scavenging through hydrogen abstraction from the carbon-bonded hydrogens (Hernandez-Marin & Martinez, 2012), combined with increased viscosity of the alginate emulsion. Increased viscosity of oil-in-water emulsions was shown by Sims et al. (Sims, Fioriti, & Trumbetas, 1979) to protect emulsified oil from oxidation due to the decreased diffusion rate of oxygen through the oil–water interface.

The mannuronate- and guluronate-rich fractions of alginate were subjected to TBARS analysis and the results are presented in Fig. 3C. The acid hydrolyzed alginate showed concentration-dependent oxidation inhibition, and was able to inhibit the formation of up to 91% of the TBARS. The antioxidant activity of the acid hydrolyzed alginate was comparable to that of polymeric alginate, and the antioxidant mechanism is suggested to be similar to that of polymeric alginate; noting that the viscosity of the acid hydrolyzed alginate emulsion was lower than the viscosity of the polymeric alginate emulsion. Importantly, the results presented in Fig. 3C showed that polymannuronate and polyguluronate had similar antioxidant activities. The antioxidant activity was hence independent of the relative content of mannuronate and guluronate, which is in accordance with the findings of Ueno et al. (Ueno et al., 2012). As the only structural difference between polymannuronate and polyguluronate is the orientation of the C5 carboxyl groups, it can hence be concluded, that the antioxidant activity of alginate is independent of the orientation of the C5 carboxyl groups.

Glucuronic acid (Fig. 1), which is a commercially available analogue to mannuronic acid and guluronic acid, was used as a representative of the monomeric forms of alginate. As shown in Fig. 3D, glucuronic acid increased the formation of TBARS by approximately 70% (>75 mg/ml). Mannuronic acid and guluronic acid are expected to exert a similar effect on lipid oxidation in emulsions, as this may possibly be due to the low pH of the emulsions (pH <2 for all concentrations of glucuronic acid). The pH values of the aqueous emulsion might affect the hydration state of ferrous/ferric ions, thus influencing their activity as oxidation initiators. At neutral pH, at which the analyses with sodium AOs and sodium alginate were performed, only the ferrous ions were fully soluble while the ferric ions were only partially soluble. At pH <3, the ferric ions, too, became fully soluble. Thus, the total amount of oxidation-initiating transition metals in solution was higher in the glucuronic acid emulsions. This is in accordance with the fact, that the pro-oxidative effect of glucuronic acid was concentration-independent. At concentrations higher than 75 mg/ml (where all iron ions were assumed to be soluble), a further decrease in pH did not lead to a further increase in the pro-oxidative effect of glucuronic acid. Hydrogen abstraction from the carbon-bonded hydrogens in glucuronic acid could have contributed with an antioxidant activity, which, however, was masked by the pro-oxidative activity of the low pH of the emulsion. In actual (food) products, the concentration of metal ions is much lower than the concentration employed in this accelerated oxidation study, and this study does not conclude that glucuronic acid, or mannuronic acid and glucuronic acid, act pro-oxidatively when added to lipid-containing food products.

To determine the influence of the state of the carboxyl groups as either acids or salts on the antioxidant activity of AOs, acidified AOs were prepared and analyzed by the TBARS assay for comparison. At low concentrations (⩽25 mg/ml), acidified AOs were found to have a pro-oxidative effect (Fig. 3E). As with glucuronic acid, all emulsions with acidified AOs had a pH <2, which enhanced the solubility of iron ions, which possibly explains the pro-oxidative effect of acidified AOs at low concentrations. At increasing concentrations (⩾50 mg/ml), however, acidified AOs inhibited the formation of TBARS similarly to sodium AOs (100% inhibition). This showed that AOs, irrespective of the state of their carboxyl groups, exerted an antioxidant activity, which can overcome the pro-oxidative effect of low pH.

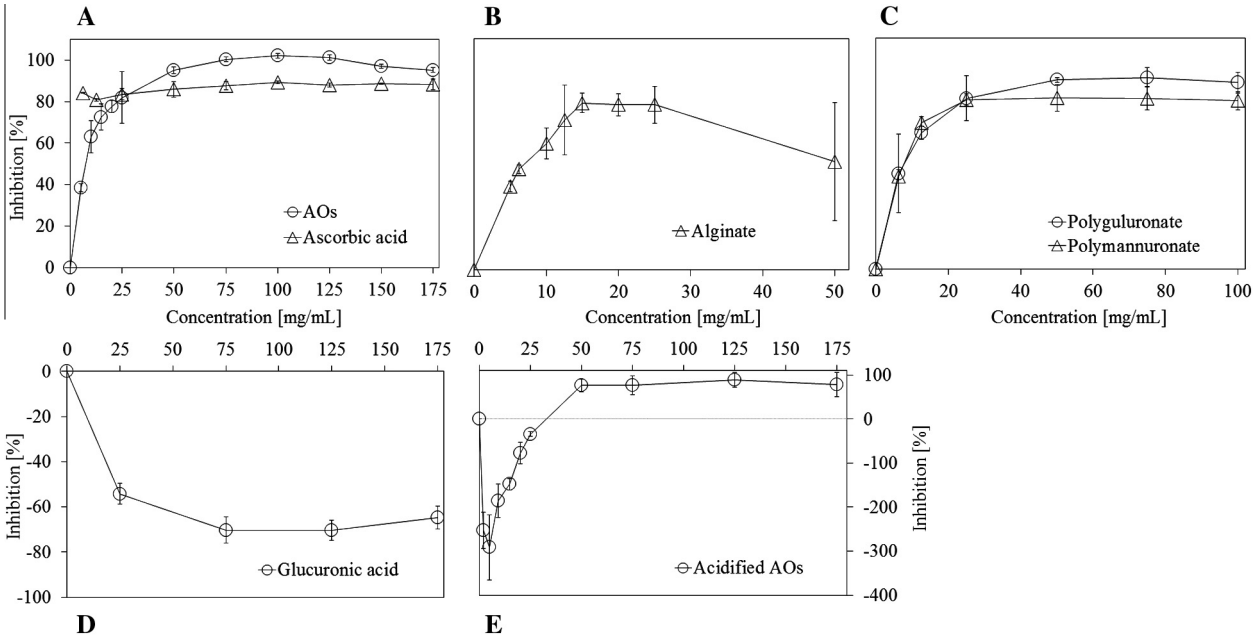

Fig. 3. Inhibition or increase (negative inhibition) of lipid oxidation *versus* saccharide or ascorbic acid concentration (TBARS).

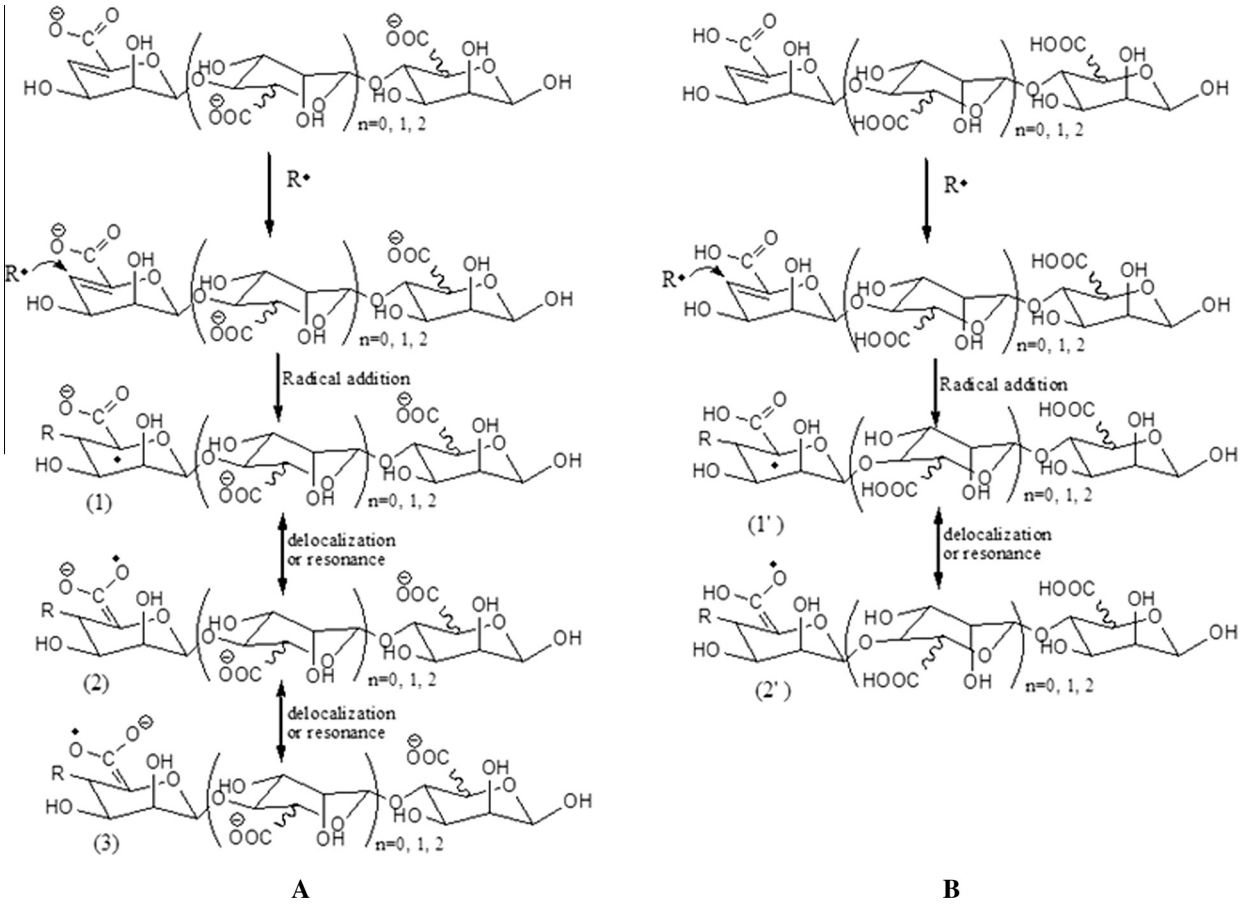

Fig. 4. Proposed mechanism for the antioxidant activity of AOs in salt form (A) and acid form (B).

To interpret the antioxidant activity of AOs, we propose a mechanism as illustrated in Fig. 4. As depicted in Fig. 4, a conjugated alkene acid structure occurred in AOs from the enzymatic depolymerization of alginate. When a radical R· is formed during lipid oxidation, it can attack the C=C and conjugate to the AOs forming a new R-AOs radical (1). This radical electron can be delocalized by resonance to R-AOs radical (2), which is in an energy-preferable state when the radical electron resides in high-electronegative oxygen. Radical (2) is very stable, and this radical can be further delocalized as R-AOs radical (3). The radicals (2) and (3) are equivalent resonance structures, and it is well known that their presence as contributing structures makes the radical intermediate very stable (Pauling, 1960). This stable radical structure cannot initiate or participate in lipid oxidation chain reactions. Neither alginate nor other mono-/polymeric alginates have conjugated alkene acid structures, which could explain the superior antioxidant activity of AOs over these structures. When AOs are present as acid form at low concentrations, it does not provide enough radical scavenging activity, and its acid property generated the pro-oxidative effect, as aforementioned. At sufficient concentrations, the AOs in acid form also exerted antioxidant activity (Fig. 3E), however, it is worthy to note that acid form R-AOs radical (2′) cannot hybrid as equivalent resonance; therefore, acid form R-AOs radicals are not as stable as salt form R-AOs radicals (Pauling, 1960).

In short, polymeric and monomeric forms of alginate, as well as acid hydrolyzed alginate (all of which lack the conjugated alkene acid structure) exhibited a lower antioxidant activity compared to AOs. This, along with the findings that the antioxidant activity was independent of the orientation of the carboxylate groups, supports the hypothesis that the antioxidant activity of AOs is enhanced due to the presence of the conjugated alkene acid structure.

3.3. Radical scavenging

The ABTS assay was used to quantify the ability of the saccharides to scavenge free radicals. Usually, the decrease in absorbance is measured a few minutes after combining the ABTS with an antioxidant, after when the absorbance stabilizes (Re et al., 1999). However, in this study it was found that the absorbance of the samples continued to decrease, even after several hours and days. The data collection was therefore continued for up to 95 h. The results are presented in Fig. 5. As shown in Fig. 5A, the absorbance remained stable during the analysis time when no scavenger was added (0 mg/ml of AOs). The AOs were found to scavenge ABTS· in a concentration and time dependent manner. For the solutions with lower AOs concentration, i.e. 1.56 mg/ml and 3.13 mg/ml, 52% and 77%, respectively, of the ABTS· were scavenged after 95 h. For the solutions with higher concentrations, >99.5% of the ABTS· were reduced to the colourless ABTS within 95 h. Assuming the average molecular weight of the AOs to be 525 g/mol (i.e. the molecular weight of the alginate trimer), these results hence showed that approximately 1.5 mole AOs could scavenge 1 mole ABTS· when allowed to react for up to 95 h ($\varepsilon_{ABTS} = 1.5 \times 10^4\ M^{-1}\ cm^{-1}$ (Re et al., 1999)). In comparison to AOs, polymeric alginate was found to have a significantly lower ABTS· scavenging capacity (Fig. 5B). After 95 h, only 23% of the ABTS· were reduced by polymeric alginate. Meanwhile, glucuronic acid was found to have no ABTS· scavenging activity (Fig. 5B), even at high concentrations (up to 100 mg/ml).

Hydroxyl radicals are extremely reactive species, which can react with and damage any biological molecule. In this study, the Fenton-type reaction was used to generate hydroxyl radicals, and the hydroxyl radical scavenging activity of the saccharides was determined using salicylic acid as molecular probe. As shown in Fig. 5C, the AOs exhibited a concentration-dependent ability to compete with salicylic acid for the reaction with hydroxyl radicals (hydroxyl radical scavenging). At the highest concentration of AOs (15 mg/ml), only 10% of the salicylic acid was hydroxylated, when defining 100% hydroxylation as the value obtained from the negative control with no scavenger. When assuming the average molecular weight of the AOs to be 525 g/mol, this result hence revealed that ~29 mM AOs (15 mg/ml) effectively protected 1 mM salicylic acid from attack by hydroxyl radicals. The scavenging ability was

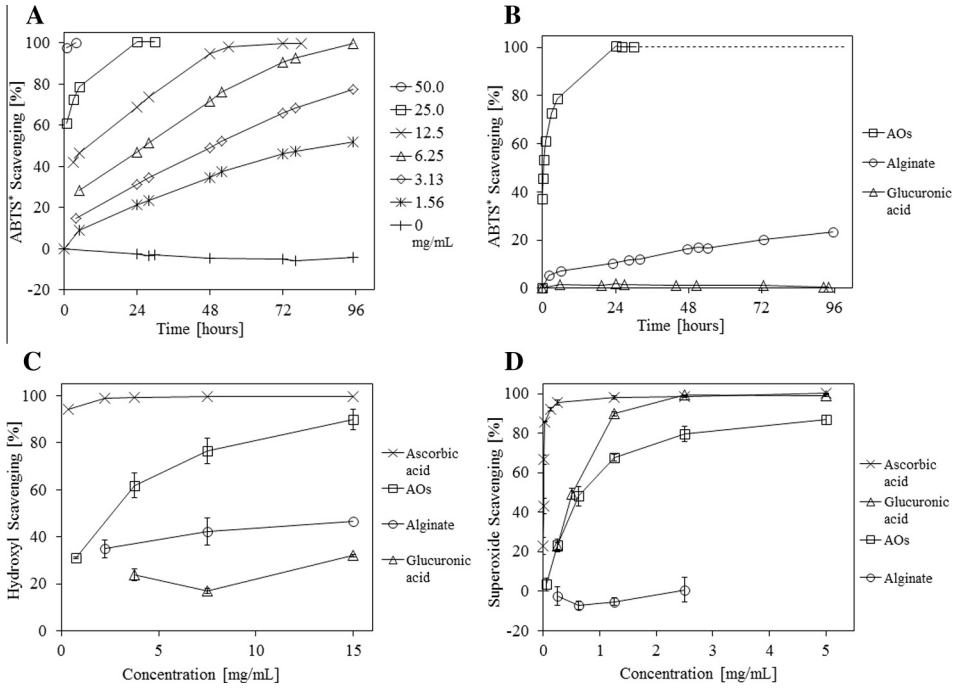

Fig. 5. Radical scavenging. (A) ABTS· scavenging of AOs at varying concentrations (mg/ml). (B) ABTS· scavenging of AOs and polymeric alginate (25 mg/ml) and glucuronic acid (100 mg/ml). The standard deviations are within the data point markers. (C) Hydroxyl radical scavenging. (D) Superoxide radical scavenging.

non-linearly concentration-dependent in accordance with the results reported by Wang et al. (Wang et al., 2007), who additionally showed that AOs (molecular weight 4388 Da) had a higher hydroxyl radical scavenging activity than chitosan and fucoidan oligosaccharides. Within the analyzed concentration range, ascorbic acid protected nearly 100% of the salicylic acid from hydroxylation. Alginate was able to scavenge up to 46% of the hydroxyl radicals, while glucuronic acid was able to scavenge only up to 23%. Hydroxyl radical scavenging by carbohydrates, in general, is suggested by Hernandez-Marin et al. (Hernandez-Marin & Martinez, 2012) to occur mainly through hydrogen abstraction from a carbon-bonded hydrogen on the carbohydrate, forming a carbon-centered radical and H_2O. Hydrogen abstraction from oxygen-bonded hydrogens is less expected due to the higher bond dissociation energy of the oxygen–hydrogen bond. Peshev et al. (Peshev et al., 2013) showed that the carbon-centered carbohydrate radicals that form after reaction with hydroxyl radicals can lead to scission into smaller carbohydrate units, as well as to random recombination to form new oligosaccharides. A similar fate is expected for the saccharides in this study; in addition to the radical stabilization by resonance schematized in Fig. 4.

As shown in Fig. 5D, AOs also showed concentration-dependent ability to compete with NBT for the reaction with superoxide radicals (superoxide radical scavenging). At the highest concentration of AOs analyzed (5 mg/ml), only 13% of the NBT was reduced to formazan, when defining 100% reduction as the value obtained from the negative control with no scavenger. Due to its high viscosity, polymeric alginate could only be analyzed at lower concentrations, at which alginate showed no superoxide scavenging activity. Within the analyzed concentration range, ascorbic acid protected nearly 100% of the NBT from reduction by superoxide. Compared to the other radical scavenging assays, glucuronic acid showed a surprisingly high superoxide scavenging activity (up to 99%). We note that the acidic nature of glucuronic acid could have inactivated the XOD and hence caused an overestimation of the decrease in formazan formation; this issue was not evaluated further in present study.

In summary, AOs showed good radical scavenging activity towards both ABTS·, hydroxyl and superoxide radicals, which can partly account for the good antioxidant properties of AOs observed in the TBARS assay. In accordance with the findings of Tsiapali et al. (Tsiapali et al., 2001), the radical scavenging activity of the polymer (alginate) was generally higher than that of the monomer (glucuronic acid). In line with Tsiapali et al. we argue that this was due to the greater ease of abstraction of the anomeric hydrogens from internal saccharides units in a polymer, compared to the anomeric hydrogen from the reducing end. The radical scavenging activity of AOs was found to be markedly higher than that of both polymeric and monomeric alginate, which indicated that the radical scavenging activity of AOs was contributed by an additional factor. We suggest that this contribution originated mainly from the conjugated alkene acid structure, to which radical addition could occur, according to Fig. 4.

3.4. Ferrous ion chelating activity

Transition metal ions are known to be catalysts of lipid oxidation, and compounds with ability to chelate transition metals ions hence possess antioxidant activity. AOs and its analogues were analyzed for their ferrous ion chelating activity by determining their ability to compete with ferrozine for chelation of aqueous ferrous ions. Fig. 6 shows the chelating activity of each saccharide and EDTA *versus* the amount of sample applied (saccharides; lower axis. EDTA; upper axis). EDTA was, as expected, able to chelate the ferrous ions in an equimolar manner, and the chelating activity was hence linearly concentration-dependent ($R^2 = 0.9881$) below

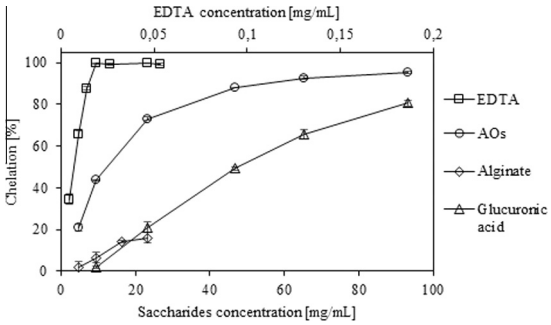

Fig. 6. Ferrous iron chelating activity *versus* saccharide (lower axis) or EDTA (upper axis) concentrations in (mg/ml).

the saturation amount (0.06 μmol – corresponding to 0.014 mg/ml) in accordance with the findings of Le et al. (Le et al., 2007). The AOs exhibited non-linear concentration-dependent ferrous ion chelating activity. 93.3 mg/ml AOs were required to chelate 95% of the 0.06 μmol ferrous ions present. By assuming the average molecular weight of the AOs to be 525 g/mol, as previously, approximately 4000–5000 mole AOs were thus required to chelate 1 mole ferrous ions. Hence, the ferrous ion chelating activity of AOs is insignificant from a food preservation point of view. This finding is in accordance with those of Wang et al. (Wang et al., 2007) and Ueno et al. (Ueno et al., 2012) who concluded that AOs and partially acid hydrolyzed alginate have no ferrous ion chelating activity.

The chelating activity observed for glucuronic acid corresponds to an estimated chelating ratio of approximately 15,000:1, and as for the AOs, this chelating effect is insignificant for antioxidant purposes.

Due to the high viscosity of polymeric alginate in water, data points could only be collected at low concentrations, at which the ferrous ion chelating activity of polymeric alginate was very low. It is well known that sodium alginate can chelate divalent metal ions (Lee & Mooney, 2012); however, according to the original findings by Haug et al. (Haug & Smidsrod, 1965), the affinity of sodium alginate for the ferrous ion is very low/undetectable, which is in accordance with present findings.

