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## Advances in chitooligosaccharides chemical modifications

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# Advances in chitooligosaccharides chemical modifications

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Camille Chapelle<sup>1</sup>, Ghislain David<sup>1</sup>, Sylvain Caillol<sup>1</sup>, Claire Negrell<sup>1</sup>, Myriam Desroches Le Foll<sup>2</sup>

<sup>1</sup> ICGM, Univ Montpellier, CNRS, ENSCM, Montpellier, France

<sup>2</sup> CST COLAS 4, Magny-les-Hameaux Cedex, France

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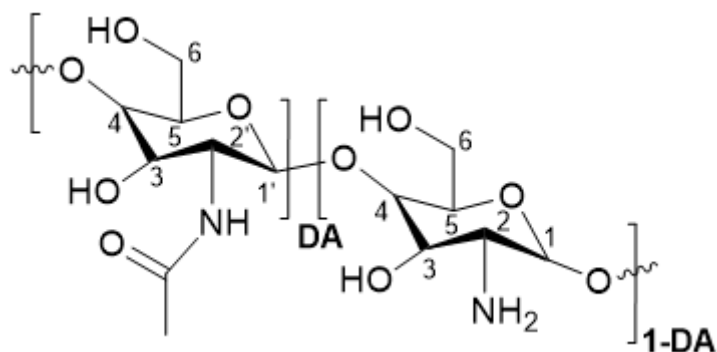
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## I. Introduction

Chitosan is a polysaccharide made of  $\beta$ -D-glucosamine (GlcN) and N-acetyl- $\beta$ -D-glucosamine (GINAc) units linked by a glycosidic bond (1 $\rightarrow$ 4) (**Erreur ! Source du renvoi introuvable.**). It is the chemical modification of chitin, a polymer extracted from shrimp shells, squids, insects or mushrooms that leads to chitosan. Chitin is the second natural polymer on earth after cellulose. Chitosan results from N-deacetylation of chitin, which is only partial in most of the cases. This polysaccharide is characterized by its acetylation degree (DA) and its molecular weight. There is a large variety of chitosans, with a wide range of molecular weights infrom 10,000 g/mol up to 2,000,000 g/mol and acetylation degree between 0 and 30 %. Nowadays, chitosan is used in various applications <sup>1</sup> like water treatment <sup>2,3</sup>, cosmetics<sup>4</sup>, food industry or drug delivery <sup>5,6</sup> due to its many interesting properties such as biodegradability, biocompatibility, hemostaticity, and antibacterial activity <sup>7,8</sup>.



**Figure 1 Chitosan and chitooligosaccharides (COS) chemical structure**

Numerous chemical reactions have been carried out on chitosan for introducing functional groups that could modify its properties<sup>9, 10</sup>. Nevertheless, according to its molecular weight, chitosan is only soluble in water and in dilute acidic media. Thus the grafting efficiency of non-hydrophilic compounds remains quite low <sup>11, 12</sup>. Furthermore, the concentration of high molecular weight chitosan in water hardly reaches 3 % (w/w) and in such conditions, the resulting viscosity becomes high enough to lower the chitosan functional groups reactivity <sup>13, 14</sup>. In order to overcome this drawback, reducing the molecular weight of native chitosans would be effective.

