 DOI: 10.1002/masy.200950303

Oligoagarose-Graft-Polycaprolactone Copolymers: Synthesis and Characterization

Archana Bhaw-Luximon,[*1] *Dhanjay Jhurry,*[1] *Mooneerah Heerah Booluck,*[1] *Gaëlle Correc,*[2] *Sabine Génicot,*[2] *William Helbert*[2]

Summary: In this paper, we combine hydrophilic oligoagarose ($DP_n = 10–15$), issued from enzymatic degradation of the natural biopolymer agarose, with polycaprolactone, a synthetic biodegradable and hydrophobic polyester. To synthesize these amphiphilic graft copolymers, we use partially acetylated oligoagarose as macroinitiator in combination with tin (II) octanoate for the bulk polymerization of ε-caprolactone. The grafting was confirmed by NMR and SEC which showed a monomodal distribution. After removal of the acetyl protecting groups, copolymers with 30–60% free hydroxyl groups were soluble in water and insoluble in chloroform, thus indicating that they probably adopt micelle-like structures in aqueous solution with a PCL hydrophobic core and spherical oligoagarose side chains.

Keywords: biopolymers; graft copolymers; oligoagarose; poly(ε-caprolactone)

Introduction

It is well established that the surface properties of polymeric biomaterials affect their biocompatibility, including protein adsorption, cell adhesion, cytotoxicity, blood compatibility, and tissue compatibility.[1] The interactions between proteins and polymer surfaces are detrimental to the performance of biomaterials. Such interactions can be minimized by various techniques, amongst which the grafting of biomolecules or biodegradable polymer chains or natural polysaccharides leads to interesting results.[2] The combination of natural polysaccharides with synthetic polymers is an area receiving growing interest by polymer and biomaterials scientists. Natural polysaccharides with a high hydroxyl content generally show good enzymatic degradation behaviour and good biocompatibility.[3] Sagnella *et al.*[4] developed chitosan-based polymers consisting of a chitosan backbone and PEG and hexanal side chains that could be used to modify the surface of existing biomaterials in order to improve their blood compatibility. Ouchi *et al.*[5,6] developed polylactide-grafted dextrans using the trimethylsilyl (TMS) protection method. While poly-L-lactide films did not absorb water at all because of their high hydrophobicity, the graft copolymer films swelled immediately after immersion in PBS. The top surface of the Dex-*g*-PLA film was suggested to be covered with hydrophilic dextran segments, thus giving rise to the wettable surface. Such a wettable surface led to the suppression of cell attachment and protein adsorption onto the film. The copolymers exhibited better hydrophilicity and cell affinity compared to pure PLA. Nouvel *et al.*[7] reported on the grafting of polylactide on dextran using a three-step synthesis. The dextran molecule was first protected via silylation step and the remaining unprotected hydroxyl groups were used to ring-open lactide using catalytic amount of $Sn(Oct)_2$. The final step involved deprotection of the silylated OH groups. Depending on the number of PLA repeat units, PLA-grafted dextrans were soluble either in water or in organic solvents.

[1] Department of Chemistry, Faculty of Science, University of Mauritius, Réduit, Mauritius
E-mail: a.luximon@uom.ac.mu
[2] Equipe Structure des Polysaccharides Marins, UMR 7139 (CNRS-UPMC) Végétaux marins et Biomolécules, Station Biologique, 29682, Roscoff, France

Well-defined ethyl cellulose-graft-polycaprolactone copolymers were synthesized via ring-opening polymerization (ROP) of ε-caprolactone (CL) with an ethyl cellulose (EC) initiator and tin 2-ethylhexanoate ($Sn(Oct)_2$) catalyst in xylene.[8] Biodegradable amphiphilic copolymers based on polycaprolactone and chitosan were synthesised and were used to successfully prepare nanoparticles.[9] The PCL-graft-chitosan copolymers were synthesized by coupling the hydroxyl end-groups on preformed PCL chains and the amino groups present on 6-O-triphenylmethyl chitosan and by removing the protective 6-O-triphenylmethyl groups in acidic aqueous solution. The PCL content in the copolymers could be controlled in the range of 10–90 wt %. The copolymers were shown to form spherical or elliptic nanoparticles in water. Amphiphilic copolymers, based on dextran grafted with polycaprolactone side chains were synthesized by Lemarchand *et al.*[10] The copolymers could form nanoparticles with a well defined core-shell structure. By virtue of their amphiphilic nature, the copolymers were able to stabilize o/w emulsions without the need of additional surfactants.