4. Conclusion

The AOs prepared in this study were found to have good antioxidant properties and were able to completely (100%) inhibit the formation of TBARS during iron-induced oxidation of emulsified linoleic acid. The degree of protection towards oxidation obtained by AOs was higher than that obtained by ascorbic acid, which was able to inhibit up to 89%. Analysis of radical scavenging activity showed that AOs could scavenge the stable free radical ABTS·, as well as the biologically relevant radicals, ·OH, and $O_2^{\cdot-}$, up to >99.5%, 90%, and 87%, respectively. Comparatively, polymeric alginate scavenged up to 23%, 46%, and <1% of the respective radicals; while monomeric alginate (represented by glucuronic acid) scavenged up to <1%, 25%, and 99%, respectively; the latter most likely being an artifact due to acidic inactivation of the xanthine oxidase. The ferrous ion chelating activity of AOs was found to be insignificant for consideration as an antioxidative mechanism. Based on these findings we conclude that the antioxidant activity of AOs mainly originates from their radical scavenging capability, which is higher compared to similar carbohydrates due to the presence of the conjugated alkene acid structure, which forms during enzymatic depolymerization. The possible mechanism of the radical scavenging may be hydrogen abstraction, presumably from the hydrogen-bonded hydrogens, combined with radical addition to

the conjugated alkene acid structure yielding an addition product stabilized by resonance. The AOs were easily prepared by enzymatic treatment of sodium alginate, which is a natural, abundant source of biomaterial. AOs can hence be considered to have potential applications in the food industry as natural antioxidants. This work gives detailed insights into the antioxidant mechanism of AOs, which are of importance in the design of antioxidant systems in e.g. food products. Furthermore, knowledge of the molecular basis of the antioxidant mechanisms allows for rational modifications of AOs in the tailoring of the antioxidant towards specific applications.

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Manuscript II

Dodecenyl Succinylated Alginate as a Novel Dual-function Emulsifier for Improved Fish Oil-in-water Emulsions

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Dodecenyl succinylated alginate (DSA) as a novel dual-function emulsifier for improved fish oil-in-water emulsions

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ABSTRACT

Alginate was chemically modified with dodecenyl succinic anhydride (SAC12) to produce an innovative emulsifier with antioxidant properties for use in fish oil-in-water emulsions. The modification reaction was conducted in an aqueous reaction system at low temperature and neutral pH. The resulting emulsifier had a degree of succinylation of 33.9 ± 3.5% and the product was structurally verified by Fourier-transformed infrared (FTIR) spectrometric analyses. The surface activity of the DSA was confirmed by contact angle determinations in static drop shape analysis on a non-polar surface. The dodecenyl succinylated alginate (DSA) was applied as stabilizer of 30% fish oil-in-water emulsions. The emulsions' creaming stability was significantly increased compared to emulsions stabilized with native alginate or β-lactoglobulin (BLG). Confocal laser scanning microscopy (CLSM) showed that DSA stabilized emulsions through adsorption to the oil-water interface, while native alginate stabilized emulsions by increasing the viscosity of the aqueous phase. Emulsions stabilized with DSA had a larger negative zeta potential compared to emulsions stabilized with native alginate or BLG, which provided repulsive forces between oil droplets, enhancing the emulsions' physical stability. DSA was found to inhibit lipid oxidation in emulsions, thus lowering the formation of secondary oxidation products by 79% compared to the control with no DSA. In all, this work presents a novel nature-based food emulsifier, which was found to have antioxidant and emulsion-stabilizing properties, thus representing a promising ingredient with application potential for efficient delivery of oxidation-susceptible oils.

1. Introduction

Increased consumption of omega-3 (n-3) polyunsaturated fatty acids (PUFAs) is associated with a number of positive health effects, and the importance of these fatty acids in human nutrition and disease prevention is scientifically recognized (Ruxton, Reed, Simpson, & Millington, 2007; Shahidi & Miraliakbari, 2004, 2005). Fish oils contain high concentrations of n-3 PUFAs, and for this reason, there is an interest in producing regularly consumed food products enriched with fish oils (Jacobsen & Nielsen, 2007; Taneja & Singh, 2012). Fish oils can be added to food products in the form of oil-in-water emulsion-based delivery systems (McClements, 2010; Taneja & Singh, 2012). Oil-in-water emulsions are heterogeneous systems of oil droplets dispersed in an aqueous continuous phase. Conventional emulsions are thermodynamically unstable and will separate into oil and water phases over time (Berton-Carabin, Ropers, & Genot, 2014; McClements, 2007). To prepare kinetically stable oil-in-water emulsions, the addition of stabilizers during the emulsification process is required. One type of stabilizer is thickening agents, which increase the viscosity of the emulsion's aqueous phase and slow the creaming of oil droplets. A second type of stabilizer is emulsifiers, which are amphiphillic molecules that can adsorb at the oil-water interface, forming a protective coating around the oil droplets, preventing aggregation, and thus stabilizing the emulsion. In food applications, a few main categories of emulsifiers are generally described, such as surfactants, phospholipids, and amphiphillic biopolymers such as proteins (Berton-Carabin et al., 2014; Hasenhuettl & Hartel, 2008).

Problems with enrichment of food with fish oils occur, as n-3 PUFAs are highly sensitive and prone to oxidation due to their high degree of unsaturation. It is hence important to inhibit oxidation of PUFAs, which can be done by the use of antioxidants, which scavenge free radicals or chelate metal ions, among other antioxidant mechanisms (Jacobsen, Let, Nielsen, & Meyer, 2008; Jacobsen & Nielsen, 2007; Shahidi & Zhong, 2010). Additionally, compared to

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bulk oil, the susceptibility of lipid oxidation in an oil-in-water emulsion is increased due to its larger interfacial area, which can promote contacts between the oil and the pro-oxidant compounds in the aqueous phase (Berton-Carabin et al., 2014; Jacobsen & Nielsen, 2007; McClements & Decker, 2000). Presence of antioxidants at the emulsion interface will hence provide optimal protection against lipid oxidation (Barreiro, Bravo-Diaz, Paiva-Martins, & Romsted, 2013; Laguerre et al., 2009). Emulsifiers are by nature present at the interface, and emulsifiers with incorporated antioxidant activity would hence provide optimal protection against lipid oxidation in emulsions. This study focused on designing and synthesizing an innovative emulsifier, which in addition to having high surface activity, possesses good antioxidant activity. An interesting starting material for such an emulsifier is alginate. Alginate is a polysaccharide originating from marine algae, composed of α-L-guluronate and β-D-mannuronate, arranged as linear homopolymeric and heteropolymeric blocks (Pawar & Edgar, 2012), which previously have been shown to have antioxidant properties (Falkeborg et al., 2014). This work presents the modification of alginate with dodecenyl succinic anhydride (SAC12) (Fig. 1) to produce an innovative emulsifier, DSA, with antioxidant properties. The new emulsifier was characterized for its interfacial activity and for its antioxidant properties, and the properties were compared to native alginate, as well as to β-lactoglobulin (BLG) representing an emulsifier commonly known for its antioxidant activity (Berton-Carabin, Genot, Gaillard, Guibert, & Ropers, 2013). To evaluate the emulsion-stabilizing properties of DSA, simple oil-in-water emulsions were prepared, which were characterized by confocal laser scanning microscopy (CLSM), dynamic light scattering (DLS), zeta potential determinations, and creaming stability studies.

2. Materials and methods

2.1. Materials

Sodium alginate (Grindsted® Alginate FD 170) was provided by DuPont, Brabrand, Denmark. This alginate originated from brown algae, and the ratio of α-L-guluronate units to β-D-mannuronate units was 40–60. Refined fish oil from cod liver (inOMEGA3™ 100, sourced and manufactured in Norway) was a gift from inOmega3, Denmark. β-lactoglobulin (BLG) was provided by Arla Foods, Videbaek, Denmark. Linoleic acid 96% was obtained from Zhongchuan Biotechnology Co Ltd. Anqing, China. 2-dodecen-1-yl succinic anhydride (SAC12), Nile Red ($C_{20}H_{18}N_2O_2$), Rhodamine 6G 99% ($C_{28}H_{31}N_2O_3Cl$), Tween® 20, trichloroacetic acid, thiobarbituric acid (TBA), potassium hydroxide (KOH), ferrous sulfate ($FeSO_4$), sodium salicylate >99.5% ($C_7H_5NaO_3$), phosphate buffered saline (PBS) 0.01 M pH 7.4 (foil pouch), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$), and hydrogen peroxide (H_2O_2), were purchased from Sigma–Aldrich.

2.2. Preparation of dodecenyl succinylated alginate (DSA)

Dodecenyl succinylation of alginate was performed with inspiration from the method of Rao, Prakasham, Rao, and Yadav (2008) and Le-Tien, Millette, Mateescu, and Lacroix (2004). Succinylation was performed in a 250 mL screw-capped flask thermostated at 30 °C with circulating water. Alginate (750 mg) was dissolved in 75 mL H_2O, followed by addition of 4.035 g SAC12, corresponding to a 4-times molar excess of SAC12 with respect to alginate monomeric units (i.e. sodium guluronate and mannuronate units with molecular weights 198.01 g/mole). The dodecenyl succinylation was conducted for 4 h with magnetic stirring at 400 rpm. The pH was adjusted hourly to 7.0 ± 0.3 using 3% NaOH. The DSA was isolated by precipitation in 300 mL ethanol and centrifugation at 8000 rpm for 20 min at 20 °C. Unreacted SAC12 and water were removed by washing four times with 200 mL acetone, and the resulting DSA was finally dried at room temperature for 48 h. The degree of succinylation (DS) was determined by titration (Section 2.3) and the modification was structurally confirmed using Fourier transformed infrared spectrometry (FTIR) (Section 2.4).

2.3. Degree of succinylation (DS)

The DS was determined with inspiration from the acidification-based method reported by Plate, Diekmann, Steinhauser, and Drusch (2012), by performing the dodecenyl succinylation of alginate downscale according to the procedure described in Section 2.2 (100 mg alginate was modified with 538 mg SAC12). The washed DSA was then dispersed in 10 mL 2.5 M HCl in isopropanol (IPA) and shaken for 10 min to convert all carboxylate groups to their acid form. Additional 20 mL IPA was added and the acidified DSA was centrifuged at 4000 rpm at 17 °C for 10 min. The precipitated DSA was washed 3 times with 40 mL IPA to remove excess acid, and then dispersed in 30 mL H_2O. The carboxyl groups were titrated with 0.1 M NaOH using phenolphthalein as an indicator. During titration, the acidified DSA slowly dissolved, and the titration volume was recorded when the DSA was completely dissolved and the pink indicator color was permanent. One hundred mg alginate was acidified and titrated correspondingly as a control. The DS was quantified as the increase in free carboxyl groups compared to unmodified alginate, divided by the number of available hydroxyl groups in the alginate, according to Equation (1), where V is the titration volume for DSA, and $V_{control}$ is the titration volume for alginate. The number of available hydroxyl groups in the alginate was defined as double the number of carboxyl groups in the native alginate.

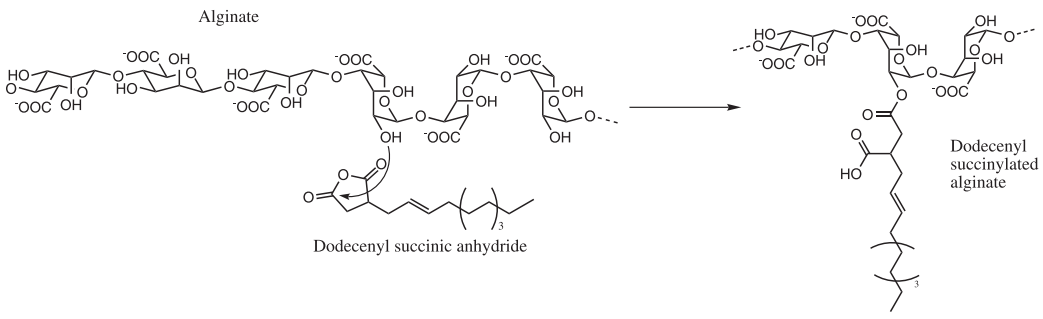

Fig. 1. Reaction scheme of dodecenyl succinylation of alginate.

$$DS = 0.5*\frac{V - V_{control}}{V_{control}}*100 \quad (1)$$

The DS was determined as the average of duplicate titrations.

2.4. Confirmation of structure modification by FTIR

FTIR spectra were recorded in absorbance mode in the 4000–650 cm^{-1} region at a resolution of 4 cm^{-1}, using a Qinterline QFAflex spectrometer equipped with a deuterium triglycine sulfate detector. The DSA was mounted in its pure solid form in a Pike attenuated total reflectance (ATR) device thermostated at 25 °C. The spectra were ratioed against a single-beam spectrum of the clean ATR crystal. Native alginate and SAC12 was analyzed correspondingly. The resulting spectra were analyzed using GRAMS/AI software.

2.5. Static drop shape analysis

The interfacial tension-lowering effect of DSA was evaluated by contact angle determinations through static axisymmetric drop shape analyses. An aqueous solution of DSA (2%) was prepared and left overnight at room temperature for complete hydration. Contact angles of drops (10 ± 1 μL) of the aqueous DSA solution on a non-polar surface were studied using a Krüss DSA10 goniometer. The surface-drop contact angles were determined approximately 15 s after the drops were placed on the non-polar surface. The nature of the non-polar surface is described in detail by Holberg and Bischoff (2014) ("coating G"). This surface was chosen, as it resembles the non-polarity of oils. The contact angles were hence measures of the tension-lowering effects of DSA in interfaces between an aqueous phase and a non-polar phase, with resemblance to common oil-in-water emulsions. Aqueous solutions of native alginate and BLG (2%) were analyzed correspondingly for comparison, and pure water was used as control. The contact angles were determined at room temperature with five repeats for each sample.

2.6. Application of DSA in oil-in-water emulsions

DSA was evaluated as stabilizer of model fish oil-in-water emulsions and compared to native alginate and BLG. Emulsions consisting of 30.0% (w/w) fish oil, 3.0% (w/w) stabilizer (DSA, native alginate, or BLG), 0.05% (w/w) sodium azide, and 66.95% deionized water were prepared. The stabilizer and sodium azide were dissolved in water, and this aqueous solution was heat-treated at 140 °C for 2 min to enhance the solubility of the stabilizers and to partly denature the BLG, followed by cooling to 5 °C. The fish oil was then added during 1 min under magnetic stirring at 1500 rpm (Stuart Stir SB161). Stirring was continued for an additional three minutes at 1500 rpm after the addition of oil. All emulsions were prepared in batch sizes of 10 g and cooled during preparation using crushed ice.

2.6.1. Confocal laser scanning microscopy (CLSM)

The emulsions stabilized with native alginate and DSA were visualized by CLSM using a Zeiss LSM 510 Meta confocal microscope to evaluate their microstructure (droplet size distribution) and the distribution of stabilizer in the emulsions. For imaging of the lipid phase, 10 μL Nile Red solution (1 mg/mL in acetone) was added to 1 mL emulsion. For imaging of the stabilizer (in separate emulsions), 10 μL Rhodamine 6G aqueous solution (1 mg/mL) was added to 1 mL emulsion. The emulsions were mixed gently to disperse the fluorescent probes evenly. The CLSM images were collected at room temperature using a 20× objective. Nile Red molecules were excited at a wavelength of 488 nm and the fluorescence emission intensity was collected over 575–625 nm. Rhodamine 6G was excited at a wavelength of 480 nm and the fluorescence emission intensity was collected at 525–560 nm. All emulsions were analyzed in duplicates. The resulting images were analyzed using Zeiss LSM Image Browser software version 4.2.0.121.

2.6.2. Emulsion characteristics (droplet size, zeta potential, pH)

The oil droplet size distribution and zeta potential were determined in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) at 25.0 °C. The emulsions were analyzed immediately after preparation and were diluted three times 1:10 in PBS buffer prior to analysis. No apparent destabilization of the emulsions were observed upon dilution during the short timespan between dilution and analysis. The zeta potential was determined as an average of three measurements. The size distribution of oil droplets was determined by DLS using non-invasive backscatter optics. The size distribution was determined as an average of three determinations on duplicate emulsions. The pH of all emulsions was determined in duplicates using a pH-meter (inoLab pH 7110, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany).

2.6.3. Effect of stabilizer on creaming stability

To examine their creaming stability, the emulsions were transferred to clear, screw-capped 15 mL vials (diameter 1.8 cm) and stored at 5 °C. By aid of daily digital photographs, the phase separation in each emulsion was monitored over 11 days. The extent of creaming was characterized by the creaming index (CI) according to Equation (2), where H_A is the height of the lower, aqueous phase, and H_E is the total height of emulsion (McClements, 2007).

$$CI = 100*\frac{H_A}{H_E} \quad (2)$$

Each data point was determined as an average of two measurements.

2.7. Antioxidant activity

2.7.1. Inhibition of lipid oxidation in emulsions

The ability of DSA to inhibit lipid oxidation in emulsions was evaluated by a modified version of the TBA reactive species (TBARS) assay as described by Zhao, Li, Xue, and Sun (2012). To minimize the influence of other factors, such as oil droplet sizes, on the oxidative stability of emulsions, a pre-emulsion was formed by mixing linoleic acid (2%) and Tween®20 (1%) in pure water. From this pre-emulsion, 50 μL was dispersed in 1.5 mL aqueous solution of stabilizer (25 mg/mL). Oxidation was accelerated by the addition of 250 μL 25 mM $FeSO_4$, and the emulsions were incubated with magnetic stirring at 200 rpm for 15 min at room temperature. The formation of secondary oxidation products was then detected by the addition of 2.5% trichloroacetic acid (0.5 ml) and 0.7% TBA in 0.05 M KOH (1 ml) to each emulsion, which were then incubated at 100 °C for 15 min. Following that, the emulsions were cooled at room temperature for 10 min and centrifuged at 4000 *g* for 1 min. TBA formed colored complexes with the secondary oxidation products, which were detected in a UV–visible spectrophotometer (Cary 50Bio, Varian, Australia) at 532 nm. The degree of oxidation in each emulsion was calculated with respect to the degree of oxidation in emulsions with no stabilizer added (i.e., only Tween®20), according to Equation (3), where A_S is the absorbance of samples with added stabilizer, and A_0 is the absorbance of samples with no added stabilizer.

$$Oxidation\ [\%] = \frac{A_S}{A_0}*100 \quad (3)$$

Native alginate and BLG were analyzed correspondingly for comparison. All data points were collected as averages of double spectrophotometric measurements of triplicate samples.

2.7.2. Hydroxyl radical scavenging

The capacity of DSA to scavenge hydroxyl radicals was examined by detecting its competition with salicylic acid for the reaction with hydroxyl radicals, as described previously (Falkeborg et al., 2014). The hydroxyl radicals were generated from H_2O_2 through the Fenton reaction in a reaction mixture containing 0.6 mM H_2O_2, 0.6 mM $FeSO_4$, and 1 mM sodium salicylate, and varying concentrations (0–10 mg/mL) of DSA (total volume 0.7 mL). The reaction mixture was incubated at 37 °C for 20 min after when the absorbance at 510 nm was determined in a UV–visible spectrophotometer (Cary 50Bio, Varian, Australia). The absorbance of negative controls, prepared by replacing salicylic acid with pure water, were withdrawn the absorbance of the respective samples. This difference in absorbance, at each sample concentration, was notated A_S, and the hydroxyl scavenging activity was calculated according to Equation (4), in which A_0 refers to the absorbance of a control with no scavenger.

$$Hydroxyl\ scavenging\ [\%] = \frac{A_0 - A_S}{A_0} * 100 \quad (4)$$

Native alginate and BLG were analyzed correspondingly for comparison. All data points were collected as averages of double spectrophotometric measurements of duplicate samples.

2.7.3. ABTS radical scavenging

The ability of DSA to scavenge ABTS radicals (ABTS*) was examined using the modified procedure of Re et al. (1999). ABTS* was generated by incomplete oxidation of ABTS with $K_2S_2O_8$ by combining equal volumes of 7 mM ABTS and 2.45 mM $K_2S_2O_8$ in deionized water, followed by incubation in the dark for 24 h. The resulting ABTS* solution was diluted in pure water to give the final ABTS* solution an absorbance of $A_{734nm} = 0.700 \pm 0.020$. DSA was dissolved in deionized water to a concentration of 25 mg/mL, and 50 μL of this solution was added to 1 mL ABTS* solution and carefully mixed. The decrease in absorbance at 734 nm was measured over 30 min at room temperature. The percentage of ABTS* scavenged at each time point was calculated according to Equation (5), in which $A_{INITIAL}$ refers to the initial absorbance ($A_{734nm} = 0.700 \pm 0.020$) and A_S refers to the absorbance after a given time of reaction with a given concentration of saccharide.

$$ABTS\ scavenging\ [\%] = \frac{A_{INITIAL} - A_S}{A_{INITIAL}} * 100 \quad (5)$$

Native alginate and BLG were analyzed correspondingly for comparison. Samples were analyzed in duplicates, and duplicate spectrophotometric measurements were made for each sample.

2.8. Statistical analysis

Data processing was performed in Microsoft Excel 2013. All measurements were conducted in duplicates/triplicates, and the results are reported as means ± standard deviations. One-way analysis of variance (one-way ANOVA) was performed using Microsoft Excel Analysis Toolpak (2010) to identify significant differences between groups ($p < 0.05$).

3. Results

3.1. Dodecenyl succinylation of alginate

Alginate was modified with SAC12, and the degree of succinylation in the final DSA was determined by titration to be 33.9 ± 3.5%. The successful succinylation was confirmed by FTIR analysis (Fig. 2). The DSA showed bands assignable to the sodium alginate backbone, as well as bands assignable to the alkane chains originating from the dodecenyl substitutions. The bands at 2925 cm^{-1} and 2854 cm^{-1} in the DSA are assigned to the alkane moieties (CH_3 and CH_2) of the dodecenyl substitutions, and corresponding bands are also seen in the spectra of the substrate SAC12 (at 2955 cm^{-1}, 2915 cm^{-1}, and 2850 cm^{-1}). In the spectrum of DSA, the C=O asymmetric stretching vibrations of the carboxylates in alginate, the free acids in the substitutions, and the ester bonds between alginate and SAC12, are not well separated, but can be identified at 1596 cm^{-1}, 1649 cm^{-1}, and 1728 cm^{-1}, respectively. The band of the carboxylate asymmetric stretching vibrations (1603 cm^{-1}) is expectedly also present in the native alginate. In the spectra of native alginate and DSA, two bands at ~1080 cm^{-1} and ~1030 cm^{-1} occur. They are assigned to the C–O and C–C stretching vibrations of the pyranose rings in alginate (Falkeborg et al., 2014). In the spectrum of SAC12, distinct bands can be assigned to the anhydride moiety, namely, 1855 cm^{-1} and 1781 cm^{-1} (C=O) and 1070 cm^{-1} (C–O). They are as expected absent in the spectrum of DSA, demonstrating that no anhydride moieties were present in the final DSA product. The bands at 943 cm^{-1} and 948 cm^{-1} in the spectra of

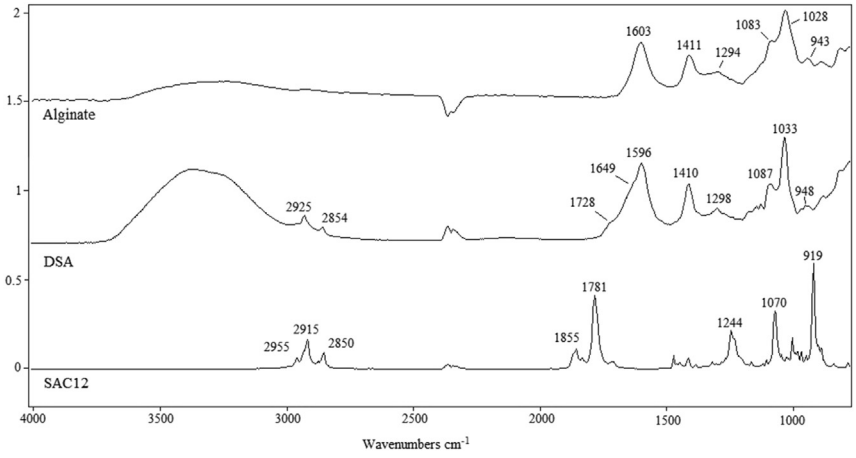

Fig. 2. FTIR spectra of native alginate, DSA, and SAC12.