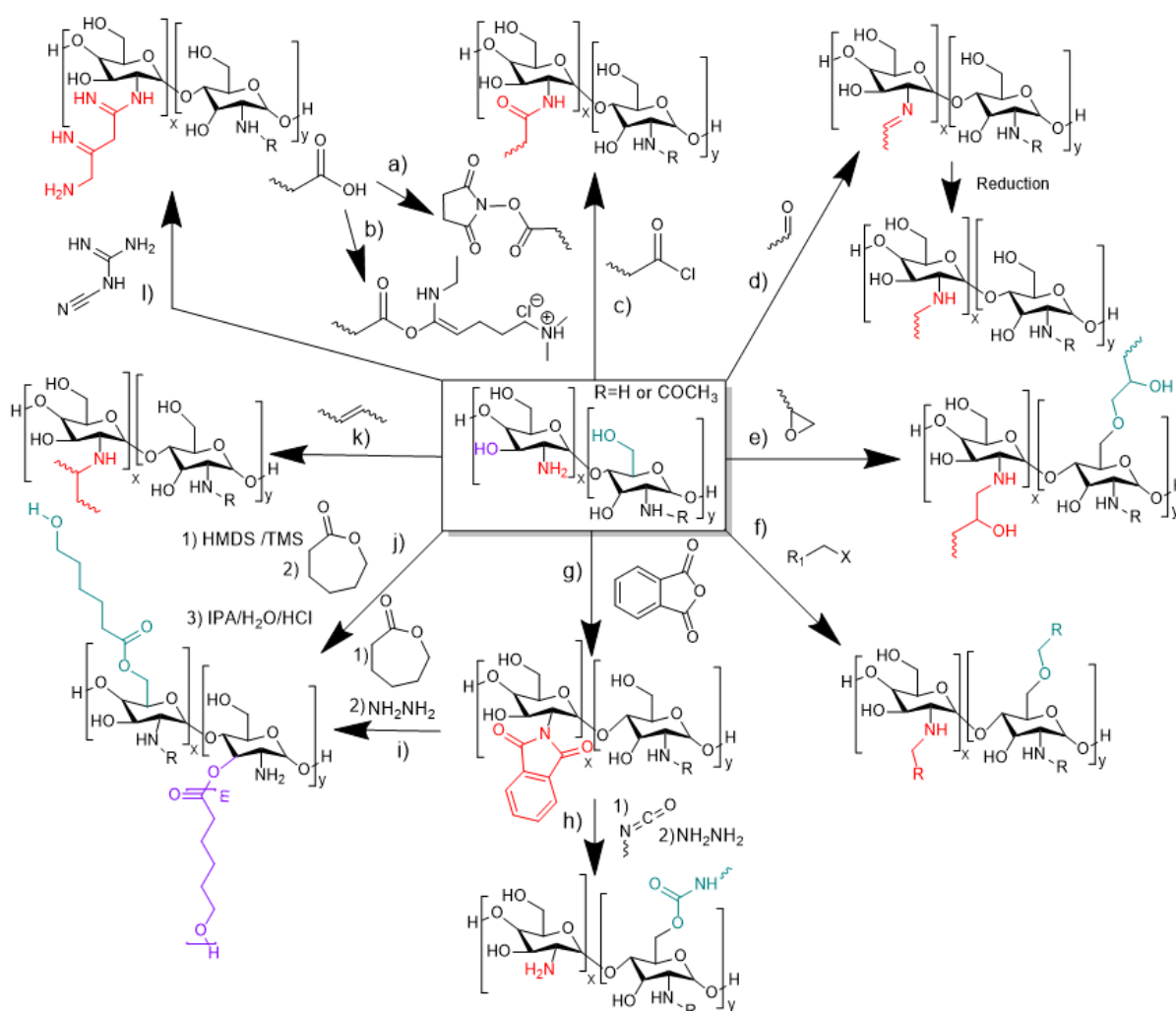
Chitooligosaccharides (COS) or chitosan oligomers are often defined as chitosans with a degree of polymerization (DP) and average molecular weight respectively lower than 20 and 5,000 g/mol<sup>15</sup>. Nevertheless, low molecular weight chitosan ( $M_n < 20$  kg/mol) are often called COS in the literature. It was shown that COS are more easily workable, due to less chain entanglement and steric hindrance which confer them a direct solubility in water for  $DP < 10$  and pH dependent solubility for  $10 < DP < 20$  while maintaining a low viscosity in solution. COS are insoluble in acetone, butanol, ethanol, ethyl acetate, propanol and pyridine but as opposed to chitosan, partially soluble in methanol and partially soluble in dimethyl sulfoxide and dimethyl formamide when heated. Hence the smaller are the oligomers, the better is the solubility<sup>15</sup>. COS have recently received considerable attention as functional biomolecules with a wide range of applications in food, agriculture, medicine, pharmaceuticals and cosmetics<sup>16, 17, 18, 19</sup>. Compared to chitin and chitosan, COS are defined by some unique properties such as water solubility, cell membrane penetrability, easy absorption and various biological activities. The water solubility limit for COS is reported to be between 3.82 to 4.67 kg/mol but those values are dependent on the DA and the dispersity of COS<sup>20</sup>. They are reported to treat asthma, prevent tumor growth, malaria, control blood pressure and also have neuroprotective effects for Alzheimer disease treatment<sup>21</sup> thanks to their biological activities that include antibacterial, antifungal, antiviral, anti-tumor and antioxidant properties<sup>22, 23</sup>, which have been reported to be better than chitosan's<sup>24</sup>. Several ways to obtain chitosan oligomers were reviewed in literature<sup>25, 26, 27</sup>. Those methods are divided in three main categories: enzymatic, physical, and chemical depolymerization. The enzymatic depolymerization of chitosan involves cleavage of the chitosan glycosidic bonds by the action of enzymes that have the particularity of being selective. Indeed, certain enzymes cleave only one type of bonds (i.e. GlcNAc-GINAc, GlcN-GlcN and GINAc-GlcN). Most commonly used enzymes are chitinase, chitosanase and lysozyme but other teams developed depolymerization of

COS with other enzymes such as lipase and papain (etc.)<sup>28, 29</sup>. Commercially available COS are generally obtained by enzymatic pathway, which explains their cost. Depolymerization of chitosan is also possible using physical methods that need an external source of energy from specific apparatus such as electron beam plasma<sup>30</sup>, gamma irradiation<sup>31</sup>, sonication<sup>32</sup>, microfluidization<sup>33</sup>, UV light<sup>34</sup> or microwaves<sup>35</sup>. In a general manner, physical methods are efficient enough to depolymerize chitosan. Interestingly, the methods based on UV, sonication, irradiations and microwaves are often used in combination with chemical methods to improve yields and kinetic<sup>36, 37, 38</sup>. Microwaves-based depolymerization allows to easily obtain oligomers but the reaction is hardly reproducible due to its dependence on many parameters (volume, glassware, time, temperature etc.)<sup>39</sup>. The chemical depolymerizations consist in acid hydrolysis<sup>40, 41</sup>, the use of oxidative agent such as hydrogen peroxide<sup>42, 43</sup> or even the action of nitrous acid<sup>44, 45</sup>. This last method was shown to be applicable and reproducible on any kind of crude chitosan leading to low molecular weights oligomers<sup>46</sup>. Though, it leads to formation of AMF end chain which is electrophilic and reactive for further reactions.

Whatever the depolymerization technique, COS show better solubility than the corresponding chitosan. Furthermore, they present very interesting biological properties even better than high molecular weight chitosan<sup>23</sup>. Most of COS are water soluble in a larger range of pH at relatively higher concentration than chitosan. Moreover, depending on their size, they are soluble in other solvents like DMSO, DMF and water/alcohol solutions. This is why there has been a growing interest on modification of those oligomers to widen their applications. Thus, COS are easily functionalizable due to the presence of free primary amines and alcohol on the sugar units. Moreover, when COS are obtained by nitrous acid depolymerization, a reactive aldehyde is present at the end of the oligomer chain. Thanks to their shorter hydrophilic chain, their functionalization can lead to amphiphilic structures with better properties than those from chitosan-based. Oligochitosan also allowed to reach nano and microgel structures<sup>47, 48</sup>. As far as we

know, there is no review in the literature on such a rich chemistry that can be done on COS. In this review, different routes of functionalization of the obtained oligomers (from 1 to 20 kg/mol) will be introduced and discussed, especially based on the grafting efficiency. Depending on the depolymerization method, oligomers might present three functional groups: amine, hydroxyl and aldehyde. This opens the way to various grafting, crosslinking, and polymerization pathways with several functional groups.