Liu *et al.*[11] have reported on the use of microwave for the grafting of ε-caprolactone onto chitosan via phthaloyl protection method. The resulting chitosan-g-polycaprolactone copolymer had free hydrophilic amino groups and hydrophobic polycaprolactone chains, as confirmed by NMR.

Agar, which consists of a mixture of agarose and agaropectin,[12] is another interesting natural biopolymer originating from red seaweeds such as the *Gracilaria* species. Our group has reported previously on the extraction and characterization of agar from *Gracilaria* species collected around Mauritius.[13] We have attempted the grafting of biodegradable polymers such as polycaprolactone directly onto agar but little control is obtained over the reaction due primarily to the high molar mass of agar and its very low solubility in organic solvents. Instead, oligoagarose ($DP_n = 10–15$) which is obtained by enzymatic degradation of agar, appears to be a better starting material for controlled reactions. In this paper, we report on the synthesis and characterization of a range of oligoagarose-grafted polycaprolactone copolymers using the hydroxyl groups of oligoagarose as initiator for the bulk polymerization of caprolactone.

Experimental Part

Hydrolysis of Agar

Recombinant β-agarase B from *Zobellia galactanivorans* was purified according to Jam *et al.*[14]. Agarose (0.5% w/v in water, Eurogentec) was molten in a boiling water bath and then cool down to 40 °C to prevent the polysaccharide from gelling. 1L of this solution, referred to as melted agarose, was incubated with 500 μL of enzymes. The amount of reducing sugars released was assayed using a ferricyanide method adapted from that of Kidby and Davidson.[15] The solution was filtered through a 30 kDa membrane in order to remove undigested fraction. Oligoagarose samples were lyophilised and stored at room temperature.

Synthesis of Partially Acetylated Oligoagarose

Oligoagarose (0.46 g), acetic anhydride (4.8 ml) and pyridine (0.8 ml) were added in a glass tube at room temperature and stirred under nitrogen. After 3 h stirring, ice was added to the mixture to hydrolyse any unreacted acetic anhydride and the acetylated oligoagarose precipitated from cold methanol. It was obtained as a white residue and was dried under vacuum before characterization by NMR and SEC.

Synthesis of Graft Copolymers: Poly(O-acetyloligoagarose-g-caprolactone)

Acetylated oligoagarose (0.07 g) was measured in a glass tube. Tin(II) octanoate (17 mg) and toluene (0.5 ml) were added. The mixture was allowed to stir for 2 h at 40 °C under nitrogen before the addition of ε-caprolactone (0.133 g). Polymerization

was allowed to proceed at 100 °C for 20 h. The crude product was then dissolved in chloroform followed by precipitation in cold methanol. The precipitate was isolated, dried under vacuum and characterized by NMR and SEC.

Deprotection of Acetyl Groups of Graft Copolymers

Poly(O-acetyloligoagarose-*g*-caprolactone) was dissolved in mixed THF/CH_3OH solvent (v/v = 1/1) along with the addition of a catalytic amount of $NaOCH_3$ (pH = 8). After stirring at room temperature for 3 h, the deprotected graft copolymer was recovered by neutralization, precipitation, filtration and drying under vacuum. The resulting product was characterized by NMR and SEC.

Characterization

Oligo-agarose samples were analysed by High Performance Anion Exchange Chromatography with pulsed amperometric detection (HPAEC-PAD), using a Dionex chromatograph DX 500 equipped with a 50 μl injection loop, a CarboPac PA100 column (4 mm × 250 mm) and an electrochemical detector with a gold electrode. Analyses were performed according to a method adapted from Weinberger *et al.*[16] as follows. Elution was carried out at 1 ml.min^{-1} with a 30 min linear gradient of 0–300 mM sodium acetate in 150 mM NaOH. The column was calibrated with neoagarotetraose and neoagarohexaose standards (Dextra Laboratory).

NMR analysis was carried out in DMSO-d_6 or D_2O or $CDCl_3$ at room temperature on a FT Bruker Spectrometer 250 MHz. Size Exclusion Chromatography was performed using a Polymer Standards Systems (PSS) apparatus with a refractive index detector. Calibration was done using polystyrene standards and, THF and water were used as eluent.

Results and Discussion

Agar consists of a mixture of agarose and agaropectin. Agarose is a linear and neutral polymer of high molar mass. It is composed of agarobiose repeating disaccharide units with alternating 1,3-linked-β-D-galactopyranose and 1,4-linked-3,6-anhydro-α-L-galactoyranose (Figure 1). Agaropectin has a backbone similar to that of agarose but is slightly branched and sulfated and may have methyl and pyruvic acid ketal substituents. Agar is insoluble in cold water but soluble in boiling water. It has the ability to form firm and resilient gels with up to 99.5% of water upon cooling of a hot solution to 30–40°C. Agar is insoluble in most commonly used organic solvents and its derivatization in homogeneous medium is thus rendered very difficult.