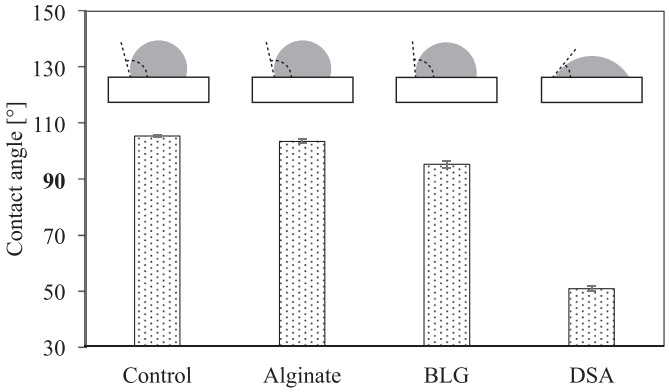

Fig. 3. Contact angles between drops of aquoues solutions (2%) of DSA, native alginate, and BLG, on a non-polar surface, compared the contact angle of pure water (control).

native alginate and DSA, respectively, are assigned to the 1 → 4 glycosidic linkages in the alginate (Chandia, Matsuhiro, & Vasquez, 2001). The DSA and the native alginate both have the characteristic broad absorbance band in the region 3200–3500 cm^{-1}, assignable to the hydroxyl stretching vibrations. The intensity of this band is higher in the DSA, as it is contributed from the O–H stretching vibration of the free carboxylic acids (Yadav, 2005).

3.2. Interfacial characteristics

The interfacial tension-lowering effects of DSA between an aqueous phase and a non-polar oil-resembling surface was evaluated by static drop shape analysis. The contact angles are presented in Fig. 3. The control was based on pure water, and as expected, the non-polar surface repelled the water (contact angle > 90°). The contact angle was only slightly lowered by native alginate (103.44 ± 0.64° versus 105.36 ± 0.40° for pure water), showing, as expected, that native alginate do not possess interfacial activity. BLG lowered the contact angle to 95.20 ± 1.35°; the BLG solution was thus slightly repelled by the non-polar surface, too. The DSA solution, however, gave rise to a contact angle of 50.68 ± 0.84°, which was significantly lower than the comparisons, and importantly, significantly lower than 90°, showing good wetting of the non-polar surface by the aqueous DSA solution, and thus good interfacial activity of the DSA. This interfacial activity is further characterized by analyses of the oil-in-water emulsions, as presented in the following.

3.3. Application in fish oil-in-water emulsions

Emulsions with 30% fish oil were prepared by employing low-energy input from magnetic stirring to mix the ingredients. All emulsions appeared white and homogenous upon preparation. The emulsions stabilized by native alginate were very viscous, resembling mayonnaise, while emulsions stabilized by DSA resembled whole milk. The alginate-based emulsions were characterized using CLSM (Fig. 4), which showed that the average oil droplet size was significantly smaller in the emulsions stabilized by DSA (Fig. 4B) compared to the emulsions stabilized by native alginate (Fig. 4A). Using the Zeiss LSM Image Browser software, the largest oil droplets in the emulsions stabilized with native alginate were determined to be up to 45 μm in diameter, while for emulsions stabilized with DSA, the largest oil droplets were only up to 15 μm. Furthermore, the droplet size distribution was apparently narrower in the emulsions stabilized with DSA. Oil droplets in both emulsions appeared well separated without indication of droplet flocculation.

The CLSM images using Rhodamine 6G as fluorescent probe provided insights into the stabilizing mechanisms of DSA and native alginate. Fig. 4C shows that native alginate distributed almost homogeneously in the aqueous phase of the emulsion and did not pack at the oil-water interface. Native alginate forms viscous solutions in water, and accordingly, the viscosity of the system increased significantly when native alginate was used as

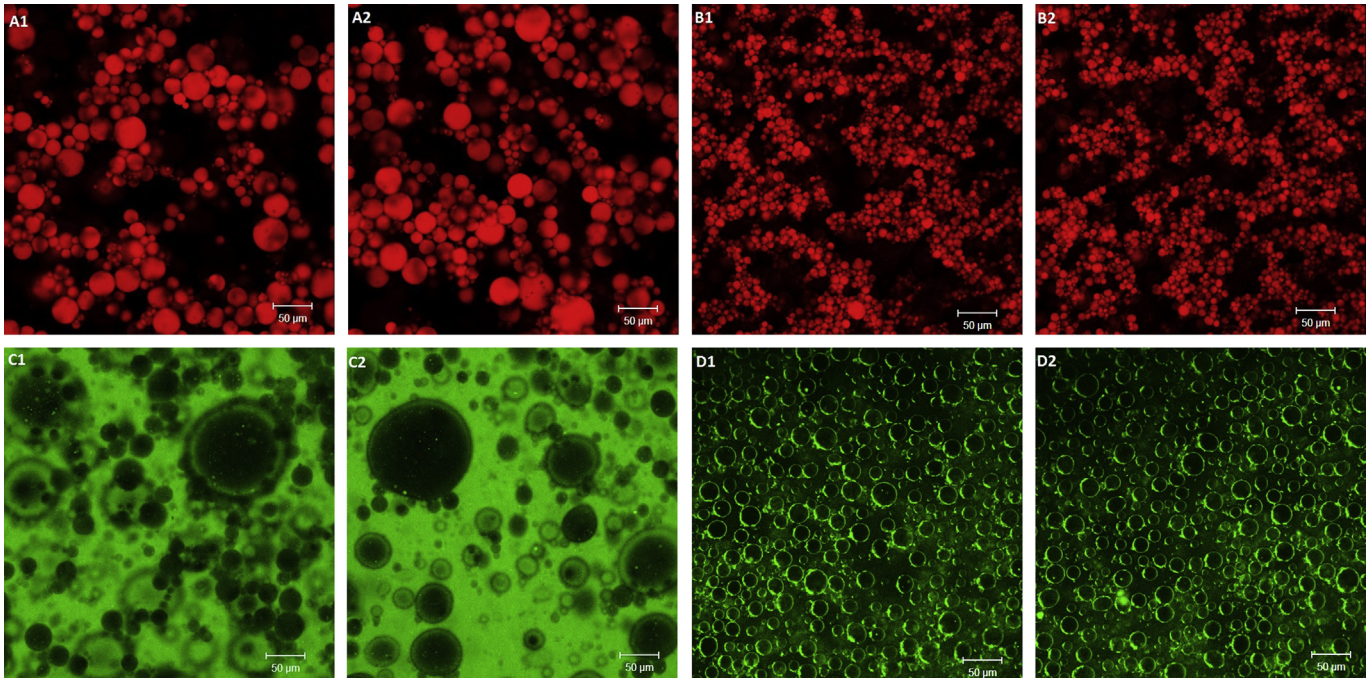

Fig. 4. CLSM images of emulsions with native alginate (A and C) and DSA (B and D) with Nile Red for oil phase imaging (A and B) and Rhodamine 6G for alginate imaging (C and D) of duplicate emulsions (1 and 2). Scalebars are 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Emulsions' characteristics.

Stabilizer	Mean droplet size (d_{43}) (μm)	PDI	pH	Zeta potential (mV)
DSA	7.64 ± 1.06	0.483 ± 0.239	6,7	−32.1 ± 0.9
Alginate	22.02 ± 10.16	0.784 ± 0.216	6,1	−22.8 ± 0.8
BLG	3.50 ± 1.18	1 ± 0	6,7	−11.6 ± 1.1

stabilizer. Hence, native alginate stabilized the emulsions mainly through its action as a thickening agent, increasing the viscosity of the emulsions' aqueous phase and slowing the creaming of oil droplets. DSA, on the other hand, was present almost exclusively in the interfacial region of the emulsions, coating the surface of the oil droplets (Fig. 4D). Only low amounts of DSA could be readily observed in the aqueous phase of the emulsions, showing that the majority of the emulsifier (3% of the total emulsion) was adsorbed at the oil-water interface.

3.3.1. Droplet size distribution, zeta potential, and pH

The oil droplet sizes were additionally determined by DLS analyses (Table 1). The size distribution was generally very broad for all emulsions, as indicated by the high PDI values and observations from the CLSM images, which decreases the accuracy of droplet size distribution determinations by DLS. The results from the DLS analyses should hence only be considered an additional characterization of the droplet size information gained from the CLSM images.

Emulsions stabilized with BLG had the smallest droplet sizes; however, it should be noted, that larger droplets were observed in the sample cell, which ascended due to their lower density, and were hence not included in the analyzed part of the sample. The use of DSA over native alginate gave rise to significantly smaller oil droplets (7.64 ± 1.06 μm compared to 22.02 ± 10.16 μm), which is consistent with the observations from CLSM.

The pH of all emulsions was slightly below neutral (Table 1). The zeta potential was negative in all emulsions, and significant differences were observed between them (Table 1). Alginate has carboxylate groups in its structure, giving rise to the observed negative surface charge. DSA has additional carboxyl groups originating from the modification (Fig. 1) and the emulsions stabilized with DSA were expectedly more negative (−32.1 ± 0.9 mV) compared to the emulsions stabilized with native alginate (−22.8 ± 0.8 mV). Emulsions stabilized with BLG had a medium negative zeta potential (−11.6 ± 1.1 mV), owing to the fact that the pH of the emulsion (6.7) was higher than the isoelectric point of BLG (5.2) (Majhi et al., 2006) hence giving the protein a net negative charge.

3.3.2. Creaming stability

The creaming stability of the emulsions was evaluated by determining the phase separation over time (Fig. 5). None of the emulsions were completely physically stable during the storage time, and their thermodynamic instability led to phase separation and increase of creaming indices to varying extents. The emulsions stabilized with BLG were very unstable, and phase separation occurred within just one day of storage at 5 °C, and reached a final creaming index of 54 after 11 days. In the emulsions stabilized with BLG, coalescence of oil droplets during storage led to formation of a clear oil layer on top of the emulsions (oiling off) (McClements, 2007), followed by an emulsified phase with a clear boundary to the lower aqueous phase. Excess protein clearly layered at the interface between the lower aqueous layer and the emulsified layer. The emulsions stabilized with native alginate were more stable, but after 11 days of storage, the creaming index had increased to 35. A narrow oil layer was observed on top of the emulsions, indicating oil droplet coalescence in addition to creaming. The emulsions stabilized with DSA showed good creaming stability during storage, and after 11 days, the creaming index was only 8, and no oil layer on top was observed.

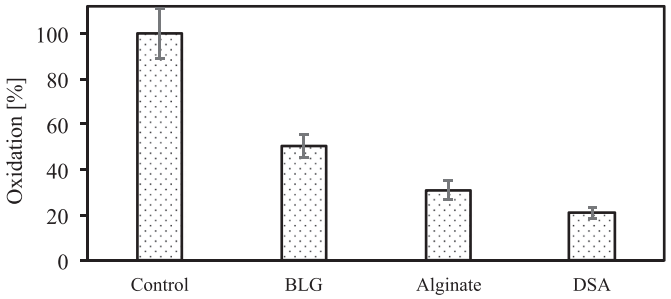

Fig. 6. Inhibition of oxidation of emulsified linoleic acid by DSA, native alginate, and BLG, compared to pure water (control, defined as 100% oxidation).

3.4. Antioxidant activity

The DSA was examined for its antioxidant activity, in terms of its ability to inhibit lipid oxidation in an emulsion, and ability to scavenge hydroxyl radicals and ABTS radicals.

Emulsions were formed using Tween® 20 as (co-) emulsifier, in order to minimize the effects of droplet sizes on the extent of lipid oxidation. Fish oil was furthermore replaced with linoleic acid to enhance the susceptibility towards oxidation. As shown in Fig. 6, all the stabilizers (BLG, native alginate, and DSA) showed inhibitory effects towards oxidation of the emulsified linoleic acid. Under the conditions employed in this study, the presence of BLG led to a decrease in oxidation to a final 50.3 ± 5.1% with respect to the degree of oxidation observed in emulsions with no co-emulsifier. The alginate showed additional decreases in degree of oxidation (31.0 ± 4.3%); and when using DSA, the oxidation was only

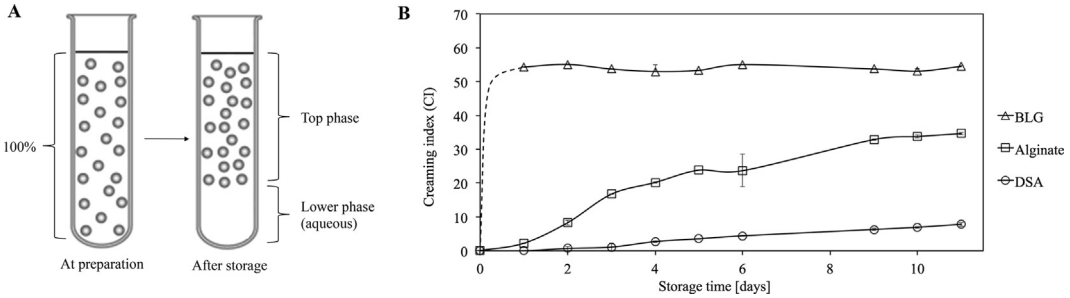

Fig. 5. Emulsion stability. A: Illustration of emulsions in vials at preparation (100% emulsified phase) and after storage at 5 °C with phase separation. The creaming index (CI) is noted daily and plotted in B.

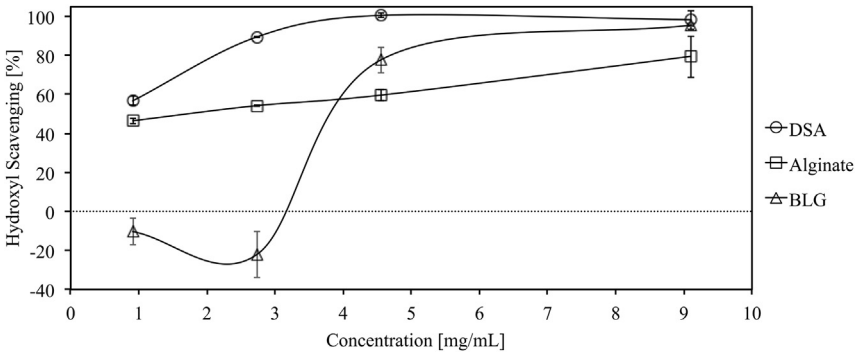

Fig. 7. Hydroxyl scavenging of DSA, native alginate, and BLG.

20.8 ± 2.7% relative to the degree of oxidation with no co-emulsifier.

To examine the hydroxyl radical scavenging activity of DSA, the Fenton-type reaction was used to generate hydroxyl radicals and salicylic acid was used as molecular probe. The hydroxyl radical has biological relevance as it is a known intermediate in lipid peroxidation, and effective scavenging of this reactive radical species can hence prevent or delay the oxidation progress. As shown in Fig. 7, DSA exhibited a concentration-dependent ability to compete with salicylic acid for the reaction with hydroxyl radicals (hydroxyl radical scavenging). At 2.7 mg/mL of DSA, only 11% of the salicylic acid was hydroxylated, when 100% hydroxylation is defined as the value obtained from the negative control with no scavenger. At 4.6 mg/mL, DSA completely (100%) inhibited the hydroxylation of salicylate. DSA was a superior hydroxyl scavenger to the native alginate. At maximum concentration tested, the native alginate inhibited 79% of the hydroxylation of salicylate. In a previous study (Falkeborg et al., 2014), native alginate showed the same trend in concentration-dependent ability to scavenge hydroxyl radicals, although to a smaller extent than observed in the present study. Slightly different operating procedures, acceptable day-to-day variations of the assay, and the fact that alginate is a natural material with batch-to-batch variations, can explain this difference. As can be seen in Fig. 7, DSA had been endowed with increased hydroxyl radical scavenging activity through the modification with SAC12. This could be caused by an increased ability of DSA to act as an acceptor in radical addition reactions, possibly due to the presence of very electronegative carboxyl groups in the modification moiety (Fig. 1). For comparison purposes, BLG was examined for its hydroxyl radical scavenging activity. At low concentrations, BLG was found to promote the hydroxylation of salicylate; while at higher concentrations, BLG showed hydroxyl radical scavenging activity and protected salicylate from hydroxylation to a similar extent as DSA.

Fig. 8 shows the ability of native alginate, DSA, and BLG, to scavenge ABTS radicals. The trend in ABTS* scavenging was opposite to the trend seen for hydroxyl radical scavenging. BLG showed the highest ability to scavenge ABTS* (up to 66%), while DSA only scavenged up to 3% of the ABTS* in 30 min. Generally, ABTS radicals can be reduced by hydrogen-donating antioxidants (Re et al., 1999) and hence only this type of radical scavenging activity can be determined. The radical scavenging activity resulting from the ability to act as an acceptor in radical addition reactions cannot influence this assay. It can be concluded that DSA possessed lower hydrogen-donation activity compared to native alginate. The fact that DSA had a higher activity towards hydroxyl radicals compared to native alginate must be a result of its increased ability to scavenge free radicals by acting as an acceptor in radical addition reactions. By either mechanism, less reactive radicals are formed that have lower probability of participating in lipid oxidation reactions.

4. Discussion

The modification of alginate with SAC12 was performed with inspiration from commercially accepted methods generally used to prepare modified starch (Phillips, Xing, Chong, Liu, & Corke, 2000; Sweedman, Tizzotti, Schafer, & Gilbert, 2013). The succinylation method has also been adopted to the modification of alginate with succinic anhydrides (no dodecenyl chain) for the preparation of a material for immobilization of bacteria (Le-Tien et al., 2004; Rao et al., 2008). The modification procedure involved an aqueous reaction medium and pH neutral conditions, and the reaction was performed at low temperature in a short reaction time. Reaction parameters such as temperature, substrate molar ratio, and

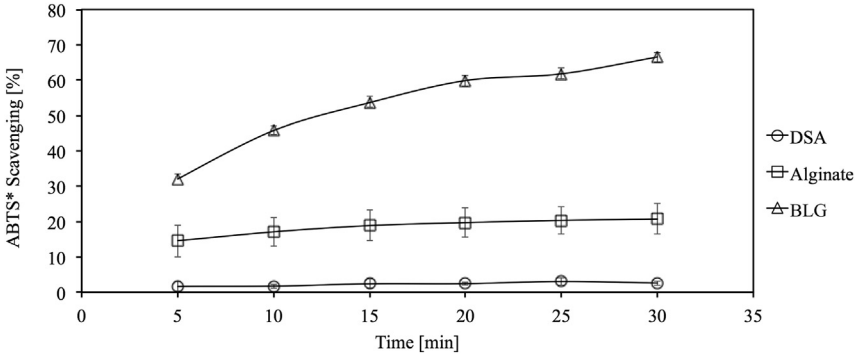

Fig. 8. ABTS* scavenging of DSA, native alginate, and BLG.

reaction time, were optimized prior to the present study to achieve an as high degree of succinylation as possible. Under the optimized conditions, the degree of succinylation in the final DSA was determined by titration to be 33.9 ± 3.5%, which interprets to that, on average, two out of three monomeric unit of alginate was modified with one hydrophobic moiety.

The use of DSA as a stabilizer of fish oil-in-water emulsion was compared to native alginate, and to BLG representing an emulsifier widely used in foods. CLSM images showed that the DSA located primarily in the oil-water interface of the emulsions, whereas native alginate was located primarily in the aqueous phase. Similar observations were made by Yang et al. (Yang, Jiang, He, & Xia, 2012), who esterified the carboxyl groups in alginate with dodecenyl alcohols. They showed that this type of modified alginate provided increased stability to oil-in-water emulsions compared to alginate, due to its ability to adsorb to the oil-water interface. In addition to Yang et al. (2012) a few others have reported similar types of hydrophobic modifications onto the carboxyl group of alginate (Broderick et al., 2006; Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000). However, in none of them, like in this work, is alginate hydrophobically modified onto the hydroxyl groups, or leading to the introduction of extra carboxyl groups. Generally, the methods reported for hydrophobic modification of alginate involve the use of organic solvents and potentially toxic reagents and coupling agents. The dodecenyl succinylation of alginate provides a green alternative to these methods, as the reaction takes place in an aqueous reaction media at low temperature and neutral pH. This is particularly attractive when the emulsifier is intended for use in food products.

The creaming stability of emulsions stabilized with DSA was increased compared to emulsions stabilized with native alginate or BLG. The increased creaming stability was caused by the surface activity of the DSA, which decreased the oil droplet sizes, as well as by the large negative zeta potential, which provided stabilizing repulsive forces between the oil droplets, preventing their aggregation (McClements, 2007). When comparing the creaming stability of these emulsions to values reported in literature it is important to note, that all emulsions in this study were prepared without input from high-energy sources such as high-pressure homogenization or ultrasound. Applying such techniques is naturally expected to decrease the average oil droplet size and thus increase the emulsions' creaming stability.

The CLSM images show that the oil droplet surface coverage by DSA was very high, and that the interfacial layer between the oil droplets and the aqueous phase was thick. When the calculations are based on the average oil droplet size found in CLSM and DLS analyses, i.e. from 5 μm to 15 μm, the oil droplet surface coverage was 38 mg/m^2 to 114 mg/m^2 when assuming that just 50% of the DSA was adsorbed to the oil-water interface; a low estimate based on the CLSM images. This is significantly higher than values generally reported in literature, which are in the range of 1.5–4.5 mg/m^2 for protein-stabilized emulsions, and only 1–2 mg/m^2 for surfactant-stabilized emulsions, as has been summarized by Berton-Carabin et al. (2014). The high surface coverage by DSA is expected to significantly affect the physical stability of the emulsions, primarily due to the high degree of steric hindrance between the oil and the water phase.