## II. Grafting onto amine and hydroxyl



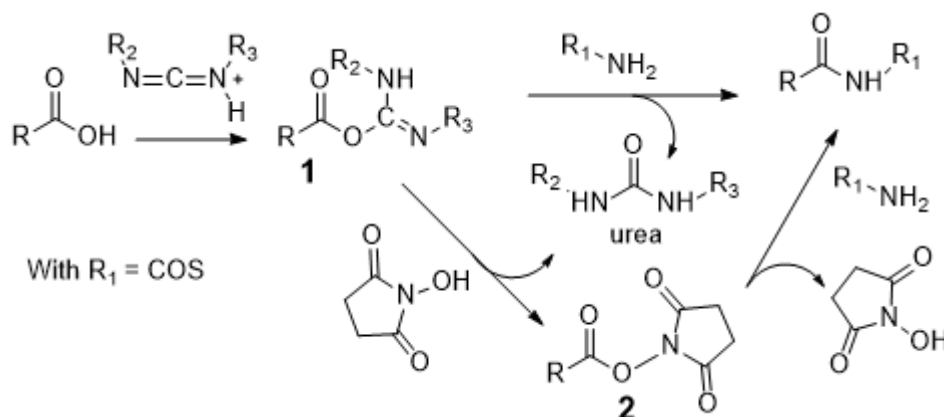
**Scheme 1.** a) Grafting of carboxylic acid by EDC/DCC NHS coupling b) Grafting of carboxylic acid by EDC coupling c) Grafting of carboxylic acid by acyl chloride coupling d) Schiff base reaction e) Epoxy-amine and epoxy-hydroxyl coupling f) Nucleophilic substitution g) *N*-phthaloylation h) Isocyanate reaction i) j) Ring opening polymerization k) Michael addition l) Guanidination

### II.1. Carboxylic acids and its derivatives



The amino and hydroxyl graftings of chitosan are widely used even on high molecular weight polysaccharides<sup>49</sup>. COS allows nevertheless larger choice of solvent, and higher substitution degree are usually reached. One of the most common reactions that can be done on the free amine of COS is amidation with a carboxylic acid. In order to react with the amine, the carboxylic acid must be activated. In general organic chemistry, carboxylic acid (CA) activated by thionyl chloride, phosphorus trichloride or pentachloride to form acyl chloride are the most reactive species.

Carboxylic acid might also be activated by formation of anhydrides or esters. In order to convert carboxylic acids into amides or esters, carbodiimides such as 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) or *N,N'*-Dicyclohexylcarbodiimide (DCC) can be used. Additives, such as *N*-hydroxysuccinimide (NHS) or 4-dimethylaminopyridine (DMAP), are often used to increase yields and avoid side reactions. In COS functionalization, the formation of an amide using a carbodiimide is straightforward, but side reactions may occur (Scheme 2). Indeed, the acid will react with the carbodiimide to produce an *O*-acylisourea, which can be viewed as a carboxylic ester with an activated leaving group. The *O*-acylisourea will react with the amine to give the desired amide and a urea. The activated carboxylic acid is very reactive and unstable which could lead to side reaction. When using NHS or DMAP, the additive will react with the activated acid to form another more stable intermediate, less suitable to side reactions. The additive is usually regenerated at the end of the reaction.



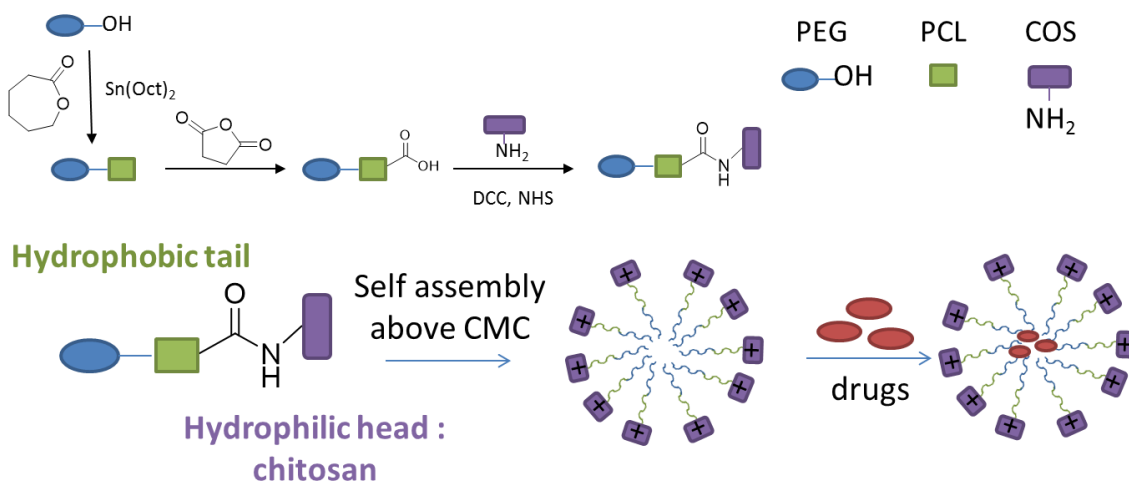
**Scheme 2** Amidation with carboxylic acid activated by carbodiimide with or without NHS as activator