From Agar to Oligoagarose

Z. galactinovorans β-agarase hydrolyzes the β (1′-4) glycosidic bond of agarose, yielding neo-oligoagarose containing neoagarobiose repeat units (Figure 2). The

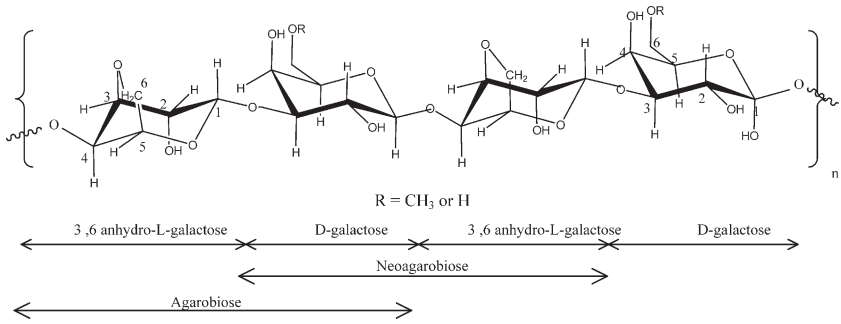

Figure 1.
Agarose repeat unit[17].

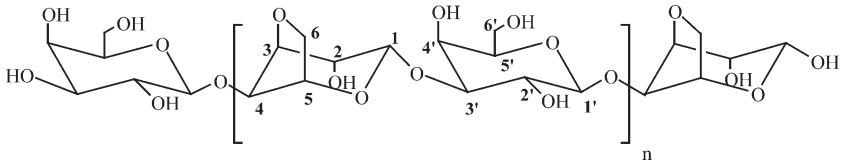

Figure 2.
Oligoagarose repeat unit.

hydroxyl content is calculated according to Equation 1 taking into account that each repeat unit contain 4 OH groups and that a total of 6 OH groups are present on sugar units at chain ends.

$$\text{Hydroxyl group content} = (n \times 4) + 6 \quad (1)$$

Characterization of Oligoagarose Samples

Oligoagarose samples were characterized by HPAEC-PAD (Figure 3). The DP_n varied from 4 to 12 depending on samples. Their average hydroxyl group content per chain was calculated from the peak areas (Table 1).

^{1}H NMR analysis of oligoagarose was performed in DMSO-d_6 (Figure 4). It was possible to distinguish between anomeric protons of the oligomer chain (doublets at 5.1 and 5.3 ppm) and anomeric protons at chain ends (doublets at 6.55 and 6.3 ppm). This assignment was based on the ^{1}H NMR of galactose in DMSO-d_6 which exhibits two signals at 6.4 ppm and 5.15 ppm. These correspond to protons H4 and H1 of the saccharide. The hydroxyl group content could also be calculated by comparison of anomeric proton intensities in the chain and in end group units, according to Equation 1. Values determined by both techniques (Table 1) are in very good agreement.

The assignment of the oligoagarose carbon atoms confirms the G(3-linked β-D-galactopyranose) and LA (4-linked 3, 6-anhydro-α-L-galactopyranose) units, according to Knutsen nomenclature.[18] (Table 2).

Partial Acetylation of Oligoagarose

The main objective of our work was to synthesize amphiphilic copolymers

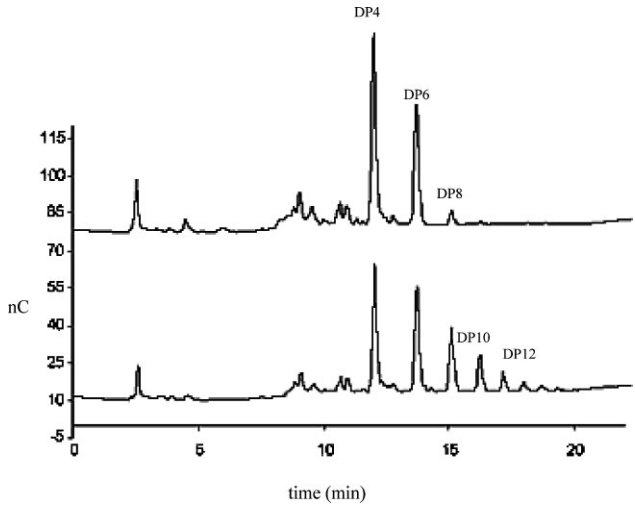

Figure 3.
HPAEC-PAD profiles of two typical oligoagarose samples used.