The high oil droplet surface coverage by DSA is also expected to lead to an enhanced oxidative stability of the emulsified oil, as it hinders direct contacts between the oil and pro-oxidants in the aqueous phase. DSA was indeed shown to inhibit lipid oxidation in a model emulsion of linoleic acid to a higher extent than native alginate or BLG. The antioxidative mechanism of DSA is suggested to be based mainly on its ability to scavenge free radicals, as has been shown for the hydroxyl radical and the ABTS radical in this study. The combined high surface activity, high oil droplet surface coverage in emulsions, and antioxidant activity, makes DSA a promising agent for use as a stabilizer of oxidation-sensitive emulsified oils. Other types modified alginates are currently accepted for food use, e.g. propylene glycol alginate (E405), as has been studied by (Baeza, Sanchez, Pilosof, & Patino, 2004). Propylene glycol alginate is accepted for food use, and the degree of esterification in commercial propylene glycol alginate is as high as 85% (Baeza et al., 2004; McHugh). As the degree of substitution of DSA is lower, it is reasonable to expect that the modified alginate presented herein can be accepted for food use.

5. Conclusion

In this study, an innovative material with good emulsification properties was prepared by modifying alginate with SAC12. The product DSA possesses good surface active properties and is able to stabilize oil-in-water emulsions by adsorbing to the oil-water interface and as a result, lowering the surface tension, and forming a protective coating around the oil droplets. Native alginate, on the other hand, stabilizes emulsions only through its action as a thickening agent, which increases the viscosity of the continuous aqueous phase and prevents creaming. Emulsions stabilized with DSA shows a significant increased creaming stability compared to emulsions stabilized with native alginate or BLG. The increased stability is in part due to the large negative oil droplet surface charge resulting from the negatively charged DSA. The viscosity of emulsions stabilized by DSA is significantly reduced compared to emulsions stabilized by native alginate, thus increasing the potential for further processes such as spray drying. The DSA possesses good antioxidant properties through its ability to scavenge free radicals, and it can reduce the extent of oxidation in a linoleic acid emulsion significantly. DSA represents a new material with potential applications in the design of systems for delivery of sensitive n-3 PUFAs from fish oils into food products.

Acknowledgments

This work was partly funded by “Innovation Consortium Multicaps” in collaboration with Danish Technological Institute, DuPont, Marine Bio products, and University of Southern Denmark. The authors thank Stefan Holberg from the Danish Technological Institute for his kind help with the static drop shape analyses. Anna Malgorzata Jurkiewicz and Wei Wei from Aarhus University are thanked for their support with the confocal laser scanning microscopy and dynamic light scattering analyses, respectively.

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Conference poster

Alginate Oligosaccharides: Enzymatic Preparation and Evaluation of their Antioxidant Properties

Mia Falkeborg, Ling-Zhi Cheong, Carlo Gianfico, Katarzyna Sztukiel, Kasper Kristensen, Marianne Glasius, Xuebing Xu, Zheng Guo, 2014.

Poster presentation at the 105th AOCS Annual Meeting and Expo in San Antonio, Texas, USA.

Recipient of "Best Poster Award" in the Lipid Oxidation and Quality section.

Alginate Oligosaccharides: Enzymatic Preparation and Evaluation of their Antioxidant Properties

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Background

The growing trend in consumer preference for natural food ingredients has increased the interest in using antioxidants from natural sources. A promising source of natural antioxidants is marine oligosaccharides, such as oligosaccharides derived from *alginate*; an abundant polysaccharide originating from marine algae. Thorough characterization of the antioxidant mechanism of new antioxidants is required for the optimal application of such compounds in food products. This study aimed at preparing alginate oligosaccharides by enzymatic treatment of alginate, followed by determination of the mechanism of their antioxidant activities trough a range of comparative studies with other monomeric-, oligomeric-, and polymeric alginates, combined with modelling of potential antioxidant mechanisms.

Highlights

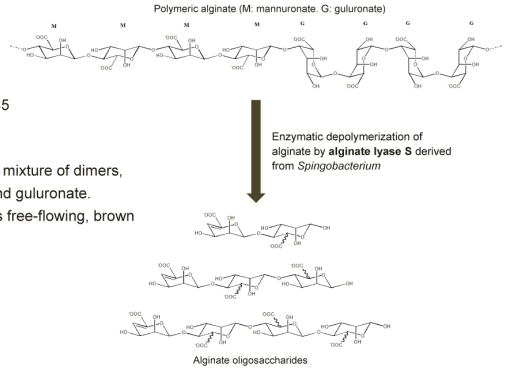
- Alginate oligosaccharides can be prepared by **simple enzymatic treatment** of the natural and abundant polysaccharide *alginate*.
- Alginate oligosaccharides are e**xcellent antioxidants** and protect emulsified oil up to 100% from oxidation.
- Alginate oligosaccharides are **strong radical scavengers** but **weak ferrous ion chelators**.
- This study proposes a **detailed molecular mechanism** that can account for the good antioxidant activities of alginate oligosaccharides. The model proposes an increased radical scavenging capability of alginate oligosaccharides due to the presence of the **conjugated alkene acid structure**.

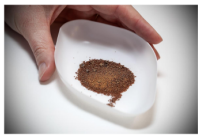
Methods

- Sodium alginate was depolymerized with **alginate lyase S**, and the composition of the resulting alginate oligosaccharides was determined with **thin layer chromatography (TLC)** and **electrospray ionization mass spectrometry (ESI-MS)**.
- The total antioxidant activity of the alginate oligosaccharides was determined by the **thiobarbituric acid reactive species (TBARS)** assay, and compared to that of polymeric and monomeric forms of alginate; of mannuronate- and guluronate-rich fractions; and of acid and salt forms of the AOs.
- Radical scavenging activities was determined against **ABTS*, superoxide (O2*), and hydroxyl (*OH)** radicals.
- **Chelating** activity was determined against **ferrous ions** and detected with **ferrozine**.

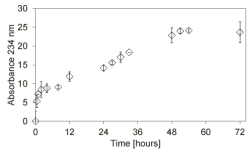
Enzymatic Depolymerization of Alginate for Preparation of Alginate Oligosaccharides

- Sodium alginate was enzymatically depolymerized by *alginate lyase S*
- The enzymatic depolymerization. proceeded through *beta*-elimination, forming double bonds between C4 and C5 of the pyranose ring in the non-reducing ends.
- The final depolymerization product was a mixture of dimers, trimers, and tetramers of mannuronate and guluronate.
- The alginate oligosaccharides appears as free-flowing, brown powder.

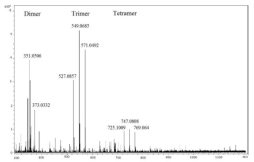


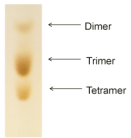


Time-course analysis of alginate depolymerization revealed that 48 hours enzymatic treatment was adequate to achieve the equilibrium oligosaccharide mixture.



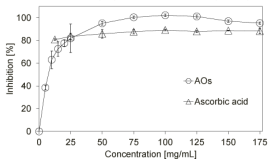
Analysis of the composition of alginate oligosaccharides by ESI-MS and TLC confirmed the presence of dimeric, trimeric and tetrameric structures.





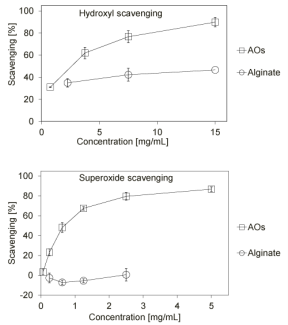
Discovering the Mechanism Behind the Antioxidant Properties of Alginate Oligosaccharides

- Alginate oligosaccharides were found to be superior antioxidants compared to ascorbic acid.
- Alginate oligosaccharides were strong radical scavengers, but weak iron chelators.
- A model for the antioxidant mechanism is proposed based on the capability of radical addition to alginate oligosaccharides due to the presence of the conjugated alkene acid structure.

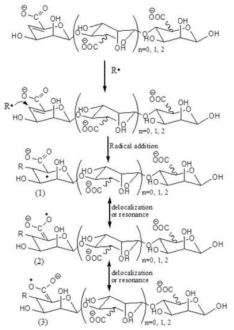


Alginate oligosaccharides are excellent antioxidants; they can provide up to 100% protection against oxidation to emulsified oil; compared to ascorbic acid, which protects up to 89%.

Alginate oligosaccharides scavenges free radicals, such as superoxide, hydroxide and ABTS radicals. Alginate oligosaccharides have higher scavenging capabilities than the parent alginate, partly due to the presence of the conjugated alkene acid structure.



Alginate oligosaccharides can scavenge free radicals through radical addition, which is uncommon for carbohydrates, which generally only have the capability to scavenge radicals trough hydrogen atom transfer.



Conclusion

Alginate oligosaccharides can protect emulsified oil up to 100% from oxidation. Their antioxidant activity mainly originates from their radical scavenging capability, which is increased due to the presence of a conjugated alkene acid structure. The possible radical scavenging mechanism may be hydrogen abstraction, combined with radical addition to the conjugated alkene acid structure. Alginate oligosaccharides have potential applications in the food industry as natural antioxidants. This work gives detailed insights into the antioxidant mechanism of alginate oligosaccharides, which are of importance in the design of antioxidant systems in food products.

Acknowledgements

This work was partly funded by "Innovation Consortium Multicaps" in collaboration with Danish Technological Institute, DuPont Denmark, Marine Bioproducts, and University of Southern Denmark. The authors thank Dr. Mauricio Mora-Pale and Prof. Jonathan Dordick, Rensselaer Polytechnic Institute, Troy, New York, USA for their support regarding the superoxide scavenging activity analyses; and Dr. Liu Xiaoli, Nagase Enzymes, Kyoto, Japan, for kindly providing the alginate lyase.
Photos: Lars Kruse, AU Communication.

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MIA FALKEBORG

AARHUS UNIVERSITY

Appendix 1

Properties of Alginate Lyase S

Provided by Nagase Enzymes, Kyoto, Japan.

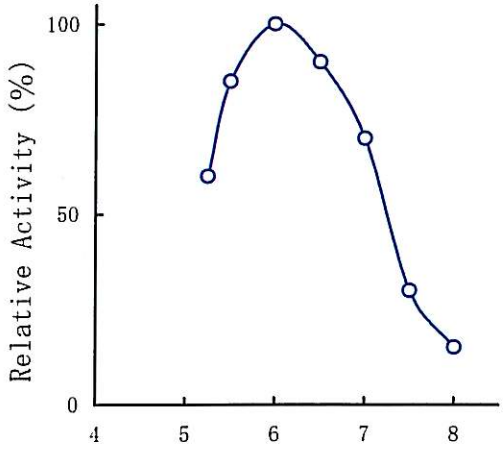
Alginate Depolymerizing Enzyme

ALGINATE LYASE S

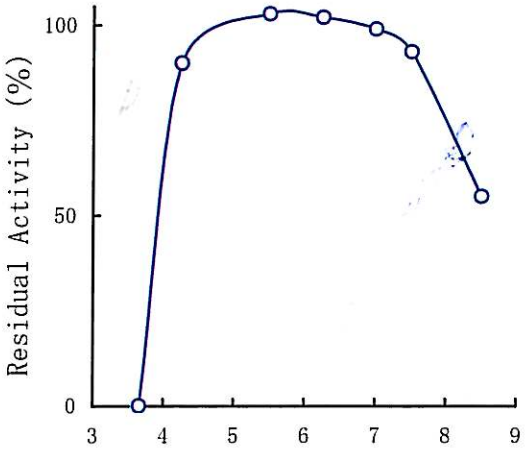
ALGINATE LYASE S catalyzes depolymerization of alginate or alginic acid and reduces viscosity of the alginate solution.

General Properties of **ALGINATE LYASE S**

(1) pH Activity

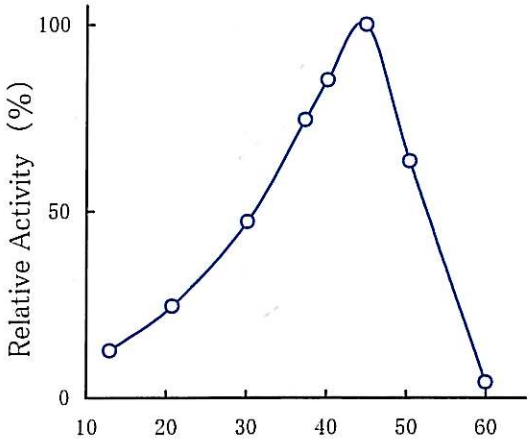


(2) pH Stability

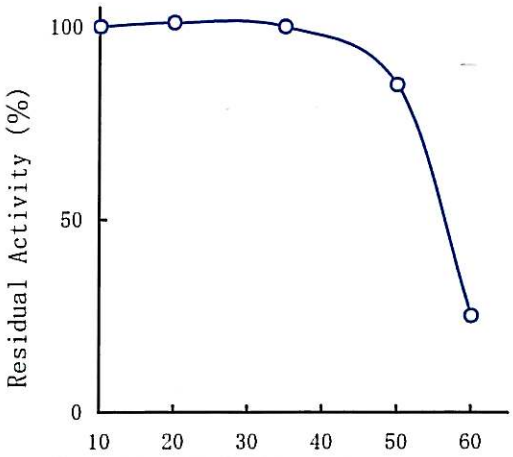


37℃, 1hr treatment

(3) Temperature Activity



(4) Thermal Stability



pH 6.3, 10min. treatment

Appendix 2

Enzymatic Synthesis of Phosphatidyl-saccharides and their Application in the Formation of Stable Liposomes

Including Paper III

Facile Synthesis of Phosphatidyl Saccharides for Preparation of Anionic Nanoliposomes with Enhanced Stability

Shuang Song, Ling-Zhi Cheong, Mia Falkeborg, Lei Liu, Mingdong Dong, Henrik Max Jensen, Kresten Bertelsen, Michael Thorsen, Tianwei Tan, Xuebing Xu, Zheng Guo, 2013.

PLoS ONE; 8(9), pp. e73891.

A2.1 INTRODUCTION

In addition to the alginate-based microencapsulation materials, which have been the primary focus of this Ph.D. study, a second group of microencapsulation ingredients was synthesized and characterized, specifically phosphatidyl-glucose (Figure A2.1), phosphatidyl-sucrose (Figure A2.2) and phosphatidyl-raffinose (Figure A2.3).

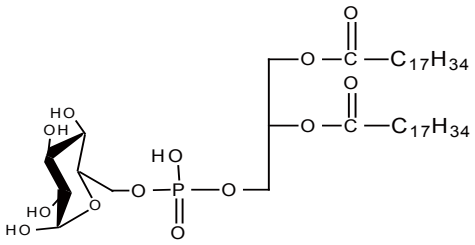


Figure A2.1: Phosphatidyl-glucose

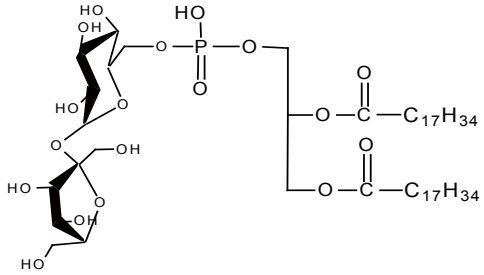


Figure A2.2: Phosphatidyl-sucrose

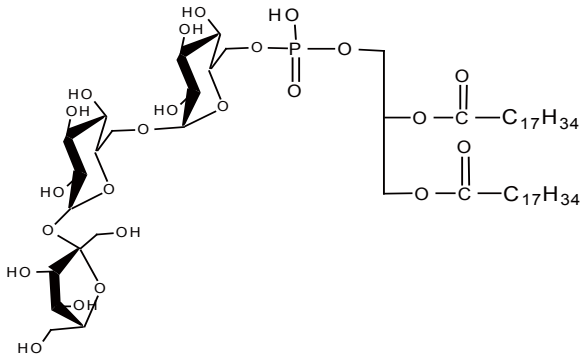


Figure A2.3: Phosphatidyl-raffinose

Phospholipids, such as the naturally abundant phosphatidyl-choline, are known to form liposomes. Liposomes are spherical bilayer vesicles, which have the potential to encapsulate sensitive ingredients. Liposomal structures find a range of applications in the pharmaceutical, cosmetic, and food industries, where they entrap, stabilize and protect sensitive ingredients (Taylor et al. 2005; Bonacucina et al. 2009).

Drying processes such as spray-drying or freeze-drying are usually required before the loaded liposomes can be properly applied (Ingvarsson et al. 2011). Such drying processes can disrupt the liposomal structure, leading to liposome fusion and formation of aggregations, and eventually, leaking of the encapsulated compounds during rehydration (Ingvarsson et al. 2011). Cryprotectants are therefore needed to stabilize the liposomes during dehydration processes. Commonly used cryoprotectants include carbohydrates such as trehalose and sucrose, which act as water replacement molecules during the drying process, replacing the hydrogen bonds to the surrounding water molecules, and thus forming a stabilizing coating on the liposomes (Ingvarsson et al. 2011).

The idea of designing phospholipids conjugated with saccharides was thus to develop compounds able to form liposomes with increased stability during storage and during dehydration / rehydration processes. By replacing the choline moiety of phosphatidyl-choline with saccharide structures, the liposomes will have an inherent coating of carbohydrates, acting as water replacement molecules and protecting the liposomal structures during drying processes.

The research presented in this appendix is the primary work of visiting Ph.D student Shuang Song. The resulting research paper is presented at the end of the appendix. My contributions to the research were focused primarily on synthesis and purification of phosphatidyl-saccharides based on the methods reported previously by Shuang Song (Song et al. 2012). Contributions to the structural identification of the compounds and their application in the formation of nanoliposomes were also made. All experimental details are presented in research paper.

A2.2 SUMMARY OF RESEARCH

Based on the methods reported by Song et al. (Song et al. 2012), phosphatidyl-glucose, phosphatidyl-sucrose and phosphatidyl-raffinose were synthesized in a single-step enzymatic reaction, in a biphasic reaction system, in which phosphatidyl-choline was dissolved in an organic solvent phase, and the saccharide moiety was dissolved in a buffer phase. The transphosphatidylation took place at the interface, catalyzed by phospholipase D. The lipids were then extracted and purified using thin layer chromatography (Song et al. 2012). Phosphatidyl-glucose, phosphatidyl-sucrose, and phosphatidyl-raffinose were obtained in molar yields 95%, 67%, and 23%, respectively. The lower yields of phosphatidyl-sucrose and phosphatidyl-raffinose were due to their bigger size, which caused steric hindrance in the active site of the phospholipase. The structures of the compounds were mapped by NMR and ESI-MS, which confirmed the expected structures of the compounds (Figures A2.1-A2.3).

Liposomes were then formed employing the phosphatidyl-saccharides as the sole component. The thin-film evaporation method was applied for the formation of liposomes, in which the phospholipids were first dissolved in a mixture of chloroform and methanol, which was then evaporated, followed by addition of water accompanied by vigorous agitation to allow formation of hydrated liposomes.

The sizes of the liposomes were determined by dynamic light scattering, which revealed that the liposomes formed from phosphatidyl-saccharides were significantly smaller than liposomes formed from phosphatidyl-choline (290.57 nm, 126.87 nm, and 141.63 nm for phosphatidyl-glucose, phosphatidyl-sucrose and phosphatidyl-raffinose, respectively, compared to 908.70 nm for phosphatidyl-choline). Furthermore, the liposomes formed from phosphatidyl-saccharides had a large negative surface charge due to the negatively charged phosphate group, whereas liposomes formed from phosphatidyl-choline were neutral due to the zwitterionic nature of phosphatidyl-choline. The negative surface charge provided repulsive forces, which prevented fusion and aggregation of the

liposomes. Additionally, the saccharide head groups could form stabilizing hydrogen bonds, which significantly enhanced the stability of the liposomes. A proposed model for the stabilizing effects of the saccharide head groups is presented in the research paper.

Studies using confocal laser scanning microscopy (CLSM) revealed that liposomes formed from phosphatidyl-saccharides demonstrated significantly enhanced stability during storage and against dehydration / rehydration processes. Liposome preparations were analyzed by CLSM immediately after preparation, and after 14 days of storage at room temperature. The CLSM images revealed that liposomes formed from phosphatidyl-choline had aggregated during the storage time, while liposomes from phosphatidyl saccharides retained their spherical structures. Similar results were obtained when the liposomes were analyzed by CLSM after first dehydration and then rehydration, indicating that the phosphatidyl-saccharides were able to form liposomes which remained stable during dehydration.

A2.3 CONCLUSION

The phosphatidyl-saccharides formed in this study were able to produce liposomes with increased stability during storage and during dehydration, compared to liposomes formed from the natural phosphatidyl-choline. The phosphatidyl-saccharides provide a useful tool in the design of specific encapsulation systems, where they can be applied as the sole components, or in combination with other polar lipids. The combining of ingredients allows for tailoring of the encapsulation system towards specific applications, where certain degrees of stability is required, in combination with desired release profiles, and compatibility with the matrix in which it is applied.

The results from this study were published in PLoS ONE in 2013 entitled "Facile Synthesis of Phosphatidyl Saccharides for Preparation of Anionic Nanoliposomes with Enhanced Stability" (Paper IV of this thesis).

The results were additionally presented at the 105th Annual Meeting and Expo in San Antonio, Texas, USA, (Falkeborg, Gao, Song, Cheong, Xu, Guo, 2014: "Biosynthesis of Surface-active Lipids"). This presentation also included results from the research regarding production of sophorolipids (Paper VI, not included in this thesis)

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Facile Synthesis of Phosphatidyl Saccharides for Preparation of Anionic Nanoliposomes with Enhanced Stability

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Abstract

Physical stability during storage and against processing such as dehyration/rehydration are the cornerstone in designing delivery vehicles. In this work, mono-, di- and tri-saccharides were enzymatically conjugated to phosphatidyl group through a facile approach namely phospholipase D (PLD) mediated transphosphatidylation in a biphasic reaction system. The purified products were structurally identified and the connectivities of carbohydrate to phosphatidyl moiety precisely mapped by ^{1}H, ^{31}P, ^{13}C NMR pulse sequences and LC-ESI-FTMS. The synthetic phosphatidyl saccharides were employed as the sole biomimetic component for preparation of nanoliposomes. It was found that the critical micelle concentration (CMC) of phosphatidyl saccharides increases as more bulky sugar moiety (mono- to tri-) is introduced. Phosphatidyl di-saccharide had the largest membrane curvature. In comparison to the zwitterionic phosphatidylcholine liposome, all phosphatidyl saccharides liposomes are anionic and demonstrated significantly enhanced stability during storage. According to the confocal laser scan microscopy (CLSM) and atom force microscopy (AFM) analyses, the nanoliposomes formed by the synthetic phosphatidyl saccharides also show excellent stability against dehydration/rehydration process in which most of the liposomal structures remained intact. The abundance hydroxyl groups in the saccharide moieties might provide sufficient H-bondings for stabilization. This work demonstrated the synthesized phosphatidyl saccharides are capable of functioning as enzymatically liable materials which can form stable nanoliposomes without addition of stabilizing excipients.