In COS functionalization, two ways for grafting carboxylic acid can be found (Table 1). In literature, the amidation of COS is mainly done with EDC because of its water solubility. Generally, the carboxylic acid is previously activated by an excess of EDC in ethanol and the mixture is added to a COS water solution from 4h to 24h at

temperature between 60 and 80 °C (Table 1). Some studies also revealed the use of both EDC and NHS<sup>50, 51, 52, 53, 54</sup>. As said previously, COS with very low molecular weight ( $M_n < 5$  kg/mol) exhibit better solubility in water with a larger pH range but also solubility in solvent such as DMSO. Thus COS amidation was performed in DMSO<sup>52, 55</sup> or in a mixture of water and DMSO<sup>56</sup>. In this case other carbodiimides can be used such as DCC<sup>56</sup> which is cheaper than EDC and gives a non-water soluble by-product. We also found other additive such as DMAP<sup>57</sup>. This wider solubility also opens the way for new solvents such as ionic liquids like 1-butyl-3-methylimidazolium acetate ([BMIM]Ac)<sup>57</sup>. At similar molecular weight, the amidation of COS with 0.5 eq of linoleic acid (C18)/NH<sub>2</sub> showed higher DS in ionic liquids (37.9 %) than DMSO (26.3 %) <sup>57</sup> and water/acetone (10.3 %) <sup>58</sup>. In addition, the reactions in ionic liquids and DMSO were performed in mild conditions at 30 °C as opposed to 60°C in water/acetone mixture. Moreover, lower coupling agent concentration was required due to the possibility of using DMAP. In addition for being a better solvent, the [BMIM]Ac is recyclable and reusable for further grafting reaction. Other reactions made in DMSO showed acceptable DS with high molecular weight carboxylic acid such as arachidic acid (C<sub>20</sub>) (10.3 %) <sup>50</sup>, modified PCL (18%) <sup>55</sup> and PEG-PCL (30.2 %) <sup>59</sup>. The use of this type of solvent then opens up the way to other kind of grafting. Reactions made only in water showed the lower DS.

In a general manner, this kind of reaction is performed on fatty acids, which are difficult to graft onto COS due to low affinity and the longer is the chain, the lower is the obtained DS. The grafting efficiency is also correlated to the molecular weight. COS with molecular weight over 12 kg/mol do not exceed 10 % DS<sup>60, 61, 62, 63</sup>. This can be ascribed to a lower solubility of the COS in water and more chain entanglement. Noteworthy, the higher is the molecular weight, the lower is the affinity with fatty acid, resulting in a very low amount of reactant that can be introduced. Indeed, the molar ratio never exceeded 0.5 eq CA/NH<sub>2</sub> with higher molecular weights COS, whereas it can reach 2 or 3 with low molecular weight (>4 kg/mol) <sup>64</sup>. The DS is also related to the carboxylic acid length. Indeed, the longer is the chain, the lower is the affinity with chitosan and the lower is the DS. The ratio of carbodiimide to carboxylic acid is also an important factor. Indeed, EDC or DCC might be used in excess because the intermediate **1** (Scheme 2) is unstable. For instance, Su et al.<sup>51</sup> only used 0.7 eq of EDC/CA and got very low DS (3.6%) working with 1 eq of acrylic acid, i.e. corresponding to a C3 fatty acid. Fernandes et al.<sup>65</sup> also obtained poor substitution degree when working at low concentration of EDC. In both cases, the low DS might also be explained by working only in water but also by the acid conditions, where the free amines of chitosan are partially protonated hence less reactive.

Broadly speaking, the products of those reactions are then precipitated either in basic conditions for the higher masses or in organic solvent. Regardless of the carbodiimide used, there is always production of urea as a side-product (Scheme 2). This urea is supposed to be toxic, hence the functionalized oligomers are generally purified by dialysis to extract both unreactive species and side products. Although this reaction is mainly described for grafting fatty acids, it can also be used with other carboxylic acids. For example, Wang et al.<sup>66</sup> grafted salicylic acid onto COS to encapsulate paclitaxel for ovarian cancer treatment. COS have also proven to be excellent drug carriers for doxorubicin<sup>53</sup>, methotrexate<sup>56</sup>, folic acid<sup>67</sup> or tocopherol<sup>68</sup>, anticancer drugs that bear carboxylic acids. Zhao et al.<sup>59, 69</sup> synthesized a carboxyl terminated PEG-PCL copolymer by ring opening polymerization of  $\epsilon$ -caprolactone initiated by hydroxyl terminated PEG with stannous 2-ethyl hexanoate catalyst and addition of succinic anhydride in presence of DMAP. A large excess of carboxylic acid chain-end (40 eq) was then activated by NHS and DCC to react with free amines of COS in DMSO and amphiphilic triblock copolymers (Figure 2 **Erreur ! Source du renvoi introuvable.**) were obtained with a maximum substitution degree of 31%. Those structures were able to self-assemble into micelles from 20 to 45 nm in aqueous solution or to form supramolecular hydrogels when combined with cyclodextrin. With the same method, Xu et al.<sup>55</sup> used hexanol instead of PEG to initiate the polymerization of  $\epsilon$ -caprolactone and grafted dithiodipropionic acid at the PCL chain end. The activation of the carboxylic acid (12.5 eq) was performed by coupling EDC and NHS in order to obtain COS grafted with 18% PCL moieties. Those carriers revealed dual responses of pH and glutathione (GSH), a molecule present in cancer cell, when releasing doxorubicin which is mainly due to the disulfide linkage. Those properties made them a great candidate for targeted drug delivery and reduction of side effects on healthy cells. Other descriptions of this type of amidation can be found in the literature. When Yin et al.<sup>54</sup> grafted COS onto liposome with EDC and NHS, Chandika et al.<sup>70</sup> used it to form a scaffold between COS and a mixture of fish collagen and alginate. Yang et al.<sup>71</sup> immersed polysulfone-poly(acrylic acid) (PSF-g-PAA) membranes in a COS/EDC solution to improve their antimicrobial activity.



**Figure 2. Synthesis of COS-PCL-PEG triblock copolymer and their self-assembly**

In comparison, only few studies were led on coupling acyl chloride to COS. To activate palmitic acid, Choudhari et al.<sup>72</sup> used thionyl chloride ( $\text{SOCl}_2$ ). The coupling reaction was carried out in water, which is quite incompatible with the use of palmitoyl chloride. Indeed, acyl chloride in water was immediately deactivated and turned back into acid by nucleophilic addition of  $\text{H}_2\text{O}$ . The amine of chitosan is not nucleophilic enough to react in an excess of water. In this condition, it is like reacting palmitic acid and the free amine of chitosan in acidic media meaning that the reaction is very unlikely to occur. Although the authors reported a grafted oligosaccharide, they could not provide any DS and the NMR data are doubtful. In comparison to the use of carbodiimide, this method is not water adaptable and should be led in aprotic solvent such as DMSO. In addition, thionyl chloride is toxic and corrosive.