Table 1.
Experimental values of hydroxyl group content of oligoagarose chains.

Sample	Average hydroxyl content	
	^{1}H NMR	HPAEC-PAD
1	36	36
2	24	28
3	28	27
4	30	30

Table 2.
Assignment of diads of oligoagarose samples using ^{13}C NMR.

Unit	C-1	C-2	C-3	C-4	C-5	C-6
Theoretical						
G	102.4	70.2	82.2	68.8	75.3	61.4
LA	98.3	69.9	80.1	77.4	75.7	69.4
Experimental						
G	101.5	70.0	80.5	68.0	75.0	60.5
LA	97.5	70.0	79.5	77.0	75.5	68.5

consisting of hydroxyl-free oligoagarose and hydrophobic grafted polycaprolactone chains. It was therefore not appropriate to use unprotected oligoagarose as a macroinitiator to polymerize caprolactone, as high hydroxyl content would have lead to a high degree of grafting and very low molar mass polycaprolactone. The hydroxyl groups of oligoagarose were therefore partially protected by conversion to acetyl groups.

Acetylation was carried out using acetic anhydride in the presence of pyridine at room temperature.[19] The degree of acetylation was determined from ^{1}H NMR (Figure 5) using the integration values of protons at 5.0–5.3 ppm (anomeric H in chain) and at 2.0 ppm (acetyl groups). The percentage acetylation was defined as the ratio of the number of acetyl groups as determined by ^{1}H NMR to the total number of hydroxyl groups present initially. Samples with percentage acetylation comprised between 30 and 70% were all soluble in chloroform and insoluble in water.

The structure of the acetylated oligoagarose was also confirmed by ^{13}C NMR. The possibles sites of acetylation are

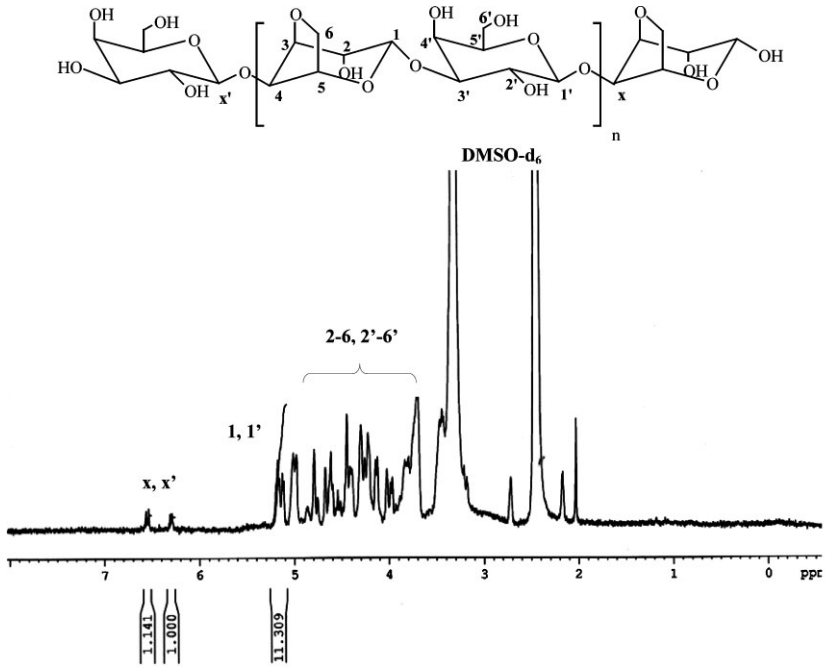

Figure 4.
^{1}H NMR spectrum of oligoagarose 3 in DMSO-d_6.