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Introduction

Phospholipids-based liposomal vesicles have evolved as a promising tool for delivery of drug, genes and nutrients due to intensive investigations for decades [1-4]. Stability of liposomes has been a key issue which limit its application as most liposomes with phosphatidylcholine (PC) as primary components have low stability [4-6]. To circumvent this problem, different strategies have been attempted including development of polymeric liposomes [5-7], functionalization of liposome surface [8-10], synthesis of novel liposome constituting components [11,12], and addition of protectants [13-15]. Addition of protectants such as trehalose and proline has been shown to significantly enhance the stability of liposomes during storage and against dehydration/rehydration process [13-17]. These protectants were hypothesized to act as water replacement molecules which form hydrogen bonding with the phospholipids to maintain head group spacing and thus prevent fusion of liposomes [18,19].

Based on this concept, we hypothesize that conjugation of phosphatidyl and sugar moiety may integrate the function of PC as building block for liposome and the function of

protectants as physical stability enhancer into phosphatidyl saccharides. In this work, we had developed a facile approach to conjugate mono-, di- and tri-saccharides to phosphatidyl group through phospholipase D mediated transphosphatidylation in a biphasic reaction system and characterized phosphatidyl saccharide-based liposomes systemically with PC-liposomes as a comparison control. The synthetic phosphatidyl saccharides were found to be capable of forming nanoliposomes with enhanced physical stability against fusion and aggregation following storage, dehydration and rehydration procedures.

This work presented a good demonstration that there is a huge potential of using natural compounds as building blocks to develop low toxic, biodegradable and biocompatible excipients for a variety of applications.

Materials and Methods

Materials

Phospholipase D (Streptomyces sp.) was a gift from Nagase ChemteX Corporation, Japan. The activity of the PLD is 600,000 U/g (1 U is defined as the activity which liberates 1 μmol choline from L-α-phosphatidylcholine per minute). Phosphatidylcholine (PC), (Epikuron 200), was a gift from Cargill Inc. (Minneapolis, MN, USA). This PC originates from soybean and consists of at least 95% PC of which 3% is the lyso- form. All solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and of HPLC grade. Glucose, sucrose, raffinose and other chemicals were of analytical grade from Sigma-Aldrich Co. (St. Louis, MO, USA).

Enzymatic Synthesis of Phosphatidyl Saccharides

PLD-catalyzed transphosphatidylation reaction between PC and saccharides was carried out using a biphasic reaction system in which 390 mg of PC was dissolved in 20 ml ethyl acetate and 60 U PLD was dissolved in 2 ml sodium acetate buffer (pH 5.6 adjusted by acetic acid). Firstly, PLD buffer solution was added to reactor with 1.8 g glucose/ 3.42 g sucrose/5.04 g raffinose and stirred for 10 min. Then, PC solvent solution was added in the reactor to initiate the reaction. The reaction was carried out at 50 °C and stirred with magnetic stirrer (600 rpm) for 2 h. At the end of the reaction, 20 ml chloroform was added to the reaction mixture to stop the reaction and extract out the lipids. The resulting organic phase was carefully separated and dried after centrifugation (4000 rpm) for 10 min. The dried mixture was re-dissolved in 10 ml chloroform for purification process.

Quantitative Analysis of Synthesized Phosphatidyl Saccharides

The molar yield of phosphatidyl saccharides was determined by HPLC analysis. The HPLC was equipped with a silica gel column (5 μm, 4.6×250 mm, Thermo Fisher Scientific Inc.) and a Sedex (Alfortville, France) model 75 ELSD; the pressure of nebulizer gas (air) was maintained at 3.2 bar and the drift tube temperature was set at 40 °C. Three kinds of eluents namely chloroform (A), methanol (B), and 1% (V/V) triethylamine buffer (titrated at pH 3 with formic acid) (C) were used. The chromatographic separation was carried out using a linear gradient: 0-5 min A/B/C (87.5/12/0.5, v/v), 5-40 min A/B/C (87.5/12/0.5, v/v) to A/B/C (28/60/12, v/v), 40-45 min back to A/B/C (87.5/12/0.5, v/v), and 45-55 min re-equilibration. The flow rate of the eluent was 0.5 ml/min. The retention time of Ptd-Glu, Ptd-Suc and Ptd-Raff were 27.1 min, 28.2 min and 29.7 min, respectively. Phosphatidyl saccharides were quantified with standard curve of purified phosphatidyl saccharides, whose structure and purity have been confirmed by LC/MS and NMR analysis.

Purification of Phosphatidyl Saccharides from Reaction Mixture

The synthesized phosphatidyl saccharides were separated from extracted polar lipids according to a reported TLC method with slight modification [20]. Around 0.5 ml of the lipid extraction from reaction mixture was separated on thin layer chromatography plates (TLC silica gel 60, 10×20, Merck, Germany) using developing solvent of chloroform/methanol/acetate acid/water (50:25:6:2). The R_f value for Ptd-Glu, Ptd-Suc and Ptd-Raff in TLC were 0.48, 0.26 and 0.09 respectively. The phosphatidyl saccharide band was scraped off and extracted by chloroform/methanol (1:1). The extract was dried after centrifugation (4000 rpm) for 20 min. The purity of extracted phosphatidyl saccharide was reconfirmed by TLC and HPLC with single plot and single peak, respectively. The structure of phosphatidyl saccharides was further identified by LC-ESI-FTMS and NMR.

Structure Conformation of Phosphatidyl Saccharides

The structures of the synthesized phosphatidyl saccharides (Ptd-Glucose, Ptd-Sucrose, and Ptd-Raffinose) were identified by NMR and LC/FTMS.

NMR investigations on Ptd-Glu was carried out at 14.1 T on a Bruker Avance III spectrometer in an inverse TXI (^{1}H,^{31}P,^{13}C) 1.7 mm probe with a z-gradient at 300 K. In order to verify the phosphor-ether bond a ^{31}P-^{1}H HMBC pulse sequence was employed. The sequence was based on the standard Bruker parameter set HMBCGPND and optimized for 4 Hz couplings. The 64 indirect increments each with four scans were acquired and zero filled to 8192 times 128. Gradients in the pulse sequence were set in the ratio 20:30:10.16 in order to select for ^{31}P-^{1}H couplings. The ^{31}P dimension was referenced externally to phosphatidylcholine in $CDCl_3$:MeOD (2:1) at 0.8 ppm and the proton dimension was references to TMS at 0 ppm in $CDCl_3$:MeOD (2:1). For assignment purposes HSQC, DQF-COSY, TOCSY and J-resolved spectra was also recorded all using standard parameters. For the Ptd-Suc and Ptd-Raff samples, cryogenic probes and RT probes where used. The following experiments, ^{13}C-^{1}H HSQC, ^{13}C-^{1}H HSQCTOCSY and ^{1}H-^{31}P TOCSY was recorded in order to determine the phosphorus linkage all based on standard bruker pulse programs. The ^{1}H-^{31}P TOCSY utilized a 60 μs spinlock for 80 ms.

LC/FTMS includes chromatographic separation (Agilent 1100 System consisting of G1376A (Cap pump), G1377A (μWell-Plate Sampler), G1316A (Column Compartment) and

FTMS detection (LTQ Orbitrap MS, Thermo Scientific). The chromatographic separation was obtained by normal-phase conditions using a Kromasil Sil column, 150 mm x 0.5 mm id., 3.5 µm. The mobile phases were: Solvent A comprised of chloroform/MeOH/NH_4OH (25%) (800/200/5 vol.) and solvent B comprised of chloroform/MeOH/H_2O/NH_4OH/ (25%) (800/200/55/5 vol.). The flow rate was 16 µl/min. The gradient is 100% A (0-5 min), 100% A-100% B (5-10 min), 100% B (10-25 min), 100% B-100% A (25-32 min), and 100% A (32-40 min) for equilibration. The total cycle time was 40 min. The MS condition was negative electrospray mode and two scan events pr cycle: 1) Full scan FTMS [200-1400] with 15 k resolution, 2) data dependant $FTMS^2$ with 7.5 k resolution.

Preparation of Nanoliposome Suspension of Phospholipids by Thin Film Hydration Technique

Solid phospholipids (1 mg) were dissolved in 1 ml chloroform: methanol (1:1). For CLSM analysis, 0.1 ml of a 0.1 mM dichloromethane solution of DIL (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) was added to the formulation. The solvent was evaporated by a stream of dry nitrogen until a dry lipid film was observed and followed by overnight vacuum drying to remove all solvent. Following overnight vacuum drying, the liposomes were suspended in Milli-Q water by vigorous vortexing for 30 mins and allowed to hydrate at room temperature for 3h.

Characterization of Physical Properties

Physical property of phosphatidyl saccharides and PC nanoliposomes were evaluated by critical micelle concentration (CMC), dynamic light scattering (DLS), confocal laser scan microscope (CLSM), and atom force microscope (AFM).

Critical Micelle Concentration (CMC) measurement was done using the pyrene assay [21]. Fluorescence spectrometer (PerkinElmer LS55 luminescence spectrometer) was used in this study. Aggregate formation was examined over a wide range of phospholipid concentration (1×10^{-7}-1 mg/ml) by using pyrene in methanol as a fluorescent probe (final concentration 1×10^{-6} M). Briefly, the ratio between the emission intensity at two wavelengths (I_1=372.5 and I_3=383 nm) upon excitation at 335 nm was determined. I_1/I_3 is around 1.0 in the absence of surfactant but increases to a plateau value of 1.42-1.53 in the presence of micelles, depending on the specific surfactant headgroups. The CMC was taken as the intersection of regression lines calculated from the linear portions of the graph.

Dynamic Light Scattering (DLS) was performed at 25 °C. The diameter and zeta potential of liposome were characterized by dynamic light scattering (DLS) using a Malvern Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK). All measurements were performed at an angle of 173°. The size distributions and zeta potentials were analyzed by using the Malvern Dispersion Software (V5.10, www.zetasizer.com). Since size changes observation is easier with dispersion with low polydispersity index (PDI), the phospholipid suspensions from shelf life evaluation were passed through a 0.45 µm PTFE syringe filter (Whatman).

Confocal Laser Scan Microscopy (CLSM) **Analysis** A Zeiss 510 Meta CLSM microscope with a 100x oil immersion 1,3 NA objective was used. The DIL dye was excited with the 543 line of the He-Ne laser and the emission was captured with the 560-620 nm BP filter. The liposome was fixed on 2% (W/V) agarose gel. To observe dehydrating procedure, the fixed liposome was dried on cover slip in ambient condition overnight. To observe rehydrating procedure, the dehydrated sample was rehydrated in Milli-Q water for 1 h.

Atom Force Microscopy (AFM) **Analysis** A commercial AFM MultiMode V (Bruker, Santa Barbra, USA) was used. For sample preparation, 5 µL of PC, Ptd-Glu, Ptd-Suc or Ptd-Raff with the concentration of 2 mg/ml were deposited on the freshly cleaned mica for 10 min, after that the residue solution were removed. All the samples were washed 3 times with Milli-Q water and dried in ambient condition before the measurement. The standard AFM images were recorded using tapping mode under ambient conditions. Commercial silicon tips from Bruker Company (Bruker, Santa Barbra, USA) with a nominal spring constant of 40 N/m and resonant frequency of 300 kHz were used in all the experiments and a normal tip radius of 15 nm were chosen to image the samples. All the AFM images were obtained in the tapping mode at a scan frequency of 1 Hz with optimized feedback parameters. The resolution of all the original AFM images was 512 x 512 pixels per image. The images were flattened and analyzed by using the commercial software Scanning Probe Image Processor (SPIPTM, Image Metrology Aps, version 5.13, Lyngby, Denmark).

Results and Discussion

Synthesis and Structural Mapping of Phosphatidyl Saccharides

One of the major challenges in synthesizing phosphatidyl saccharides is the selectivity because of presence of multiple hydroxyl groups in sugar ring. Taking advantage of high selectivity of biocatalysis, we employed PLD to catalyze the transphosphatidylation of PC with saccharides (glucose, sucrose and raffinose) in a binary ethyl acetate/water system. This binary solvent system is able to dissolve both phophostidylcholine and sugar, respectively [22]. 95 mol% of Ptd-Glu can be obtained at optimum condition. Substrates with bigger molecular size have higher steric hindrance and thus difficult to access the active site of enzyme. Therefore lower molar yields were obtained for Ptd-Suc (67 mol%) and Ptd-Raff (23 mol%). Figure 1 (a) shows the structures of the synthesized anionic phosphatidyl saccharides.

The chemical structures of the purified phosphatidyl saccharides were confirmed by LC/FTMS and NMR (Figure 2 for Ptd-Raff; Figures S1 and S2 in Supplementary Materials for Ptd-Glu and Ptd-Suc, respectively). LC/FTMS analysis showed all samples were of high purity and the molecular mass for all purified phosphatidyl saccharides were as predicted. Two abundant isomers can be found for all phosphatidyl saccharides which can be attributed to the differences in the fatty acid chains of the substrate PC ([18:2, 18:2], [16: 0, 18:2]). For example, Ptd-Raff presented m/z 1181.62 [18:2, 18:2] and m/z 1157.62 [18:2, 16: 0] (Figure 2 A). MS^2 spectra further

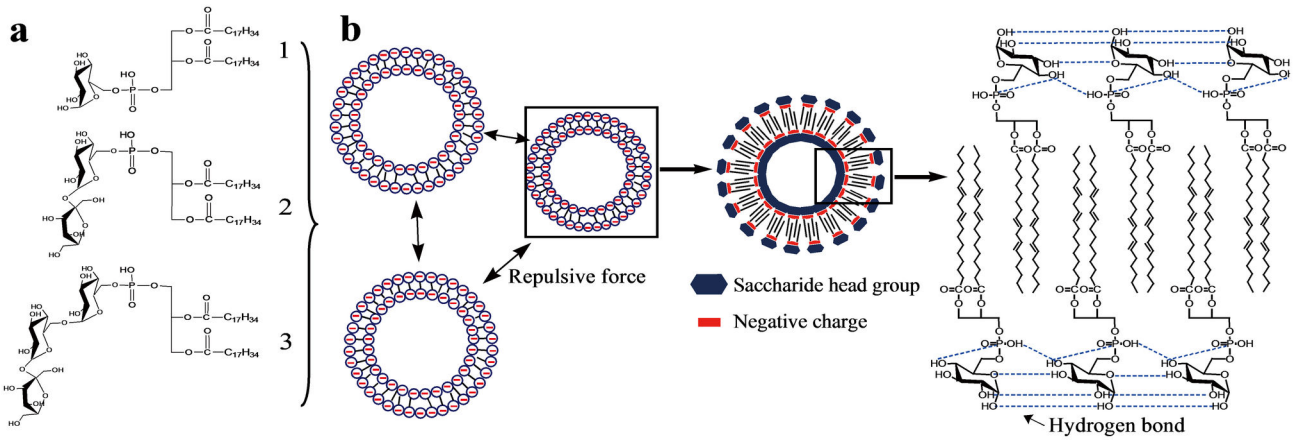

Figure 1. Phosphatidyl saccharides and the mechanism concerning stabilization of phosphatidyl saccharides nanoliposomes during storage and dehydration/rehydration. (a) Structure of enzymatic synthesized phosphatidyl saccharides (1:Ptd-Glu; 2:Ptd-Suc; 3:Ptd-Raff). (b) Both the electrostatic repulsive force caused by negative surface charge and the enhanced hydrogen bond between saccharide head groups contributed to the improved stability of phosphatidyl saccharides nanoliposomes.
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verified the structure of all the phosphatidyl saccharides as the typical fragments include loss of the glycan unit, fatty acids and $C_2H_2O_2$ by glucan-ring cleavage. For example, fragments of 1019.57 or 1001.56 (fatty acids-glycan unit-C $_2H_2O_2$) and 959.55 (fatty acids-glycan unit) were observed in Ptd-Raff (Figure 2 B-C). NMR Spectroscopy was used to assign the molecular moieties of the phosphatidyl saccharides. ^{31}P-^{1}H HMBC, ^{1}H-^{31}P TOCSY and ^{13}C-^{1}H HSQC NMR pulse sequences verified the connectivities of the carbohydrate functionality to the Ptd-backbone (Figure 2 D). For Ptd-Glu and Ptd-Raff, the phosphate ester of the Ptd-backbone binds to glucose-C6 and galactose-C6, respectively. A similar connectivity is dominant for Ptd-Suc, phosphorus was more selectively linked with glucose-C6 of sucrose, but less intense correlations (~20%), are also observed to fructose-C2 of sucrose (Figure S2D).

Physical Characteristics and Storage Stability of Phosphatidylsaccharide-based Nanoliposomes

Commercial PC and the purified synthetic phosphatidyl saccharides were used to prepare nanoliposomes. The physical characteristics of nanoliposomes were evaluated (Table 1). Phosphatidyl saccharide-based nanoliposomes were smaller in size than the PC based ones. Saccharides have higher optimal headgroup area as compared to choline; therefore, phosphatidyl saccharides have smaller packing parameter which resulted in higher membrane curvature and smaller vesicle size [23]. In terms of surface charge, all the phosphatidyl saccharides-based nanoliposomes had negative surface charge (negative zeta potential) due to the negatively-charged phosphate group. Meanwhile, PC nanoliposomes had neutral charge due to the zwitterionic structure of PC. The anionic phosphatidyl saccharide nanoliposomes provided repulsive forces which prevent fusion and aggregation. A possible mechanism has been proposed as indicated in Figure 1(b).

Both phosphatidyl saccharides and PC nanoliposomes had similar critical micelle concentration (CMC). As CMC is strongly dependent on the alkyl chain length (hydrophobicity), the property change in headgroup despite insignificant still influences the CMC [24]. Increasing amount of sugar ring from glucose to raffinose (hydrophilic moiety) resulted in 1.1 to 1.3 times increments in CMC per glycan unit (Table 1).

Storage Stability of Phophatidyl Saccharide-based Nanoliposomes

The nanoliposomes of phosphatidyl saccharides and PC loaded with hydrophobic fluorochrome marker 1,1-Dioctadecyl-3,3,3',3'-tetramethyl indocarvicyanine perchlorate (DIL) were prepared and stored for 14 days at room temperature. For phosphatidyl saccharides nanoliposomes, no apparent precipitation and aggregation is observed after 14 days' storage in comparison with the freshly prepared samples (Figure 3). Meanwhile, PC nanoliposomes aggregated and fused into bigger droplets which eventually precipitated (Figure 3). Materials with small particle size distribution of less than 10 nm can be observed in the aqueous suspension of PC which may represents the collapsed membrane pieces or micelle. However, only a little bit difference of the size distribution of Ptd-saccharide based nanoliposomes between the fresh sample and the sample after storing 14 days was observed (Figure 4). The stability of phosphatidyl saccharides nanoliposomes against aggregation and fusion during storage was further verified using Confocal Light Scattering Microscopy analysis (CLSM). The nanoliposomes loaded with hydrophobic fluorochrome marker DIL were fixed on agarose gel. After storage for 14 days, the DIL-labelled phosphatidyl saccharides nanoliposomes remained intact which can be shown by the unilamellar red-stained spherical morphology (Figure 4). In

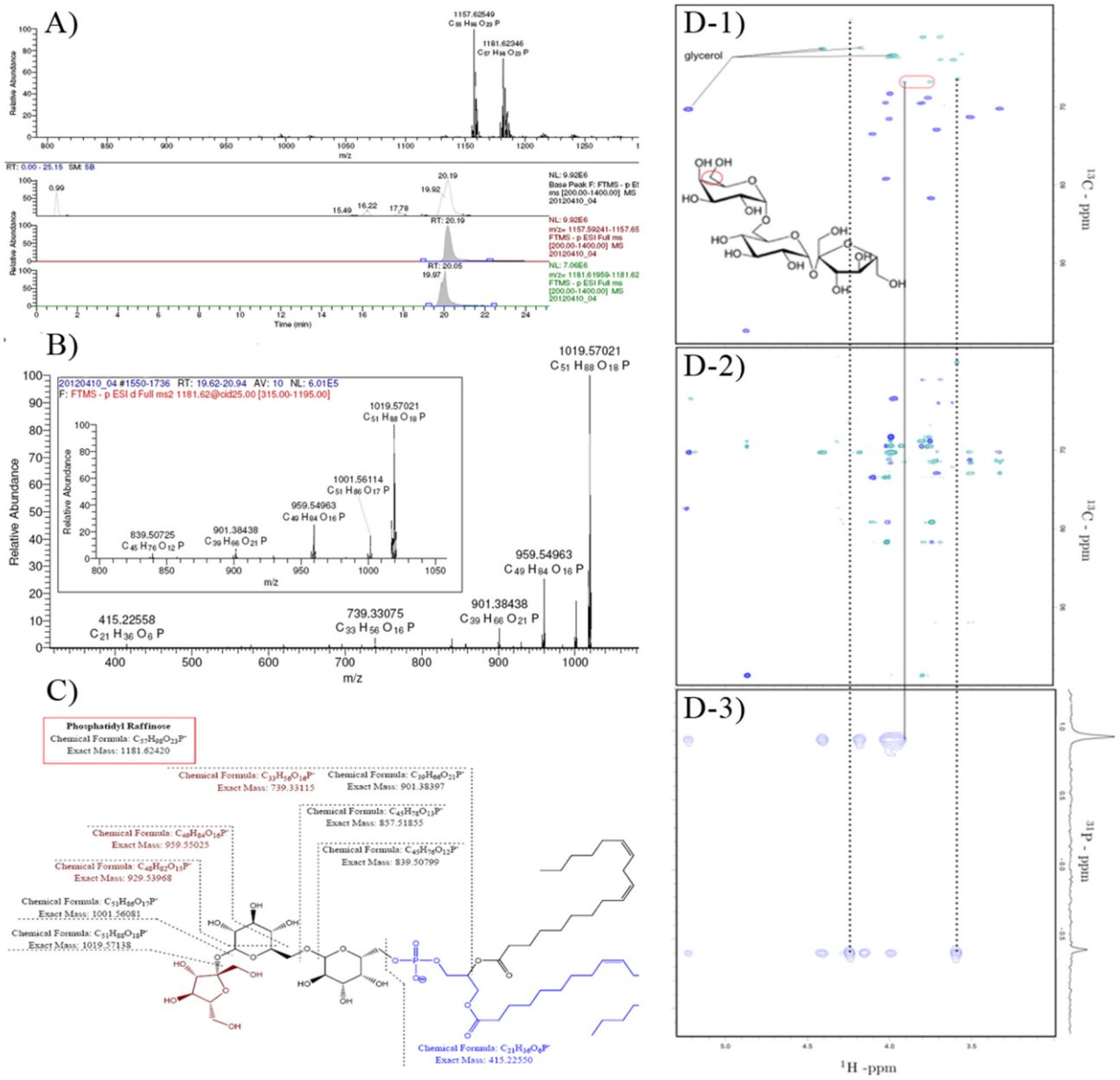

Figure 2. Structural elucidation of Ptd-Raff. A) Base-peak and extracted ion chromatograms and full FTMS spectrum of Ptd-Raff. The two major isomers are m/z 1181 [18:2, 18:2] and m/z 1157 [18:2, 16: 0]. B) MS^2 spectra of the two major isomers. The primary fragments are explained in C). C) Chemical structure of Ptd-Raff. D-1) multiplicity edited ^{13}C-^{1}H HSQC, D-2) multiplicity edited ^{13}C-^{1}H HSQC-TOCSY and D-3) ^{1}H-^{31}P TOCSY with ^{31}P detection spectra of Ptd-Raff. Dotted lines shows correlation confirming small amount of phosphatidylcholine in the Ptd-raffinose sample (Ratio estimated by quant. ^{31}P NMR to 80:20 Ptd-Raff/PC. The solid line in shows the major phosphor species in Ptd-raffinose conjugate on the galactose unit of raffinose.