Another team reported crosslinking of oligochitosan with malic acid prior grafting onto PET film without activating the carboxylic acid<sup>73</sup>. They observed by FTIR the appearance of the amide band confirming the grafting of both malic acid and PET. Nevertheless, the band is not very well defined and is more likely to show the free amines of chitosan. They did not confirm the grafting by  $^1\text{H}$  NMR but it seems unlikely that the carboxylic acid reacted without previous activation because the amines of COS are not nucleophilic enough. Hydrogen and/or ionic bonding probably occurred between the amine and hydroxyl groups of COS and the two carboxylic acids.

In conclusion of this part, for amidation reaction, a lot of working conditions were described in the literature. Parameters such as temperature, time, solvent, and coupling agent play a crucial role for reaching high DS. In addition, the initial molecular weights of COS, the length or size of the carboxylic acid are also important factors. Furthermore, very low molecular weight COS ( $M_n < 5 \text{ kg/mol}$ ) are soluble in other solvents such as

DMSO and hydroalcoholic solutions, which allows using other coupling agents and led to higher DS.

Mn of COS (kg/mol)	DA (%)	Carboxylic acid (CA)	Equivalent /NH <sub>2</sub>	Activation /CA	Solvent	Conditions (h; °C)	DS Measurement	DS (%)	Ref
18	5		0.2	EDC (8.5 eq)	Water/ethanol (50/50 v/v)	5h; 80 °C	TNBS method	3.8	60
15	5		1	EDC (10 eq)	Water/ethanol (50/50 v/v)	4h; 80 °C	TNBS method	6.5	61
5	5		N/A	EDC NHS	DMSO	6h; 55°C	N/A	N/A	52
18			0.25					9.8	
11		Stearic acid (C18)	0.5					49.5	
8	5		0.5	EDC (10 eq)	Water/ethanol (70/30 v/v)	24h ; 60°C	TNBS method	47.6	74
3			0.25					13.9	
3			0.5					45	
3			0.8					63	
18	5		0.2	EDC (3.2 eq)	Water/ethanol (60/40 v/v)	12h; 60°C	TNBS method	3.8	75
3	2		0.3	EDC (5 eq)	Water/ethanol (60/40 v/v)	6h; 60°C	Ninhydrin	6	76
			0.1					2.6	
20	10		0.2	EDC (1 eq)	Water/ethanol (95/5 v/v)	12h; rt	<sup>1</sup> H NMR	4.4	62
			0.3					8.5	
3.7	5	Linoleic acid (C18)	0.5	EDC DMAP (2.5 eq)	([BMIM]Ac, DMSO)	6h; 30°C	<sup>1</sup> H NMR Molecular weight	37.9	57
			0.5					26.3	
5	5		0.5	EDC (10 eq)	Water/acetone (65/35 v/v)	6h; 60°C	<sup>1</sup> H NMR	10.3	58
1	N/A	Linolenic acid (C18)	2	EDC NHS (1.2 eq)	DMSO	24h; rt	<sup>1</sup> H NMR	90	64
5	10	Decanoic acid (C10)	1	EDC (2 eq)	Water/methanol (60/40 v/v)	5h; 80°C	TNBS method	24	77
5	10	Arachidic acid (C20)	3	EDC NHS (1.5 eq)	DMSO	12h; 50°C	Linear Regression	9.8	50
5	5	NAC (C5)	1	EDC (2 eq)	Water	8 h; rt	N/A	N/A	52
2.5	25		2.2	SOCl <sub>2</sub>	Water	24h; rt	N/A	N/A	72
15	0.3	Palmitic acid	4	EDC	Water/ethanol (90/10)	6h ; 80°C	TNBS	3.4	63
>10	15	Acrylic acid (C3)	1	EDC NHS (0.7 eq)	Water (HCl)	72h; rt	Double bond titration	3.5	51
15	13	N-acetyl-L-cysteine	10	EDC (0.1 eq)	Water (AcOH)	3h; rt	Ellman's reagent <sup>78</sup>	1-5	65
3	8	PCL	12.5	EDC NHS (3 eq)	DMSO	24h; rt	Elemental analysis	18	55
10	5	PCL-PEG	20	DCC NHS (2 and 4 eq)	DMSO	48h; rt	<sup>1</sup> H NMR	13.8	59
			40					30.2	
1	8	PET film	N/A	Sodium	1 % Acetic	rt	N/A	N/A	73

		Malic acid	2	acetate	acid				
8.6	10	Methotrexate	0.625 1.25 2.5	DCC NHS (1.5 eq)	Water/ DMSO (10/90 v/v)	72h; rt	<sup>1</sup> H NMR	4.5 8.9 13.5	56
1.2	25	PSF-g-PAA membranes	N/A	EDC	1% acetic acid solution (v/v)	N/A	Acid orange 7 dye complex	16.5 µg/cm <sup>2</sup>	71
1-10	N/A	Fish collagen/alginate	N/A	EDC NHS	2-morpholinoethane sulfonic acid buffer	20h; rt	N/A	N/A	70
4	10	Tocopheryl	1	EDC NHS (8 eq)	Water/DMF	24h; rt	Mass spectroscopy	4	68
1.5	N/A	Salicylic acid	0.5	EDC (5 eq)	Water/ethanol (50/50)	53h; rt	N/A	N/A	66
1-3	N/A	Doxorubicin	4	EDC NHS (4 eq)	Water	12h; 20°C	UV (480 nm)	18	53
3	N/A	Folic acid	N/A	EDC NHS (2.8 eq)	PBS	16h; rt	N/A	N/A	67
2-5	25	Liposome	N/A	EDC NHS	PBS	N/A	N/A	N/A	54