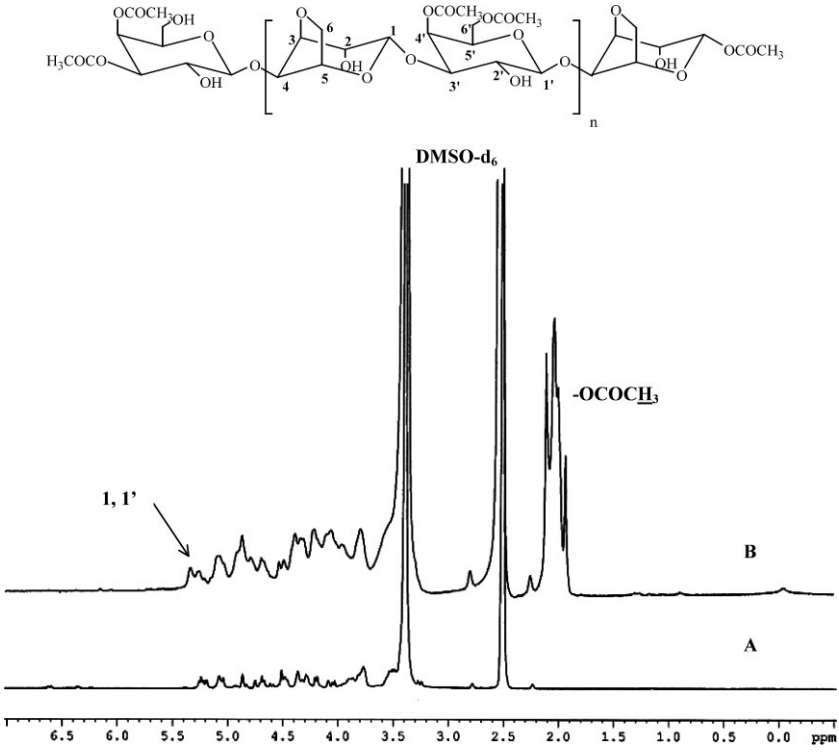

Figure 5.
^{1}H NMR spectra of (A) oligoagarose 3 (B) AcO-oligoagarose derivative in DMSO-d_6.

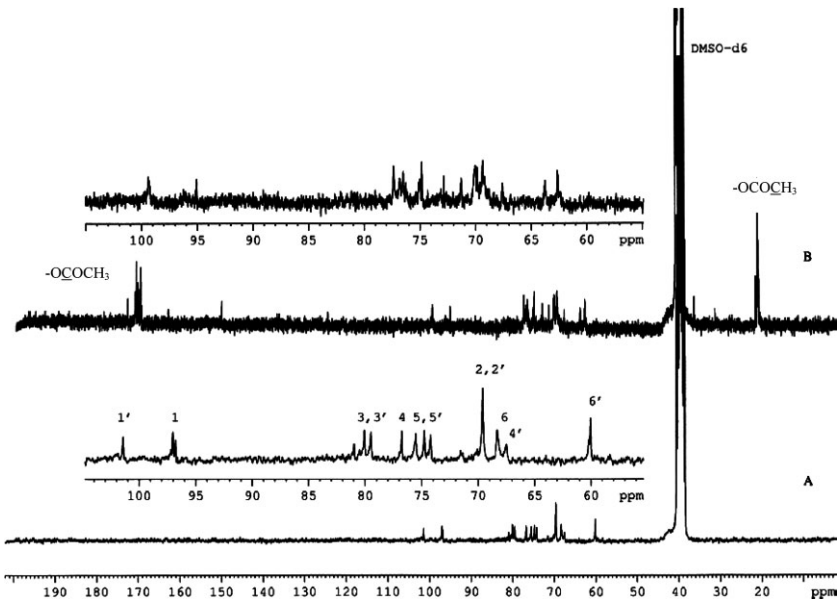

Figure 6.
^{13}C NMR spectra of (A) oligoagarose (B) partially AcO oligoagarose in DMSO-d_6.

secondary hydroxyl groups on C-2, C-2′, C-4′ and primary hydroxyl group on C-6′. As compared to the spectrum of unprotected oligoagarose where one signal is observed at 60.5 ppm corresponding to C-6′ (Figure 6), two distinct signals (62.7 and 63.8 ppm) are now present in the acetylated oligoagarose spectrum, assigned to unacetylated and acetylated C-6′ ($\underline{C}H_2OH$ and $\underline{C}H_2O$-$COCH_3$) respectively. The intense signal at 70.0 ppm due to C-2 and C-2′ initially present in oligoagarose has considerably decreased in intensity with the appearance of a new signal at 73 ppm. The spectrum of a 1:1 mixture of oligoagarose and acetylated oligoagarose was recorded. The C-2/C-2′ signal at 70.0 ppm showed a net increase in intensity, thus confirming the above assignment. Moreover, the presence of a splitting pattern in the carbonyl region indicates the absence of selectivity of the acetylation reaction vis-à-vis primary or secondary hydroxyl groups. Kulicke and Heinze have found comparable reactivity at C-2 carbon in the acetylation of starch.[20]

Synthesis of Graft Copolymers: poly(oligoagarose-*g*-caprolactone)