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contrast, DIL-labelled PC nanoliposomes were characterized as red-stained elongated and irregular morphology which indicate aggregation and fusion of the nanoliposomes. One of the possible reasons for higher stability of phosphatidyl saccharide nanoliposomes against aggregation and fusion during storage is the OH groups of saccharide moiety. The OH-groups are capable of forming abundant hydrogen bond in the polar region of the membrane resulting in enhanced membrane mechanical stability. Surface charge also plays an important role. Liposomes formed by neutral sugar esters were found to aggregate during storage due to the low head group repulsive forces [25]. Thus, the anionic phosphatidyl saccharides

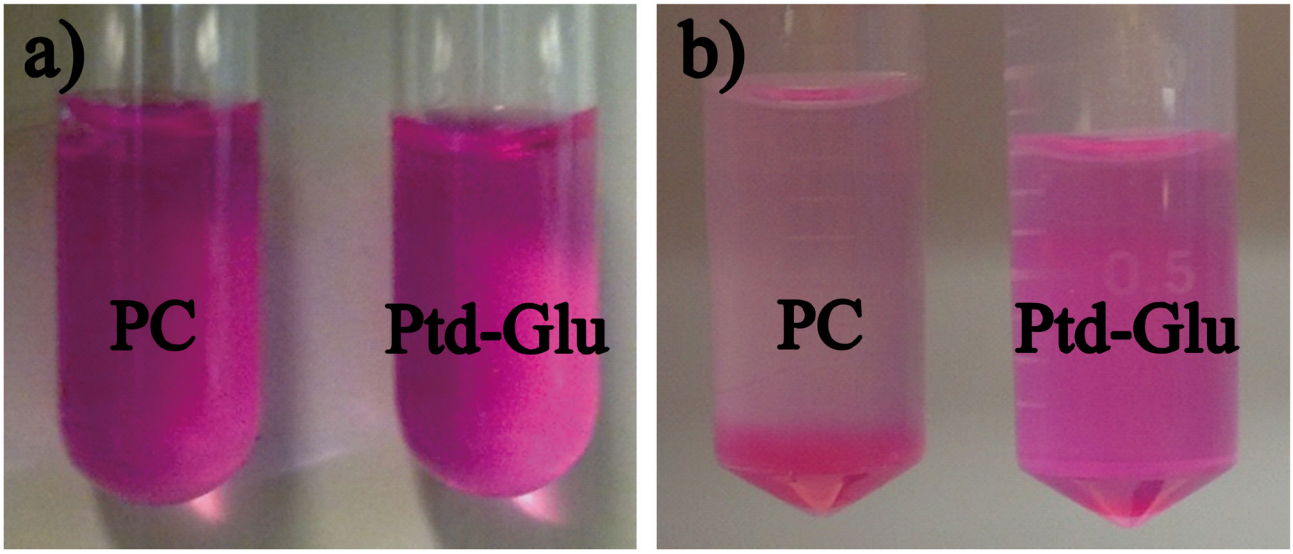

Figure 3. Physical stability of PC and phsphatidyl saccharide nanoliposomes following prolonged storage: a) freshly prepared nanoliposomes, b) nanoliposome after storage for 14 days at room temperature.
doi: 10.1371/journal.pone.0073891.g003

Table 1. Critical micelle concentration (CMC), liposome average size and zeta potential of PC, Ptd-Glu, Ptd-Suc and Ptd-Raff.

	CMC (nM)	Vesicle Size (nm)	Zeta (mV)
PC	0.12	908.70 ± 1.21	-9.50 ± 0.65
Ptd-Glu	0.16	290.57 ± 2.56	-92.10 ± 3.50
Ptd-Suc	0.18	126.87 ± 4.74	-91.20 ± 2.56
Ptd-Raff	0.20	141.63 ± 1.66	-91.43 ± 0.51

doi: 10.1371/journal.pone.0073891.t001

nanoliposomes provided repulsive forces between the headgroups to prevent aggregation and fusion of nanoliposomes (Figure 1b).

Stability of Phosphatidylsaccharide-based Nanoliposomes against Dehydration/Rehydration

Phosphatidyl saccharide nanoliposomes also demonstrated improved stability against dehydration and rehydration procedure. Following dehydration, most of the phosphatidyl saccharide nanoliposomes remained intact on the hydrophilic mica surface. Both Ptd-Glu and Ptd-Suc nanoliposomes had a height distribution within 18-80 nm and 30-130 nm, respectively. Ptd-Raff nanoliposomes had a height distribution of less than 20 nm. In comparison, nearly all of the PC nanoliposomes collapsed to form bilayer structure on the mica surface (height<10 nm) (Figure 5). Similar findings can be observed using the CLSM (Figure 6). The DIL-labelled phosphatidyl saccharide nanoliposomes remained intact with apparent red-stained spherical morphology following dehydration. Upon rehydration, the DIL-labelled phosphatidyl saccharide nanoliposomes can be recovered. Nevertheless, Ptd-Raff had reduced vesicle size upon rehydration procedure which is in agreement with the aforementioned AFM findings. Stark et al. [26] found stabilizing effects of protectants is strongly dependent on its water solubility. Protectants with higher water solubility have higher OH bonding capability. In present case, sucrose (2000g/L) has the highest water solubility followed by glucose (910g/L) and raffinose (143g/L). Thus, Ptd-Suc has the highest stabilizing effect followed by Ptd-Glu and Ptd-Raff. In comparison, the DIL-labelled PC nanoliposomes aggregated and fused (red-stained elongated and irregular morphology) upon dehydration. Rehydration procedure was not able to recover the liposomal structures of the DIL-labelled PC nanoliposomes.

Conclusions

In summary, a series of anionic phosphatidyl saccharides were successfully synthesized through enzymatic transphosphatidylation. Phosphatidyl saccharide nanoliposomes were smaller in size and had higher negative surface charge as compared to PC nanoliposomes. Presence of abundant OH groups in phosphatidyl saccharides renders them stabilizing effects. Anionic phosphatidyl saccharides nanoliposomes demonstrated enhanced physical stability against fusion and aggregation following storage, dehydration and rehydration procedures. The findings from this study can be used to overcome the physical instability of liposomes. And the synthetic phosphatidyl saccharides may provide an alternative anionic liposome for non-cytotoxic and serum stable delivery system [27]. In addition, sugar-coated nanoparticles as delivery system are becoming an area of interest since their reported cell internalization ability [28] and specific molecular recognition properties [29]. Thus, phosphatidyl saccharide nanoliposomes may have similar effects and can be used for

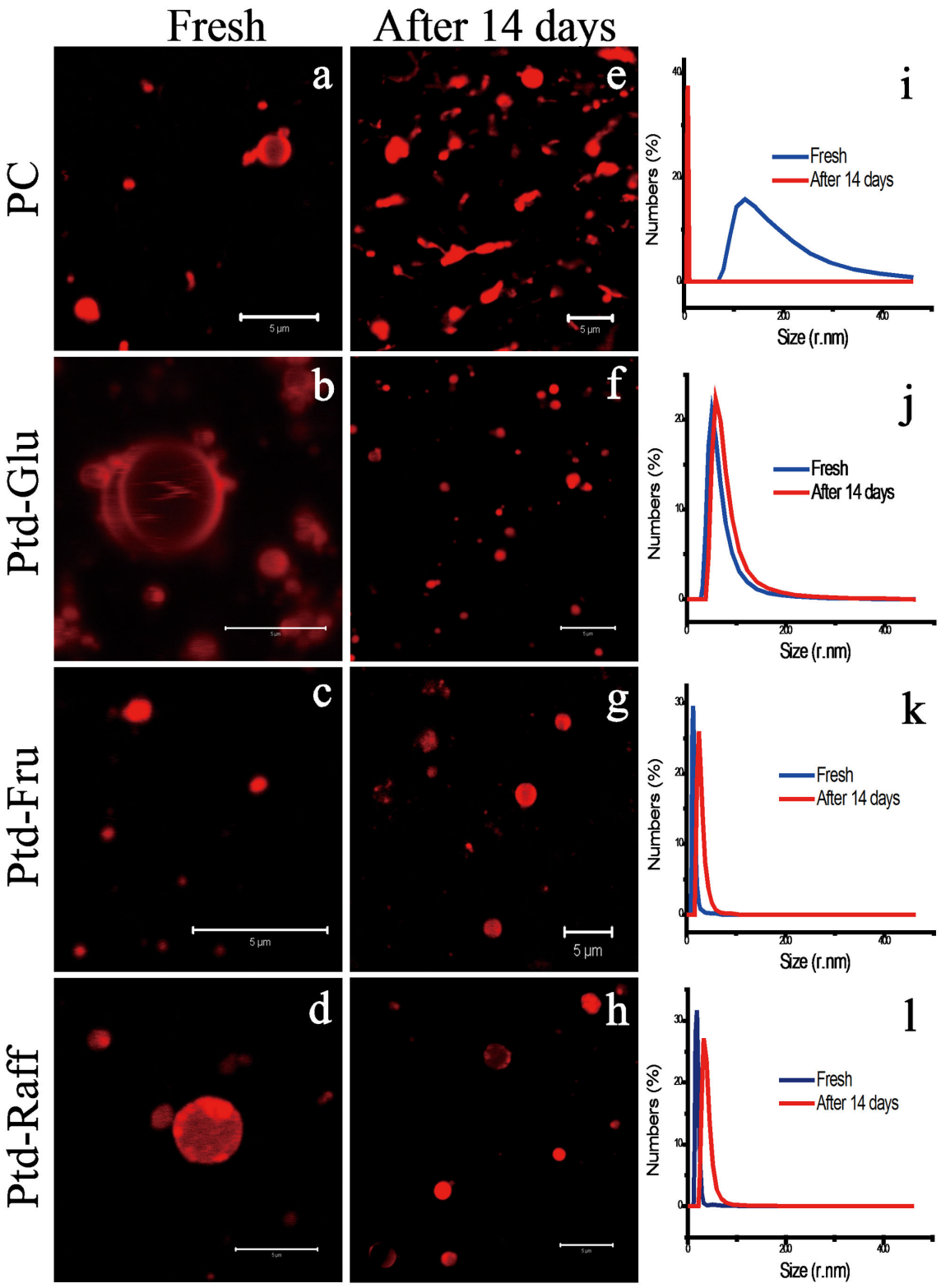

Figure 4. Characterization of nanoliposomes of PC, Ptd-Glu, Ptd-Suc and Ptd-Raff following prolonged storage by CLSM and DLS. (a-d) Morphology of freshly prepared nanoliposomes (size bar: 5 μm). (e-h) Morphology of nanoliposomes following storage for 14 days at room temperature (size bar: 5 μm). (i-l) Size dispersion of freshly prepared nanoliposomes and nanoliposomes stored for 14 days.

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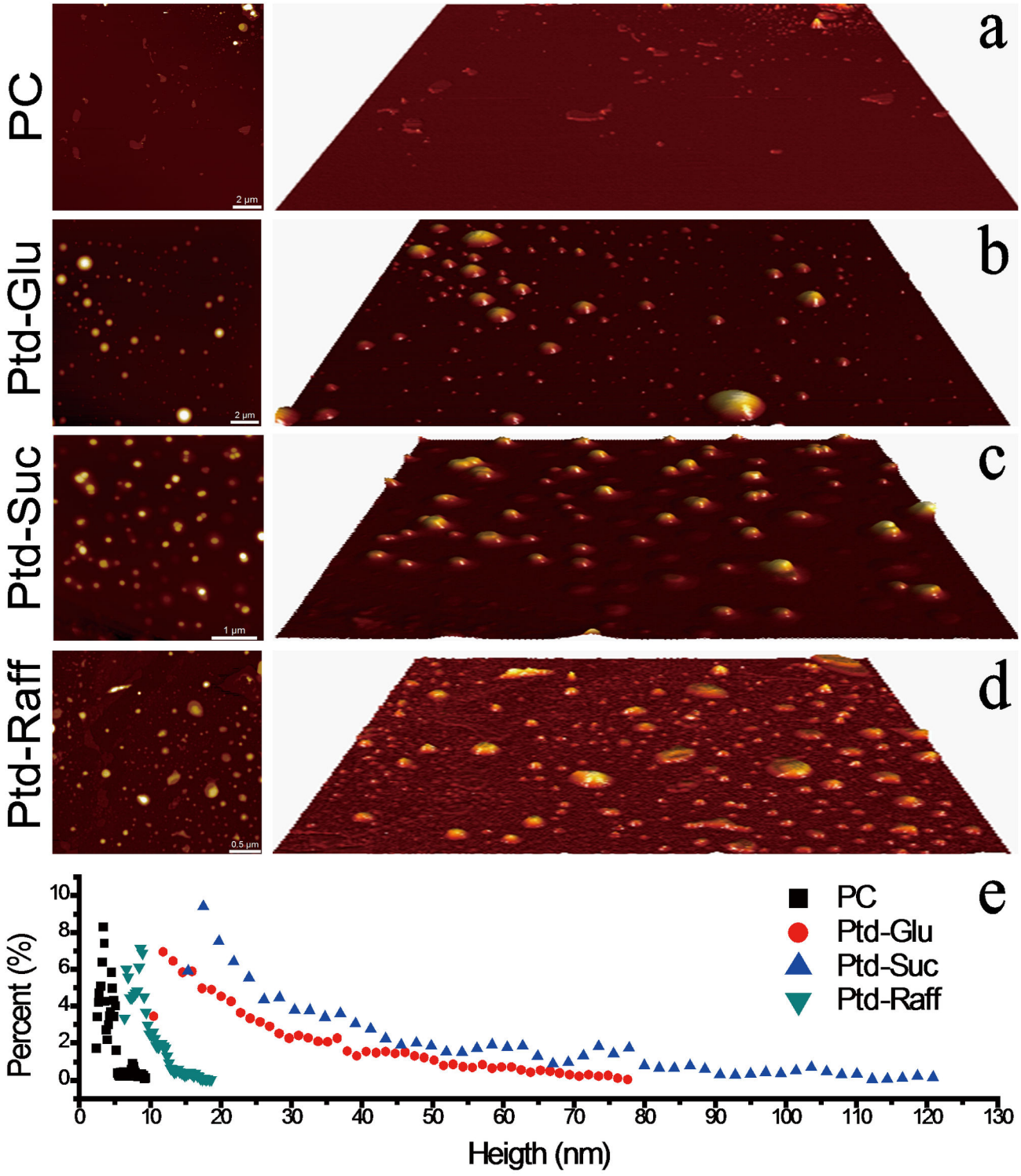

Figure 5. AFM topographic images of the nanoliposomes of PC (a), Ptd-Glu (b), Ptd-Suc (c) and Ptd-Raff (d) on mica surface following dehydration procedure. The height distribution of these structures were depicted in (e).

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targeted drug or functional ingredients delivery. More study on functional molecular encapsulation and cell internalization of the phosphatidyl saccharide liposomes will be further carried out.

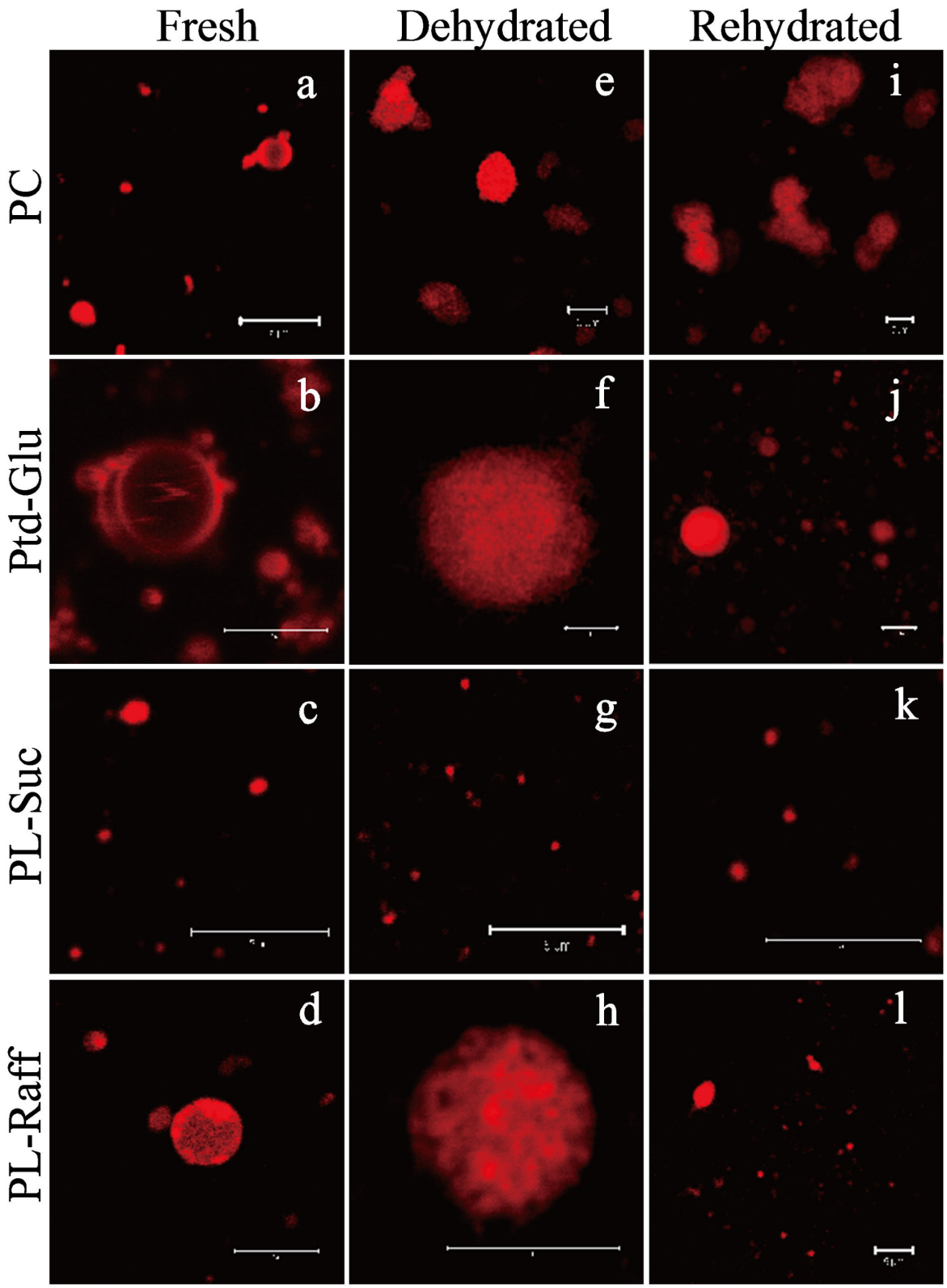

Figure 6. CLSM images of nanoliposomes following dehydration and rehydration procedure (size bar: 5 μm). (a-d) Morphology of freshly prepared nanoliposomes (e-h) Morphology of nanoliposomes following dehydration procedure (i-l) Morphology of nanoliposomes following rehydration procedure.

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Supporting Information

Figure S1. Structural elucidation of Ptd-Glu. (**A**) Base-peak and extracted ion chromatograms and full FTMS spectrum of Ptd-Glu. (**B**) MS^2 spectra of the two major isomers. The primary fragments are the loss of the glycan-unit, loss of $C_2H_4O_2$ from the glycan ring and loss of glycan and one fatty acid. (**C**) Structure of Ptd-Glu (D-1). ^{13}C-^{1}H HSQC spectrum of Ptd-Glu, showing chemical shifts of the α- and β-conformer of the glucose unit (D-2). ^{31}P-^{1}H HMBC spectrum of Ptd-Glu, showing correlations between phosphorous and theH^6_α, H^6_β and the $H^{glycerol}_\alpha$ protons.
(TIF)

Figure S2. Structural elucidation of Ptd-Suc. (**A**) Base-peak and extracted ion chromatograms and full FTMS spectrum of Ptd-Suc. (**B**) MS^2 spectra of the two major isomers. The primary fragments are the loss of the glycan-unit, loss of $C_2H_4O_2$ from the glycan ring and loss of glycan and one fatty acid (**C**) Structure of Ptd-Suc (**D-1**). multiplicity edited ^{13}C-^{1}H HSQC, (**D-2**) multiplicity edited ^{13}C-^{1}H HSQC-TOCSY and (**D-3**) ^{1}H-^{31}P TOCSY with ^{31}P detection spectra of Ptd-Suc. The solid line shows the major phosphor species in Ptd-Suc conjugate on 6-OH (C6) of glucose unit and only small amount of phosphor species conjugate on 2-OH (C2) of fructose unit. The phosphor peaks are in 72:20:8 ratio measured by quantitative ^{31}P NMR.
(TIF)

Author Contributions

Conceived and designed the experiments: SS LZC MD TT XX ZG. Performed the experiments: SS MF LL HMJ KB MT. Analyzed the data: SS LZC ZG. Contributed reagents/ materials/analysis tools: MD HMJ. Wrote the manuscript: SS LZC LL HMJ KB MT XX ZG.

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Appendix 3

Expression and Characterization of Antimicrobial Enzymes

Summary of research conducted at Rensselaer Polytechnic Institute in Troy, New York, USA, from September 2013 to February 2014.

PREFACE

As a part of my Ph.D. study, I got the opportunity to visit the Rensselaer Polytechnic (RPI) Institute in Troy, New York, USA, and work under the supervision of Professor Jonathan Dordick in the Center for Biotechnology and Interdisciplinary Studies. During my stay, I was involved in interdisciplinary projects regarding identification, expression, and characterization of enzymes with antibacterial activity. In close corporation with Ph.D. student Krunal Mehta and Postdoc Elena Paskaleva, I was involved in two such research projects, regarding expression and characterization of novel enzymes with antibacterial activity against *Staphylococcus aureus* and *Bacillus anthracis*. The identification of potential antibacterial enzymes by sequence homology studies and construction of the associated expression plasmids were completed prior to the initiation of my work. All results and observations presented herein are obtained by undersigned, and after my leave, the work continued at RPI to characterize the identified antibacterial enzymes further. My work is expected to result in the co-authorship of the resulting two publications.

The stay was financially supported by the Department of Engineering, Aarhus University, and by travel grants from Foundation Idella, Knud Højgaards Foundation, and IDA og Berg Nielsens Studie- og Støttefond.

A special acknowledgement is extended to Professor Jonathan Dordick and Assoc. Professor Zheng Guo, for giving me the opportunity to expand my research experience in the microbiology and molecular biology areas.