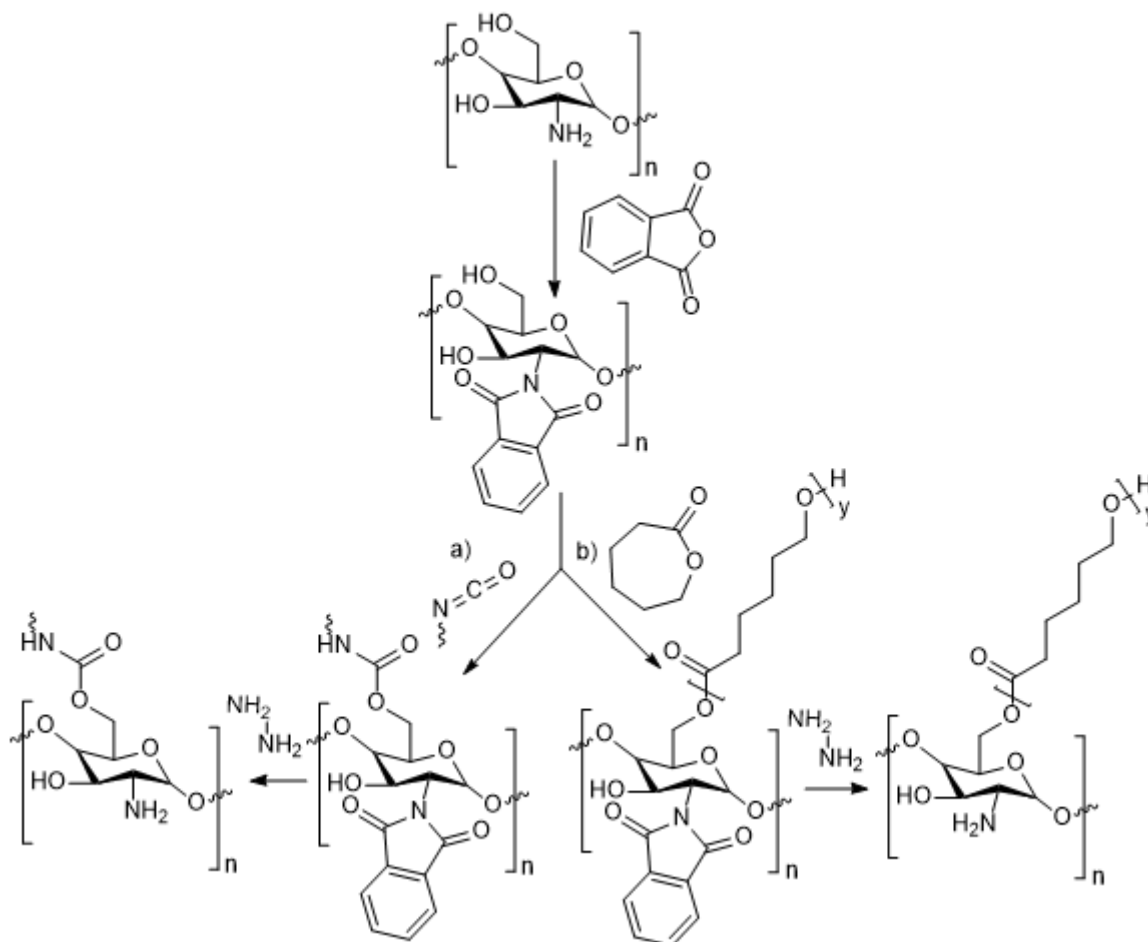
**Table 1.** Substitution degrees according to different carboxylic acid, coupling agent, reaction conditions in the amidation of COS

## II.2. Anhydrides

Acid anhydrides are compounds that bear two acyl groups bonded to the same oxygen atom. They are a source of reactive acyl groups which tend to be less electrophilic than acyl chlorides. Only one acyl group is transferred per molecule of acid anhydride, which leads to a lower atom efficiency. However cyclic anhydrides can act dually as acylating agents and find a large range of applications <sup>79</sup>.

One of the most common reaction of anhydride and chitosan is the *N*-phthaloylation with phthalic anhydride. This reaction was also widely described on high molecular weight chitosan because it led to an interesting change of solubility, i.e. chitosan becoming soluble in organic solvents such as DMF <sup>80</sup>. This reaction is thus one of the most common techniques for solubilization as well as protection of multifunctional chitosan to prepare various derivatives. Since treatment of chitosan with phthalic anhydride results in partial *O*-phthaloylation in addition to the *N*-substitution, a controlled procedure should be followed in order to selectively protect the amine. In the case of

COS, the main research aspect is the selective amino protection for further functionalization of the primary alcohol<sup>81, 82, 83</sup> (Scheme 3). The deprotection of the amino group is generally done with hydrazine hydrate.

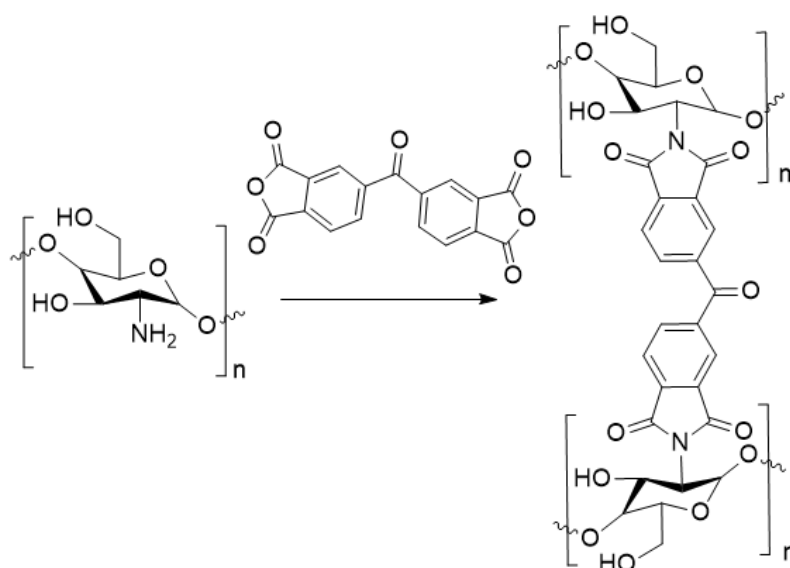


**Scheme 3** N-phthaloylation of COS followed by a) Urethane formation b) ROP both followed by deprotection by hydrazine hydrate