The polymerization of ε-caprolactone in the presence of an initiator system consisting of an alcohol and tin(II) octanoate has been well established previously.[21,22] Basically, a complex is first formed between the alcohol

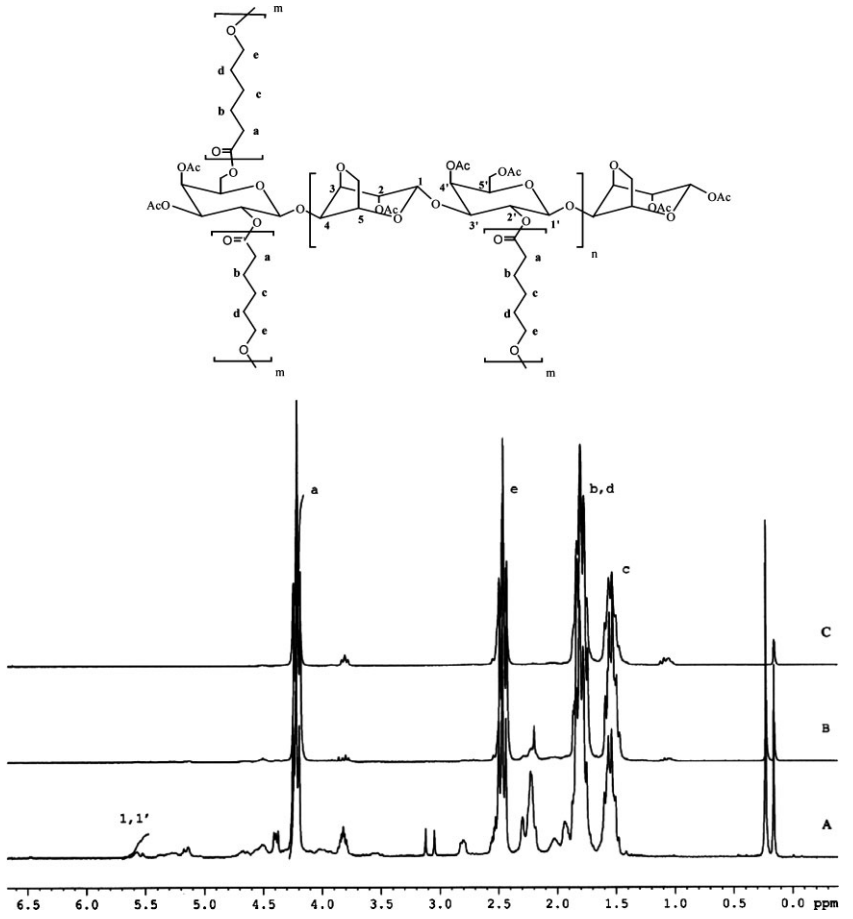

Figure 7.
^{1}H NMR spectra ($CDCl_3$) of poly(oligoagarose-*g*-caprolactone) using oligoagarose-OH as macroinitiator (A) 70% acetylated (B) 40% acetylated (c) 100% free OH.

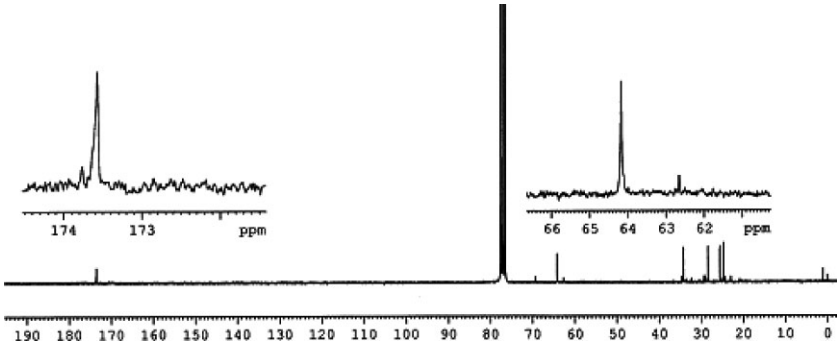

Figure 8.
^{13}C NMR spectrum of 70% AcO-oligoagarose-PCL graft copolymer recorded in $CDCl_3$.

and the tin (II) salt and polymerization proceeds via a coordination-insertion mechanism. In a first instance, oligoagarose containing 100% free hydroxyl groups was used to polymerize ε-caprolactone in bulk at 110 °C. The resulting product was completely insoluble in DMSO and water but partially dissolved in $CHCl_3$ and THF. Analysis of this product by SEC showed a bimodal distribution indicating that the presence of ungrafted oligoagarose, homo-PCL or both.