Aarhus, August 2014

Mia Falkeborg

LIST OF ABBREVIATIONS

CBD:	Cell-wall binding domain
CFU:	Colony forming units
BLAST:	Basic local alignment search tool
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
BCA:	Bicinchoninic acid (protein assay)
IPTG:	Isopropyl-β-D-thiogalactopy-ranoside
EDTA:	Ethylenediaminetetraacetic acid
OD_{600}:	Optical density at 600 nm
PBS:	Phosphate buffered saline, referring to 150 mM sodium/potassium phosphate buffer at pH 7.4
NPB:	Native purification buffer, referring to 50 mM sodium phosphate buffer at pH 8.0 containing 350 mM NaCl

A3.1 INTROCUTION

A3.1.1 PEPTIDOGLYCAN HYDROLASES AS ANTIBACTERIALS

By means of selective and rapid killing of pathogenic bacteria, recombinant peptidoglycan hydrolases can be used as antibacterial agents (Borysowski et al. 2006). They offer a range of advantages over common antibiotics, particularly in terms of high specificity towards single strains, or species, of (pathogenic) bacteria, with no effect on the commensal microflora; as well as a low risk of bacterial resistance. This study was focused on expressing and characterizing novel peptidoglycan hydrolases with lytic activity against *Staphylococcus aureus* and *Bacillus anthracis*.

A3.1.2 PEPTIDOGLYCAN HYDROLASES

Peptidoglycan hydrolases are lytic enzymes that degrade bacterial peptidoglycan. Peptidoglycan is the main structural component of the bacterial cell wall, composed of glycan (an aminosugar polymer of alternating N-acetylglucosamine and N-acetylmuramic acid, joined by β-1,4 glycosidic bonds) covalently linked to peptide side chains through amide bonds. Opposing peptides on separate glycan polymers may be cross-linked through interpeptide bridges (in gram-positive bacteria) or interpeptide bonds (in gram-negative bacteria) (Borysowski et al. 2006; Nelson et al. 2012).

Based on their origin, peptidoglycan hydrolases are classified as autolysins, exolysins, or endolysins. Autolysins are produced by bacteria for growth and repair of its own peptidoglycan, while exolysins are secreted by bacteria to lyse the peptidoglycan of other bacteria. Endolysins are encoded by bacteriophages and are produced during the late phase of the phage lytic cycle, i.e. after phage replication inside the bacterial host, for release of the progeny phages (Nelson et al. 2012).

Lysins are modular proteins, with one or more catalytic domain(s) at the N-terminus and a cell wall binding domain (CBD) at the C-terminus (Borysowski et al. 2006). The CBD recognizes epitopes on the cell wall of susceptible bacteria, giving rise to strain- or species-specific binding (Fischetti 2008; Nelson et al. 2012). Depending on its nature as a glycosidase, peptidase, or amidase, the catalytic domain of peptidoglycan hydrolases cleaves the aminosugar moieties, the peptide moiety, or breaks the amide bond, respectively, of the peptidoglycan. Additional catalytic domains have been identified, including non-hydrolases (Borysowski et al. 2006).

A3.1.3 *STAPHYLOCOCCUS AUREUS*

S. aureus is a gram-positive coccus. It is a human pathogen capable of causing a variety of health problems, and treatment of *S. aureus* infections can be difficult due to the resistance to common antibiotics showed by many strains (Durai et al. 2010; Mehta et al. 2013).

Lysostaphin is a well-studied exolysin with activity against *S. aureus*. Lysostaphin is secreted by *S. simulans* and cleaves the peptidoglycan of *S. aureus*, without affecting the peptidoglycan of *S. simulans*. The CBD of lysostaphin is an SH3 domain, and the catalytic domain, termed M23, belongs to the peptidase family. In this study, the SH3-CBD of lysostaphin was used to probe the genome of *S. aureus* and its phages (BLAST) to identify novel lytic enzymes with specificity for *S. aureus*. A series of novel enzymes was identified, and the first focus of this study was to express and characterize one of these novel enzymes and compare its activity to that of lysostaphin.

A3.1.4 *BACILLUS ANTHRACIS*

B. anthracis is a gram-positive, spore-forming bacillus. The spore form of *B. anthracis* is classified as a category A biological weapon (Öncü et al. 2003). The dormant spores are very durable, and once inhaled, the spores germinate and release toxins into the blood, killing up to 99% of untreated victims. Antibiotic-resistant strains have been identified, and it is believed,

that antibiotic resistance could be intentionally engineered, which increases the need for an antibiotic-independent treatment of *B. anthracis* (Schuch et al. 2002).

In 2002, Schuch et al. (2002) identified the first lysin with potent lytic effect on *B. anthracis*, and named it PlyG after its origin in the *γ*-phage. They showed that the PlyG lysin specifically kills *B. anthracis* in vitro and in vivo, and that both vegetative cells and germinating spores were susceptible to the treatment. They concluded that PlyG could be a tool for the treatment of *B. anthracis*. In 2006, the same group published the finding of another lytic enzyme against *B. anthracis*, named PlyPH due to its activity in a broad pH-range, which was identified through a BLAST search of genomes of *B. anthracis* strains with the amino acid sequence of PlyG as the query sequence (Yoong et al. 2006). PlyPH and PlyG only show homology in their CBDs; their catalytic domains differ (PlyPH is a muramidase and PlyG is an amidase).

Previously to this study, research at RPI had led to the identification of a lysin named AmiBA2446; an N-acetylmuramoyl-L-alanine amidase with a CBD homologous to PlyPH (Mehta et al. 2013). However, AmiBA2446 and PlyPH are only active against cells in their growing phases, whereas cells in their stationary phases are not affected by the enzymatic treatment. Activity against *Bacillus* cells in all life stages is an important feature, if these enzymes are to be used in the treatment of bacterial infections. It was hypothesized, that the low activity towards stationary cells was due to a change in the cell surface in the stationary phase compared to the growth phase. To find an enzyme with activity against cells in their stationary phases, the catalytic domain of PlyPH was used as a probe to search for peptidoglycan hydrolases with different binding domains. A novel enzyme was identified, and named PlyBeta after its origin in the *β*-phage. Second part of this study was focused on expressing PlyBeta and characterization of its activity against *B. cereus* 4342 cells in growth and stationary phases. *B. cereus* 4342 is a commonly used surrogate for pathogenic *B. anthracis* strains (Greenberg et al. 2010).

A3.2 SUMMARY OF MAIN RESULTS

A3.2.1 EXPRESSION AND CHARACTERIZATION OF ANTIMICROBIAL ENZYMES – *S. AUREUS*

In an attempt to find novel enzymes with antimicrobial activity against *S. aureus*, the genomes of *S. aureus* and its phages were probed with the sequence of the SH3 binding domain of lysostaphin. Novel enzymes were identified and termed SA1-SA4 (Figure A2.1); focus of current work was limited to the SA1 enzyme. SA1 and lysostaphin had similar binding domains (SH3), but SA1 contained a putative amidase domain (PGRP) and a CHAP domain instead of the M23 catalytic domain of lysostaphin.

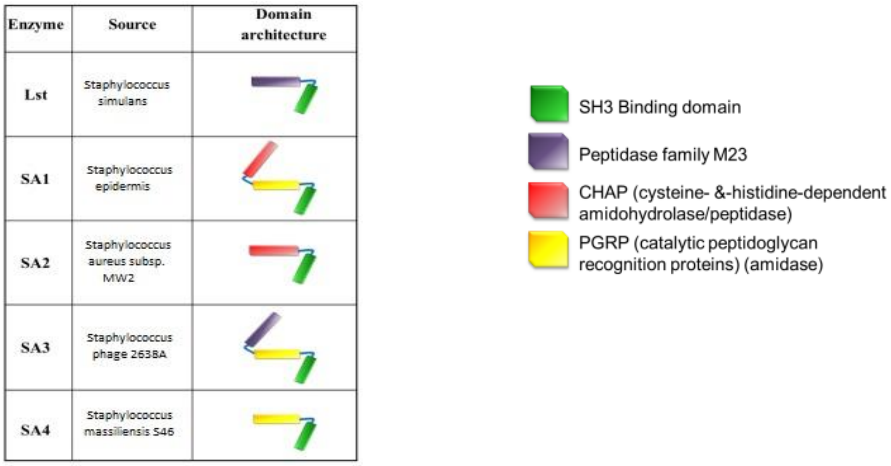

Figure A2.1: Modular structures of lysostaphin and SA1-SA4.

Histidine-tagged SA1 was successfully expressed in soluble form by IPTG-induced expression in *E. coli* expression-cells and purified from the cell lysate under native conditions using nickel affinity chromatography. The final concentration of SA1 after purification and concentration with ultracentrifugation was determined using the standard bicinchoninic acid (BCA) protein

assay to be 0.10 mg/mL. The bacteriolytic activity of the soluble cell lysate containing the SA1, and the purified SA1, was tested on *S. aureus* cells by a 'plating assay', shortly as follows. *S. aureus* cells were grown in nutrient media overnight, harvested, washed with PBS buffer to remove the nutrient media, and diluted to a final concentration of 10^7 colony forming units (CFU)/mL. In 1 mL sterile tubes, 450 µL sample (soluble cell lysate, or purified SA1 at 0.10 mg/mL) was combined with 50 µL *S. aureus* cells, leading to a final cell concentration 10^6 CFU/mL. The samples were incubated for 3 hours at room temperature at 200 rpm and then plated onto nutrient agar for colony enumeration; 100% viable cells was defined as the number of viable colonies resulting from treatment with 450 µL buffer.

As shown in Figure A2.2, the SA1 soluble cell lysate showed >3-log (99.9%) killing against 10^6 CFU/ml of *S. aureus*; however, once purified, SA1 only reduced the number of viable cells less than 1-log.

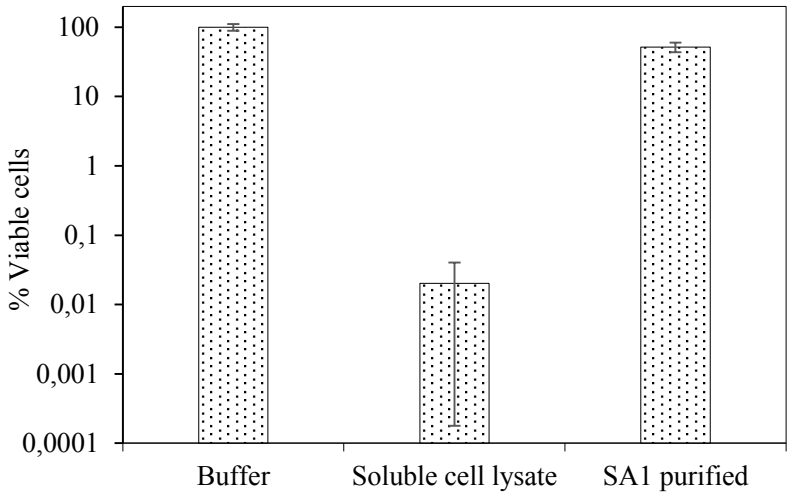

Figure A2.2: Viable cells after treatment with soluble cell lysate containing SA1, or purified SA1.

Cell lysates are reducing environments, and it was hypothesized that the loss in reducing environment during purification led to formation of inactivating disulfide bonds in SA1. To overcome this, *β*-mercaptoethanol was included in the treatment of the *S. aureus* cells to reduce such disulfide bonds. As shown in Figure A2.3, the purified SA1 showed >2-log (99%) killing

against 10^6 CFU/ml of *S. aureus* in presence of 1.25 mM *β*-mercaptoethanol, and at higher concentrations of *β*-mercaptoethanol, no viable cells were observed (indicated by stars, 100% killing, 6-log reduction). This is comparable to the effect of treatment with lysostaphin (data not presented). For later reference it is noted, that the occurrence of one colony would reflect a calculated >4-log reduction in viable *S. aureus* cells, and the occurrence of three colonies would reflect a calculated >3-log reduction. Occurrence of 0-3 viable colonies was generally accepted as day-to-day variations in this assay.

Controls using only buffer (i.e. no SA1) confirmed that *β*-mercaptoethanol alone, in the concentrations employed in this study, did not kill the *S. aureus* cells (buffer controls in Figure A2.3).

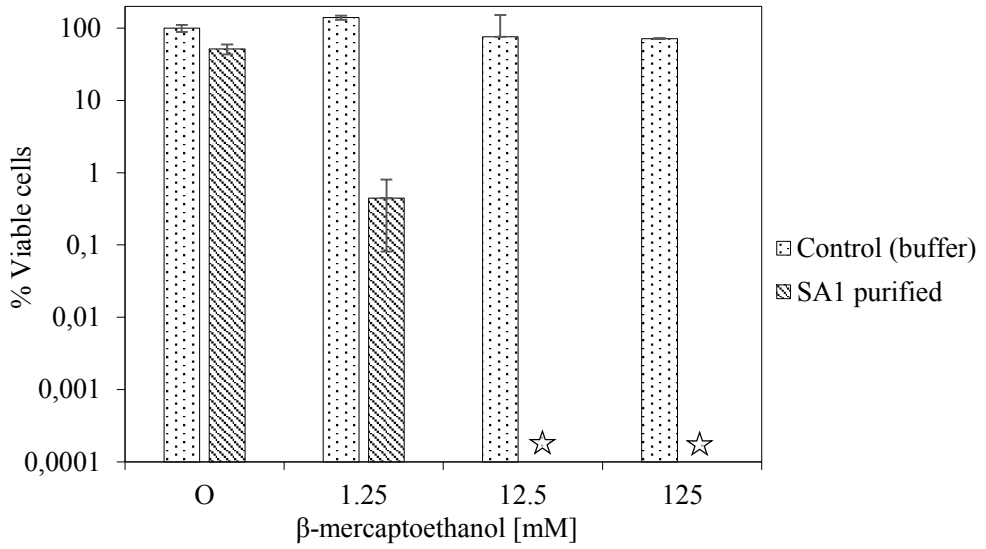

Figure A2.3: Viable cells after treatment with purified SA1 with varying concentrations of β-mercaptoethanol, and β-mercaptoethanol with no SA1.
Stars indicate no viable colonies (100 % killing).

The observations obtained from the plating assays were confirmed by a 'cell degradation assay', in which the optical density at 600 nm (OD_{600}) was determined over time of samples containing varying combinations of *S. aureus* cells and purified SA1, and/or β-mercaptoethanol. Shortly, in a total volume of 1 mL, *S. aureus* cells were combined with purified SA1 and/or *β*-mercaptoethanol, or buffer alone as control; and the OD_{600} of these samples were determined over time. The decrease in OD_{600} reflects the decrease in viable cells, *cf*. Mayer et al. (2008).

The results are presented in Figure A2.4, which shows that purified SA1 alone (triangles), and *β*-mercaptoethanol alone (squares), only affected the *S. aureus* cells to a similar extent as the buffer alone (diamonds); and only the combination of SA1 and *β*-mercaptoethanol led to a decrease in OD_{600} (circles).

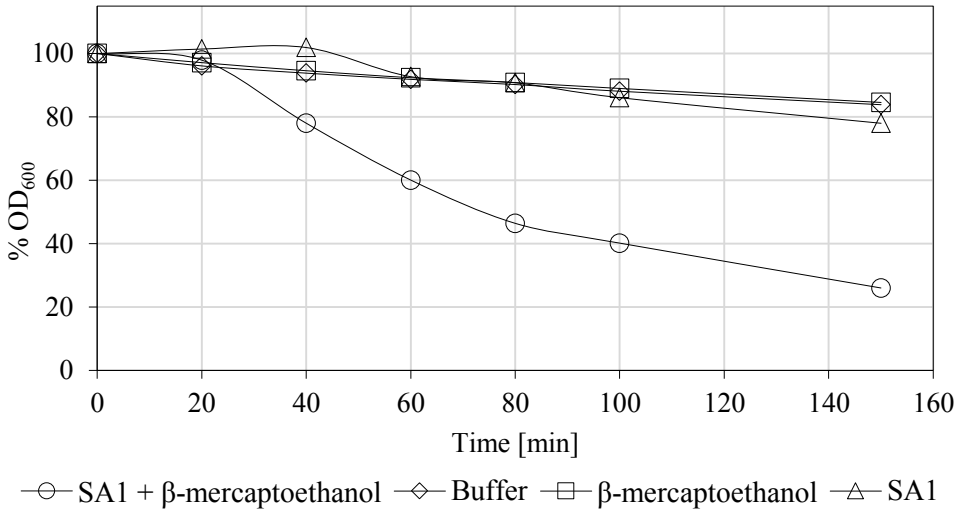

Figure A2.4: Cell degradation assay. % OD_{600nm} over time of S. aureus cells treated with SA1 and/or β-mercaptoethanol.

The effect of *β*-mercaptoethanol was hypothesized to be due to its influence on the SA1 enzyme, most likely through the reduction of disulfide bonds in the purified enzyme. However, the effect of *β*-mercaptoethanol could also be exerted directly on the *S. aureus* cells, e.g. by altering the cell surface, making it more susceptible to enzymatic degradation. To test this latter hypothesis, a set of experiments were designed, in which the *S. aureus* cells were pretreated with 125 mM *β*-mercaptoethanol (or buffer for control) for three hours, after when the *β*-mercaptoethanol was carefully removed by repeated pelleting and suspension of the cells in PBS buffer. These pretreated cells were then subjected to treatments with SA1 in the presence or absence of 125 mM *β*-mercaptoethanol. The results are presented in Figure A2.5 (*β*-mercaptoethanol pretreatment) and Figure A2.6 (control, buffer pretreatment).

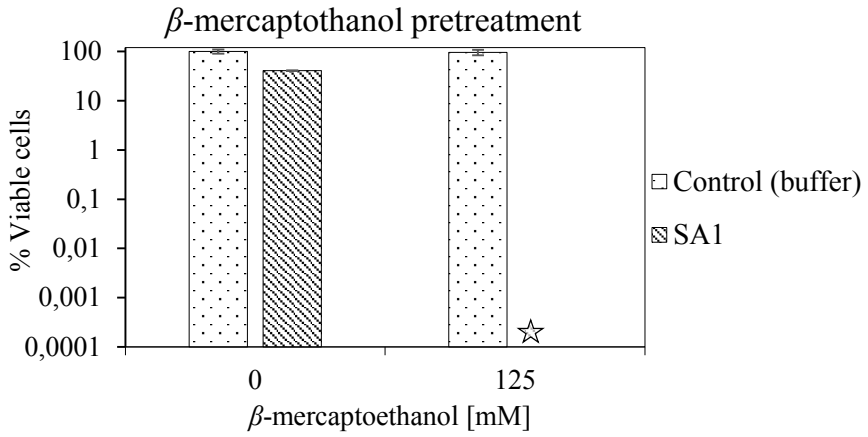

Figure A2.5: Viable cells [%], pretreated with β-mercaptoethanol, after treatment with SA1 or buffer with 0 or 125 mM β-mercaptoethanol.
The star indicates no viable colonies (100 % killing).

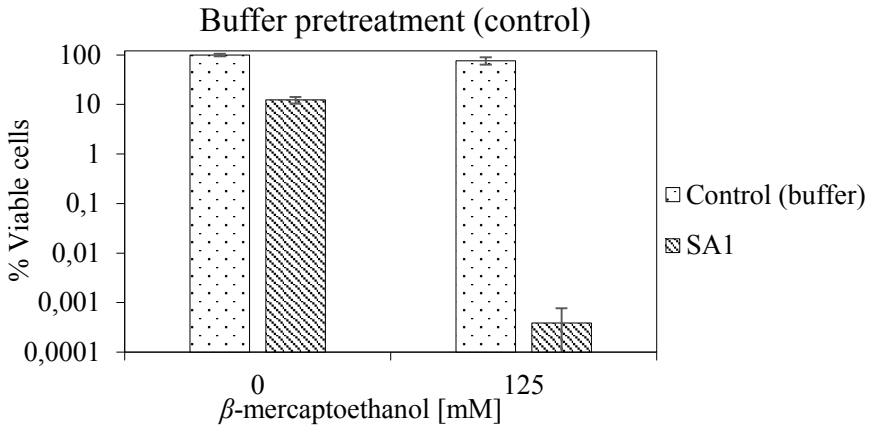

Figure A2.6: Viable cells [%], pretreated with buffer, after treatment with SA1 or buffer with 0 or 125 mM β-mercaptoethanol.

No difference was observed between the two pretreatments. SA1 alone, and *β*-mercaptoethanol alone, only reduced the *S. aureus* cells by 1-log; whereas the combination of SA1 and *β*-mercaptoethanol (125 mM) reduced the *S. aureus* cells by >5-log, as observed when the non-pretreated cells. The findings were confirmed with a cell degradation assay as presented in Figure A2.7.

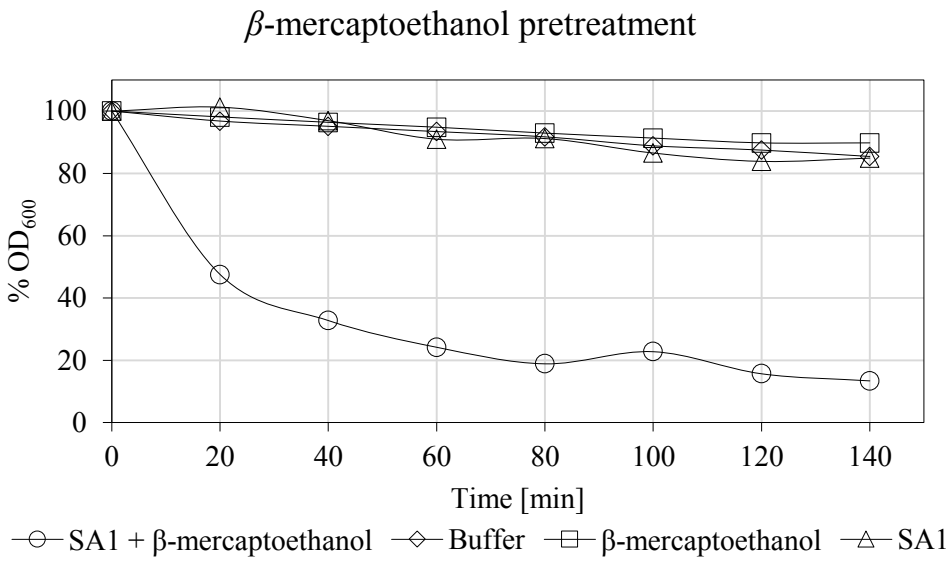

Figure A2.7: Cell degradation assay. % OD_{600nm} over time of S. aureus cells pretreated with β-mercaptoethanol, treated with SA1 and/or β-mercaptoethanol or buffer for control.