Following the same idea, Fu et al.<sup>84</sup> grafted 3,3',4,4'-benzophenonetetracarboxylic anhydride (BTDA) onto 1.5 kg/mol COS (Scheme 4). They proved the formation of the imide group by IR with the characteristic signal at  $1380\text{ cm}^{-1}$  assigned to the C-N stretching vibration. They used several BTDA/COS ratios and showed an increase of the molecular weight when this ratio was raised; i.e. 2.5 kg/mol for 1:20, 3.5 kg/mol for 1:10, 4.5 kg/mol for 1:5. This represents a maximum DS of 93% if no crosslinking occurred. Nevertheless, a higher amount of BTDA participating in the grafting reaction may increase the chance of crosslinking between CO and BTDA, which explains the higher molecular weights. Though, due to the low molecular weight of COS, the crosslinking was not sufficient because the product remained soluble in water. This is why they used



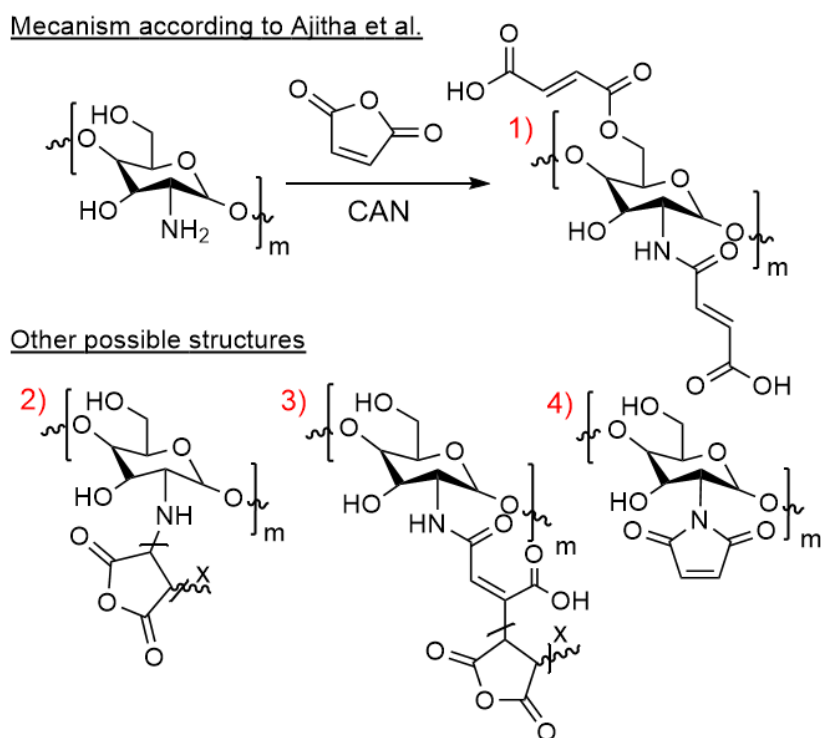
diphenyl methane-4,4'-diisocyanate (MDI) for further emulsion polymerization leading to microcapsules formation.



**Scheme 4. Crosslinking of COS with BTDA**

Ajitha et al.<sup>85</sup> used ceric ammonium nitrate (CAN) to graft maleic anhydride (MA) onto COS. They claimed that the amine and the primary alcohol both opened the anhydride to obtain a grafted COS (Scheme 5 1)). MA was used in low molar ratio (0.8/1 MA/COS). As specified before, the primary amine is much more reactive than the primary alcohol and with this ratio conditions, the amide formation is more likely to occur. Nevertheless, CAN was described as an esterification as well as amidation catalyst<sup>86</sup> which gave confidence that both reactions may occur. However, CAN was also used as catalyst for radical polymerization of methyl acrylate<sup>87</sup> and acrylic acid<sup>88</sup>. Besides, the acrylonitrile polymerization from chitosan was described and CAN led to a radical formation by degradation of the sugar units with quite different structures than the one described here<sup>89</sup>. In addition, MA may polymerize in presence of a radical initiator<sup>90</sup> and the radical polymerization of MA from starch and cellulose, two polysaccharides with structures similar to chitosan, was described before<sup>91,92</sup>. It means that the radical polymerization of maleic anhydride could occur in the used conditions (Scheme 5 2)). Indeed, CAN was added within a mixture of MA and COS, meaning that radical can be formed on both entities. The reaction could happen as described in the case of cellulose, with a radical formed on the amine or alcohol of COS and propagation on the double bond of MA. One can also imagine that the maleic anhydride can homopolymerize and be grafted onto chitosan by ring opening, or in the other way around, at first the ring opening followed by MA polymerization (Scheme 5 3)). The addition of MA onto the

primary amine can also be followed by a ring-closing process, i.e. a traditional route for maleimide synthesis (Scheme 5 4)). In addition, CAN was reported to be a catalyst for aza-Michael reaction<sup>93</sup>. In this work, as well as lot of work on this subject, only FTIR spectra are provided. This analysis can only prove that maleic anhydride was grafted onto COS but not the nature of the grafting. NMR spectroscopy combined with mass spectroscopy and molecular weights measurement could lead to the identification of the formed structure.