Two partially acetylated oligoagarose samples containing 30% and 60% free hydroxyl groups were then used as macro-initiator in the presence of tin (II) octanoate under similar conditions for the polymerization of ε-caprolactone. The resulting products are now completely soluble in THF or $CHCl_3$. ^1H NMR shows both the presence of signals corresponding to oligoagarose and PCL chains (Figure 7). The DP_n of polycaprolactone could also be calculated from the proton intensities. The signal due to $\underline{CH_2}$ 6 and 6′ now appears at 62.5 ppm in the ^{13}C NMR spectrum while the CH_2OAc is merged with CH_2 of PCL (Figure 8). The carbonyl region contained peaks in the region 170–174 ppm. The signals at 173.6 and 173.8 ppm have been attributed to the CO of the main PCL chain and penultimate repeat unit. The peak due to CO of the acetyl group can be seen 170–171 ppm. However, the formation of a graft copolymer cannot be fully established at this stage.

The SEC trace of the crude product (Figure 9a) was compared with the O-acetyloligoagarose macroinitiator (Figure 9b). As can be seen, the chromatogram of the copolymer is shifted to higher molar masses and is a monomodal trace, thus confirming the formation of a graft copolymer of oligoagarose and polycaprolactone. However, the presence of a small amount of the macroinitiator cannot be ruled out completely due to overlapping of chromatograms.

Deprotection of AcO-oligoagarose-PCL Graft Copolymer

The 70% acetylated poly(oligoagarose-*g*-PCL) was subjected to hydrolysis under

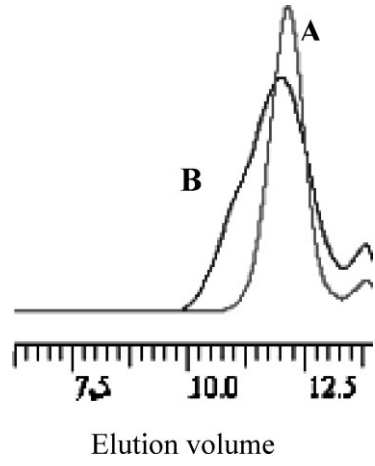

Figure 9.
SEC chromatograms of (A) 70% AcO-oligoagarose, (B) 70% AcO-poly(oligoagarose-*g*-caprolactone) copolymer in THF as eluent.

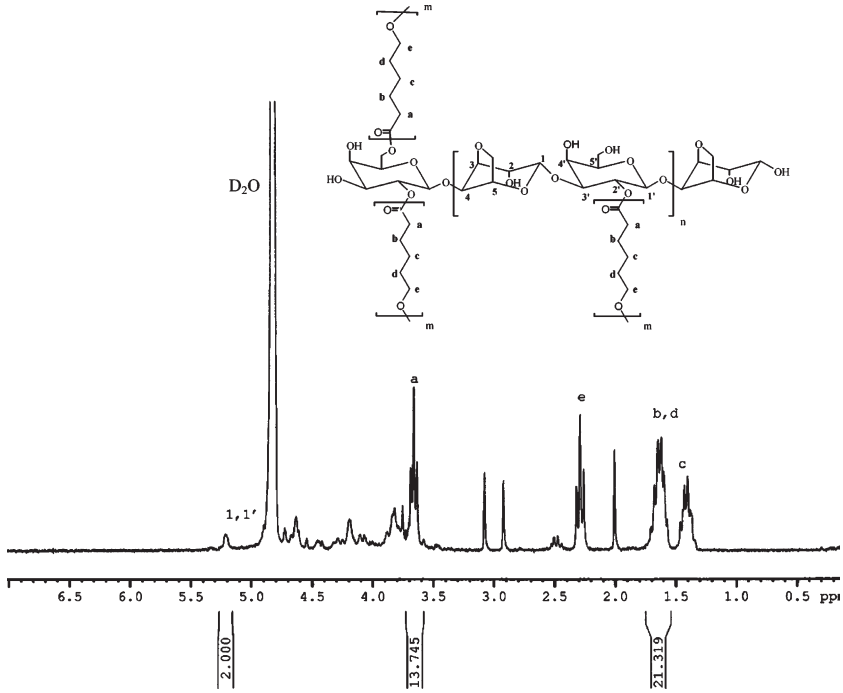

Figure 10.
^{1}H NMR spectrum of deprotected poly(oligoagarose-*g*-caprolactone) recorded in D_2O.

mild conditions to remove completely the acetyl groups and to avoid cleavage of PCL chains. After deprotection, the copolymer was soluble in water and insoluble in chloroform. ^{1}H NMR spectrum, recorded (Figure 10) in D_2O, shows absence of the multiplet due to acetyl groups (previously centered at 1.9 ppm). The ratio of PCL to oligoagarose units remained almost unchanged after deprotection.