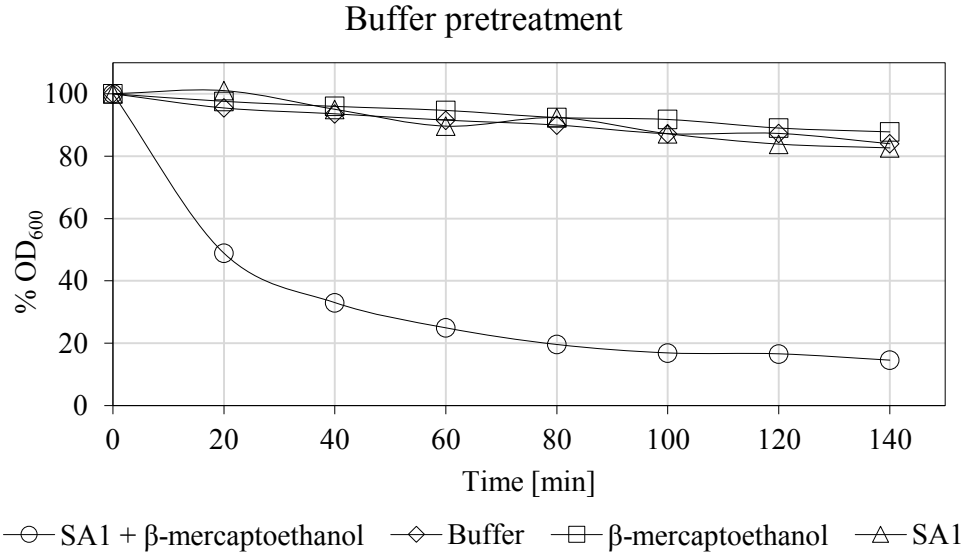

Figure A2.8: Cell degradation assay. % OD_{600nm} over time of S. aureus cells pretreated with buffer, treated with SA1 and/or β-mercaptoethanol or buffer for control.

As in the plating assay, no difference was observed between the two pretreatments. Only the combination of SA1 and *β*-mercaptoethanol exerts a bacteriolytic effect on the *S*. *aureus*, irrespective of the pretreatment of the *S*. *aureus* cells. It can hence be concluded, that *β*-mercaptoethanol does not affect the surface of the *S*. *aureus* cells.

In an attempt to confirm that the effect of *β*-mercaptoethanol was due to its effect on the SA1, SA1 was incubated in 125 mM *β*-mercaptoethanol for 3 hours, followed by dialysis against NPB buffer to remove the *β*-mercaptoethanol. EDTA was included in this system, as EDTA was hypothesized to prevent reformation of the disulfide bridges by chelating the metal ions that could catalyze oxidation of the cysteines. The results are presented in Figure A2.9. It was confirmed that EDTA did not influence the extent of killing of the *S*. *aureus* cells. EDTA alone (bar 2) showed similar killing as buffer alone, and SA1 with EDTA (bar 3) showed similar killing as SA1 alone (i.e. > 1-log reduction). Treatment with SA1 and 125 mM *β*-mercaptoethanol led to a 3-log reduction in viable *S*. *aureus* cells (bar 4), i.e. in a similar range as previously observed without EDTA. However, once *β*-mercaptoethanol was removed from the SA1 through dialysis, the bacteriolytic activity decreases. After a 1000x decrease in *β*-mercaptoethanol concentration (125 μM, bar 5), >2-log reduction in viable cells was observed; and after an additional 1000x decrease in *β*-mercaptoethanol concentration (125 nM, bar 6), the SA1 only showed same bacteriolytic activity as before treatment with *β*-mercaptoethanol (1-log reduction).

It is concluded that once the *β*-mercaptoethanol has been removed, the broken disulfide bridges in the SA1 reform, leading to a loss in activity. Preliminary experiments (not presented herein) using SDS-PAGE analysis of purified SA1 with and without *β*-mercaptoethanol indicated that multimeric complexes of SA1 formed in the absence of *β*-mercaptoethanol. Work is now ongoing at RPI to elucidate these observations.

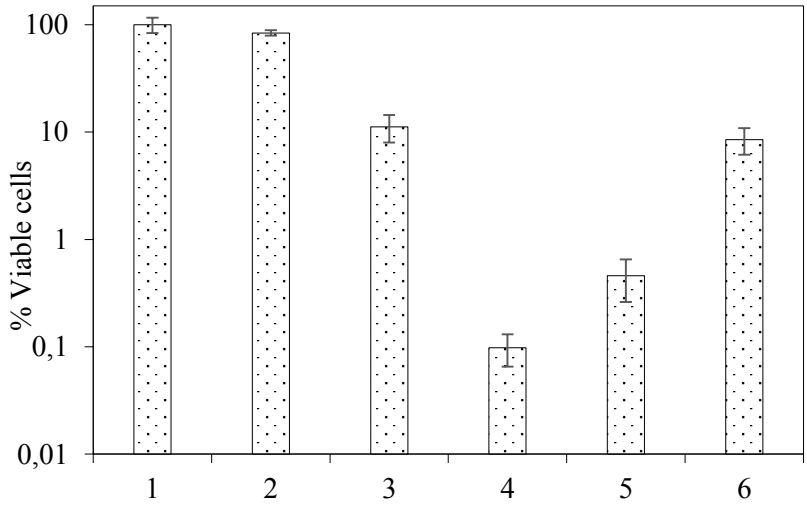

*Figure A2.9: Viable cells after treatment with **1**: NPB buffer (control), **2**: NPB buffer with 10 mM EDTA, **3**: SA1 with 10 mM EDTA, **4**: SA1 treated with 125 mM β-mercaptoethanol and 10 mM EDTA, **5**: SA1 after 1000x dialysis (125 µM β-mercaptoethanol and 10 mM EDTA), **6**: SA1 after additional 1000x dialysis (125 nM β-mercaptoethanol and 10 mM EDTA).*

Mutating the binding domain of SA1 in position 429 from a cysteine to serine was hypothesized to overcome the dependence of SA1 for *β*-mercaptoethanol, as this cysteine might participate in a cysteine bridge, which was broken in the presence of *β*-mercaptoethanol. Hence, the SA1 variant "SA1(C429S)" was constructed and expressed by IPTG-induced expression in *E. coli* expression-cells. The expression temperature and time, as well as the IPTG concentration, were optimized for high expression of soluble enzymes (Table A2.1 rows 1-9). The degree of expression and solubility was determined by SDS-PAGE analysis of the total cell lysates and the soluble cell lysates; in Table A2.1, the intensities of the respective protein bands in the gels are symbolized by '+'. After a series of experiments (rows 1-7 in Table A2.1) optimal expression of soluble SA1(C429S) was observed at overnight expression at 15°C using 20 µM IPTG (experiment 7). Scale-up of the expression system, however, led to overexpression of insoluble protein (experiment 8 and 9). During purification of the histidine-tagged SA1(C429S) from the soluble cell lysate in experiment 7, the protein was lost in the column flow through. The soluble cell lysates containing soluble enzymes did not have any bacteriolytic effect on *S. aureus* cells (0% killing, not presented). Work is now ongoing at RPI to evaluate this mutant

further in terms of sequence (has the correct sequence been synthesized), folding (is the enzyme folding correctly), formation of multeric complexes, etc.

To examine the functions of the individual domains of SA1, four additional constructs composed of the individual domains in various combinations (Figure A2.10) were attempted expressed.

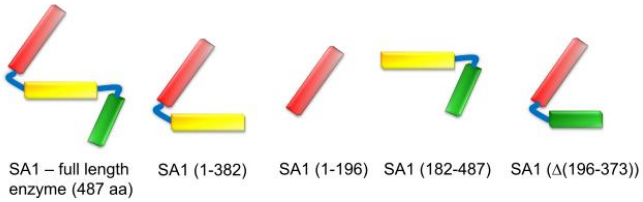

Figure A2.10: Variants of SA1 – refer to Figure A2.1 for color codes.

As for SA1(C429S), successful expression of these variants was challenging, and optimization of the expression variables was required. A summary of this expression optimization study is given in Table 1 rows 10-35.

Only SA1(1-196) was successfully expressed in soluble form, but this domain did not have antibacterial activity in itself (0% killing). SA1(1-382), SA1(Δ196-373), and SA1(182-487) were not successfully expressed in soluble form during the course of this study. Work is ongoing at RPI to examine the reasons for this, which could be that parts of the gene mistakenly were missing, the enzymes folded incorrectly, the gene was synthesized with an inaccurate reading frame, or other reasons.

*Table A2.1: Summary of expression conditions of SA1 variants. RT = Room temperature. ON = Overnight. *Optimized conditions. LS: Large scale (expression in 1 L medium).*

Enzyme	Experiment number	IPTG [μM]	Temperature [°C]	Time [hours]	Expression	Solubility
SA1 (C429S)	1	100	RT	4	++++	-
55 kDa	2	20	RT	4	++++	-
	3	5	15	4	+	-
	4	5	15	ON	+	+
	5	20	15	4	++	+
	6	5	RT	4	++	+
	7*	20	15	ON	+++	++
	8 (LS)	20	15	ON	++++	-
	9 (LS)	20	15	ON	++++	-
SA1 (1-196)	10	50	15	4	-	-
22 kDa	11	20	15	8	+	-
	12	20	15	ON	++	+
	13	10	RT	4	++	+
	14	10	RT	4	++	+
	15	10	RT	4	++	+
	16	100	RT	4	++++	+
	17*	20	RT	4	++++	++
SA1 (1-382)	18	100	RT	4	++++	-
43 kDa	19	20	RT	4	++++	-
	20	50	15	4	+	-
	21	10	15	8	+	-
	22	10	15	ON	++	-
	23	5	RT	4	++	-
	24	10	RT	4	+	-
	25	10	RT	ON	++	-
	26	5	15	ON	+	-
	27	5	15	ON	+	-
SA1 (182-487)	28	100	RT	4	++++	-
	29	20	RT	4	++++	-
35 kDa	30	50	15	4	-	-
	31	50	15	4	+	-
SA1 (Δ196-373)	32	100	RT	4	++++	-
	33	20	RT	4	++++	-
35 kDa	34	50	15	4	-	-
	35	50	15	4	+	-

In summary, SA1 was successfully expressed and purified in soluble form, but only the soluble cell lysate had antibacterial activity, whereas the purified SA1 had lost its activity. Loss of reducing environment during purification is believed to be the reason, and activity could be regained in the presence of *β*-mercaptoethanol. The effect of *β*-mercaptoethanol was exerted on the SA1, and not on the bacterial cell walls, as determined during the pre-treatment studies. Work is ongoing to elucidate this observation, and to design a method to overcome the need for *β*-mercaptoethanol in the treatment.

Several variants of the SA1 were attempted expressed, with varying extents of success. The mutant SA1(C429S) was expressed, but not purified, and the soluble cell lysate containing SA1(C429S) did not show antibacterial activity. Attempts were made to express the individual domains of SA1, alone or in combination, to examine their activities. SA1(1-382), SA1(Δ196-373), and SA1(182-487), were not successfully expressed by IPTG-induced expression in *E. coli* expression-cells. Work is ongoing at RPI to express these enzymes, if possible, and characterize their antibacterial activity. SA1(1-196) was expressed in soluble form, but this domain did not have antibacterial activity in itself (0% killing).

A3.2.2 EXPRESSION AND CHARACTERIZATION OF ANTIMICROBIAL ENZYMES – *B. ANTHRACIS*

PlyBeta was expressed in *E. coli* expression cells induced with 0.5 mM IPTG for 4 hours at room temperature. The enzyme was successfully purified from the soluble cell lysate under native conditions using nickel affinity chromatography, as evidence by SDS-PAGE analysis (Figure A2.11). PlyBeta was then dialyzed and concentrated using ultrafiltration with a 10-kDa cut-off membrane to a final concentration of 0.66 mg/mL.

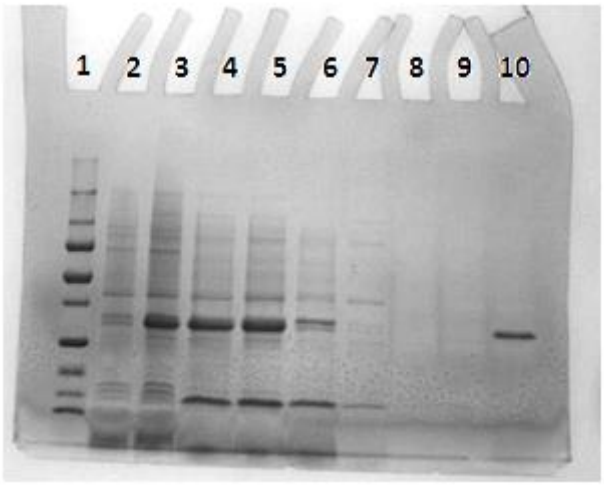

*Figure A2.11: SDS-PAGE analysis. **1.** Marker. **2**. Before IPTG induction. **3**. After IPTG induction. **4**. Total cell lysate. **5**. Soluble cell lysate. **6.** Purification flow through. 7. Purification wash one. **8.** Purification wash two. **9.** Purification wash three. **10**. Purification elution.*

The bacteriolytic activity of the concentrated PlyBeta was tested against *Bacillus cereus*, which represents a model organism for *B. anthracis*. *B. cereus* cells were grown in standard medium and harvested at different growth stages. The cell density at each stage was determined by OD_{600} measurements. The cells were washed with PBS buffer and diluted to 10^7 CFU/mL. Fifty µL cells were treated with 450 µL concentrated PlyBeta leading to a final cell concentration was 10^6 CFU/mL, in 1 mL sterile tubes, which were incubated for 3 hours at room temperature and

plated onto nutrient agar for colony enumeration. The cell density and effect of PlyBeta at each stage is depicted in Figure A2.12.

Treatment with PlyBeta only led to a <1-log reduction in viable cells (~90% killing), however, oppositely to PlyPH and AmiBA2446, PlyBeta was active against *B. cereus* cells in both their growth and stationary phases. This promising observation forms the basis for the continuous work at RPI to analyze the activity of PlyBeta under varying conditions.

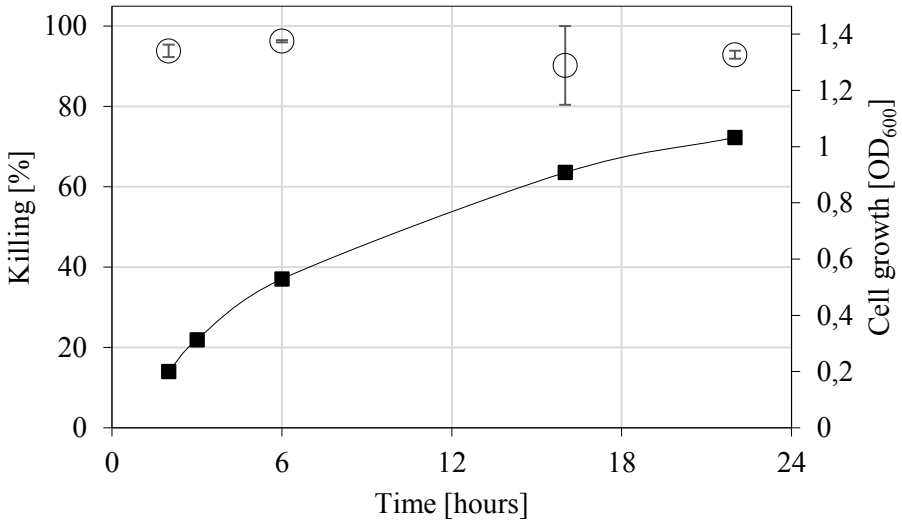

Figure A2.12: Cell density (OD_{600}, squares) and influence of PlyBeta treatment (% killing of B. cereus, circles) after varying times of cell growth.

A3.3 CONCLUSION

Infections with *B*. *anthracis* and *S*. *aureus* represent threats that cannot be readily overcome using common antibiotics, and alternatives are required in order to treat and/or prevent such infections. The use of peptidoglycan hydrolases provides a platform for the generation of new systems to combat the problems with *B*. *anthracis* and *S*. *aureus*, and other pathogenic bacteria. In this study, peptidoglycan hydrolases with activity against *B*. *anthracis* and *S*. *aureus* were expressed and characterized, and promising observations were made. Treatment with SA1, in the presence of *β*-mercaptoethanol, was effective in killing *S*. *aureus* cells, and SA1 represents a promising new agent to use in the treatment and prevention of *S*. *aureus* infections. The dependency of SA1 for *β*-mercaptoethanol presents a challenge, and work is ongoing to elucidate the reasons for this dependency, and to find ways to overcome it.

Several antibacterial enzymes with activity against *B*. *anthracis* have been identified, but they only have activity against *B*. *anthracis* in their growing phases. In this study, PlyBeta was expressed and characterized for its antibacterial activity, and though it only showed a low antibacterial effect (~1-log killing), the activity was independent of the life cycle of the *B*. *cereus* cells, serving as model cells for *B*. *anthracis*. Further studies on PlyBeta can give information on the life-cycle dependency observed for treatment with other antibacterial enzymes, and from such studies, ways to overcome this life-cycle dependency can be found. As for the study with *S*. *aureus*, this study was only a part of larger ongoing projects, and the obtained results presented herein form the basis for further analyses, currently ongoing.

A3.4 EXPERIMENTAL METHODS

A3.4.1 PROTEIN EXPRESSION AND PURIFICATION

E. coli strain BL21 Star (DE3) was transformed with plasmids: pGS-21a/SA1, pGS-21a/SA1(C429S), pGS-21a/SA1(1-196), pGS-21a/SA1(1-382), pGS-21a/SA1(182-487), pGS-21a/SA1(Δ196-373), pGS-21a/SA3(CD-LstBD), or pGS-21a/PlyBeta, and grown on LB-agar plates with 100 μg/mL ampicillin; LB-media containing (in grams per liter): tryptone 10, yeast extract 5, and NaCl 10. Overnight cultures were grown from single colonies at 37˚C and 220 rpm in 4 mL LB medium containing 100 μg/mL ampicillin and subcultured into 250 mL (or 1 L for large scale) LB-medium containing ampicillin and grown to reach an OD_{600} of 0.4-0.6 (SpetraMax M5 plate reader). Protein expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopy-ranoside) followed by incubation according to Table A2.1. Cells were harvested by centrifugation at 4,000 rpm for 10 min at 4°C and resuspended in 5 mL native purification buffer (NPB, 50 mM sodium phosphate buffer at pH 8.0 containing 350 mM NaCl). After one freeze-thaw cycle (-80˚C and 42˚C) the cell suspensions were incubated with lysozyme (100 μg/ml) and DNase (10 μg/ml), followed by probe-sonication for 5 min at 20% amplitude using a 5 sec on / 5 sec off cycle. After an additional freeze-thaw cycle, the cell suspensions were clarified for 20 min at 4,000 rpm at 4˚C. The histidine-tagged proteins were purified from the soluble cell lysate under native conditions using nickel-chelating resins and dialyzed against NPB. Successful expression, solubility, and purification of the recombinant enzymes were evidenced by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis under reducing conditions, as summarized in Table A2.1. Protein concentrations were determined using the bicinchoninic acid (BCA) standard protein assay (Pierce BCA Protein Assay) using bovine serum albumin as reference.

A3.4.2 BACTERIOLYTIC ACTIVITY AGAINST *S. AUREUS*.

A3.4.2.1 Plating assay

Staphylococcus aureus ATCC 33807 was stored at -80°C in 20% (vol/vol) glycerol. Overnight cultures were grown from the glycerol stocks at 37°C and 220 rpm in 4 mL NB medium containing (in grams per liter): beef extract 3, and peptone 5. Fresh NB-medium (4 mL) was inoculated with the overnight cultures, and cells were grown at 37°C to reach an optical density corresponding to approximately 10^9 CFU/mL (1 unit OD_{600} corresponded to 10^9 CFU/mL for *S. aureus*). One mL cell culture was centrifuged at 12,000 rpm for 3 min; the pellet was washed with and resuspended in phosphate-buffered saline (150 mM, pH 7.4, PBS), and diluted to 10^7 CFU/mL. In 1 mL sterile tubes, 450 µL of sample (soluble cell lysates of SA1 and SA1 variants, purified SA1, *β*-mercaptoethanol or EDTA in NPB, or NPB for control) was combined with 50 µL *S. aureus* cells (final cell concentration was 10^6 CFU/mL). The cells were incubated for 3 hours at room temperature, after when they were appropriately diluted and plated onto nutrient agar for overnight colony growth overnight at 37°C. The viable colonies were enumerated; 100 % viable cells were defined as the number of viable colonies resulting from treatment with buffer.

A3.4.2.2 Cell degradation assay

The lysis of *S. aureus* cells was additionally assessed based on the method described by Mayer *et al*. (2008). Cells of *S. aureus* were grown to mid-log phase and 2-mL aliquots were harvested by centrifugation (12,000 g for 3 min) in sterile capped tubes. Pellets were resuspended in 1 mL PBS, and 150-µL aliquots were added to 850 µL of either SA1, *β*-mercaptoethanol, SA1 and *β*-mercaptoethanol, or NPB buffer, and incubated at room temperature for 150 minutes; the OD_{600} was determined periodically to determine the degree of cell lysis.

A3.4.2.3 Pretreatment of *S. aureus* cells

S. aureus cells were grown overnight in NB-media. 3-mL aliquots were harvested and the cells were isolated by centrifugation and resuspended in 1 mL PBS, containing 0 or 125 mM β-mercaptoethanol, and incubated at room temperature for 3 hours at 200 rpm. The cells were then harvested by centrifugation and washed four times with 1 mL PBS, and subjected to the plating assay and cell degradation assay as described above.

A3.4.2.4 Pretreatment of SA1 enzyme

SA1 (0.10 mg/mL) was incubated with 10 mM EDTA and 125 mM β-mercaptoethanol (total volume 10 mL) for 3 hours at 4°C and 200 rpm, followed by dialysis against 2 x 1L NPB buffer containing 10 mM EDTA. The bacteriolytic activity of the SA1 before, and after each dialysis, was tested by the plating assay as described above.

A3.4.3 BACTERIOLYTIC ACTIVITY AGAINST *BACILLUS* SPECIES

B. cereus ATCC 4342 was stored at - 80°C in 20% (vol/vol) glycerol. Overnight cultures were grown from the glycerol stocks at 37°C and 220 rpm in 4 mL NB medium. Fresh NB-medium was inoculated with the overnight cultures, and cells were grown at 37°C to reach the required cell density (varying, according to Figure A2.12). One mL cell culture was centrifuged at 12,000 rpm for 3 min; the pellet was washed with and resuspended in PBS buffer. The number of bacterial cells was determined by optical density measurements; 1 unit OD_{600} corresponded to 2 x 10^8 CFU/mL for *B. cereus*. To test the bacteriolytic activity of PlyBeta, 450 µL of purified PlyBeta was combined with 50 µL cells in various growth stages (final concentration of cells was 10^6 CFU/mL in all experiments), and incubated for 3 hours at room temperature and 200 rpm. Following this, the cells were appropriately diluted in PBS, plated onto NB-agar plates, and incubated overnight at 37°C, followed by colony enumeration.

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