The graft copolymer probably organizes itself into micelles of the nanometer range in aqueous solution with the PCL chains assembling themselves into a core structure. An in-depth study of the solution properties of the poly(oligoagarose-*g*-caprolactone) is currently underway.

Conclusion

In this paper, we have shown that oligoagarose-*g*-polycaprolactone can be synthesized using a protection/deprotection method. Prior to grafting, hydroxyl groups of oligoagarose were partially protected by conversion to acetyl groups. The remaining hydroxyl groups were then successfully used as initiators in the presence of tin(II) octanoate as catalyst to graft caprolactone. The acetyl groups were then removed after grafting without hydrolysis of polycaprolactone chains, resulting in a hydrophilic copolymer containing agarose and caprolactone repeat units as evidenced by NMR and SEC analysis.

Acknowledgements: We acknowledge financial support from the International Foundation for Science (Sweden) and the Organization against the Proliferation of Chemical Weapons for this research work.

[1] Y. X. Wang, J. L. Robertson, W. B. Spillman, Jr, R. O. Claus, *Pharm. Res.* **2004**, *21*(8), 1362.
[2] B. Montdargent, D. Letourneur, *Infect. Control Hosp. Epidemiol.* **2000**, *21*(6), 404.
[3] R. Chandra, R. Rustgi, *Prog. Polym. Sci.* **1998**, *23*, 1273, and references therein.
[4] S. Sagnella, K. Mai-Ngam, *Colloids Surf. B. Biointerfaces* **2005**, *42*(2), 147.

[5] Y. Ohya, S. Maruhashi, T. Ouchi, *Macromol. Chem. Phys.* **1998**, *199*, 2017.
[6] T. Ouchi, T. Kontani, T. Saito, Y. Ohya, *J. Biomater. Sci. Polym. Ed.* **2005**, *16*(8), 1035.
[7] C. Nouvel, Ph. Dubois, Ed. Dellacherie, J. L. Six, *J. Polym Sci.,Part A* **2004**, *42*, 2577.
[8] W. Yuan, J. Yuan, F. Zhang, X. Xie, *Biomacromolecules* **2007**, *8*(4), 1101.
[9] H. Yu, W. Wang, X. Chen, C. Deng, X. Jing, Biopolymers. **2006**, *83*(3), 233.
[10] C. Lemarchand, P. Couvreur, M. Besnard, D. Costantini, R. Gref, *Pharm. Res.* **2003**, *20*(8), 1284.
[11] L. Liu, Y-e. Li, Y. Fang, L. Chen, *Carbohydrate Polymers* **2005**, *60*, 351.
[12] C. Araki, *J. Chem. Soc. Japan* **1937**, *58*, 1338.
[13] D. Jhurry, A. Bhaw-Luximon, T. Mardamootoo, A. Ramanjooloo, *Macromol. Symp.* **2006**, *231*, 16.
[14] M. Jam, D. Flament, J. Allouch, P. Potin, L. Thion, B. Kloareg, M. Czjzek, W. Helbert, G. Michel, T. Barbeyron, *Biochemical J.* **2005**, *385*, 703.
[15] D. K. Kidby, D. J. Davidson, *Anal. Biochem.* **1973**, *55*, 321.
[16] F. Weinberger, C. Richard, B. Kloareg, Y. Kashman, H.-G. Hoppe, M. Friedlander, *J. Phycol.* **2001**, *137*, 418.
[17] R. Armisen, F. Galatas, Production and utilization of products from commercial seaweeds. Chapter 1: Production, properties and uses of agar, FAO publications, Italy **1987**, 288.
[18] F. V. Velde, S. H. Knutsen, A. I. Usov, H. S. Rollena, A. S. Cerezo, *Trends in Food Science and Technology* **2002**, 73.
[19] R. B. Garcia, R. R. L. Vidal, M. Rinaudo, *Polímeros: Ciência e Tecnologia* **2000**, *10*(3), 155.
[20] W.-M. Kulicke, T. Heinze, *Macrmol. Symp.* **2006**, *231*, 47.
[21] A. J. Nijenhuis, D. W. Grijpma, A. J. Pennings, *Macromolecules* **1992**, *25*, 6419.
[22] A. Kowalski, A. Duda, S. Penczek, *Macromolecules* **2000**, *33*, 689.