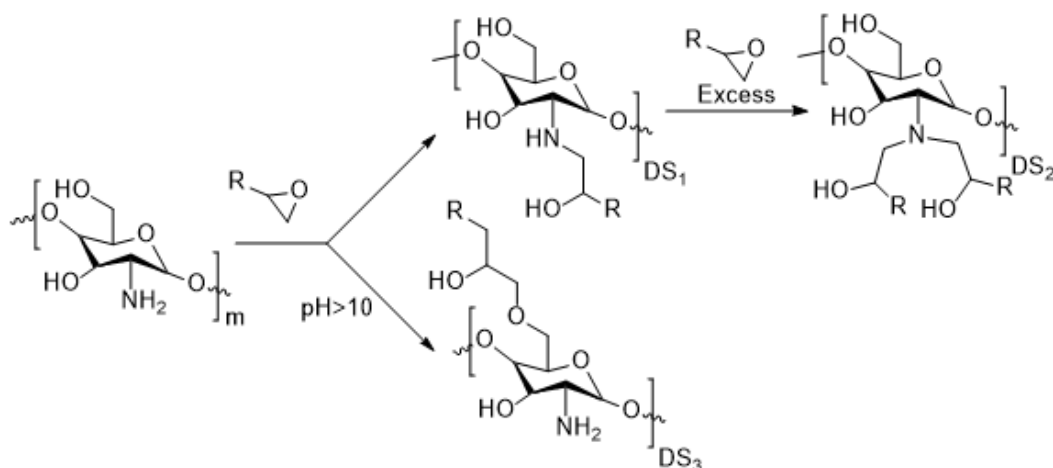
**Scheme 5. Possible structures for maleic acid grafting on COS**

Other anhydride can be grafted on COS. For example, Huang et al.<sup>94</sup> grafted succinic anhydride on 6-7 kg/mol COS with 36 to 90 % DS, measured by elemental analysis. Boesel et al.<sup>95</sup> added methacrylic anhydride in PBS solution on COS of DP 4 to 8. They showed that high substitution degree led to insoluble material and concluded that DS range of 16-21% were ideal. Afterwards, the methacrylated COS were cross-linked by free radical polymerization of the pendant methacrylic groups by redox initiation. The created gel was degradable and nontoxic, and could be potentially used in the biomedical field for tissue engineering or drug delivery.

In conclusion of this part, the reaction between anhydride and chitosan is quantitative. It also leads to the selective protection of amine for O-functionalization and the use of dianhydride can also lead to branched structures as well as cross-linked networks.

### II.3. Epoxides

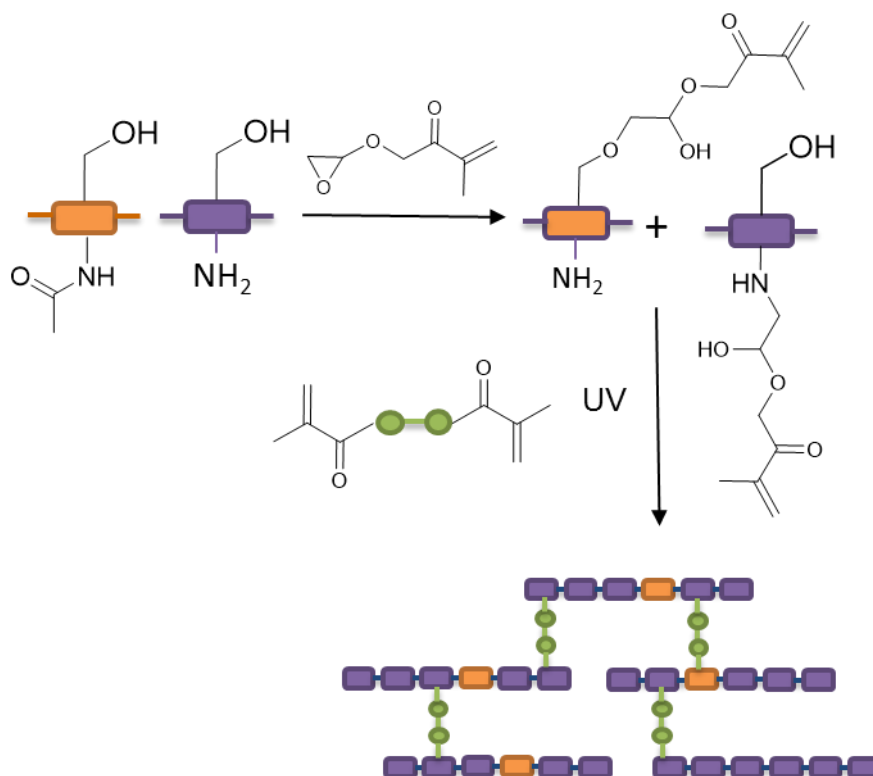
Another common reaction that can occur onto COS is the epoxy-amine or epoxy-alcohol reaction. The carbons in an epoxide group are very reactive electrophiles, due in large part to the fact that substantial ring strain is relieved when the ring opens upon nucleophilic attack. There are two electrophilic carbons in the epoxide, but the best target for the nucleophile especially with chitosan which already produced steric hindrance, is the least hindered carbon. COS has two nucleophiles; the primary amine in position 2 and the primary alcohol in position 6 of the sugar unit (Scheme 6). The reaction between an epoxide and a primary amine leads to the formation of a secondary alcohol and a secondary amine ( $DS_1$ ). The secondary amine might in turn react with another epoxide and form another alcohol and a tertiary amine ( $DS_2$ ). In chitosan and COS, this second addition is not likely to occur due to steric hindrance and poor nucleophilic strength of the secondary amine. The epoxy will therefore react with another amine of the polymeric chain, though, it could occur when working in epoxide excess. The second nucleophilic group from chitosan is the primary alcohol. It can be activated when working at  $pH > 10$ <sup>96</sup> ( $DS_3$ ). Nevertheless, the electronegativity of the oxygen implies a lower nucleophilic strength than the primary amine. Both additions are described in the literature and discussed below.



**Scheme 6. Possible reactions between an epoxide and chitosan**

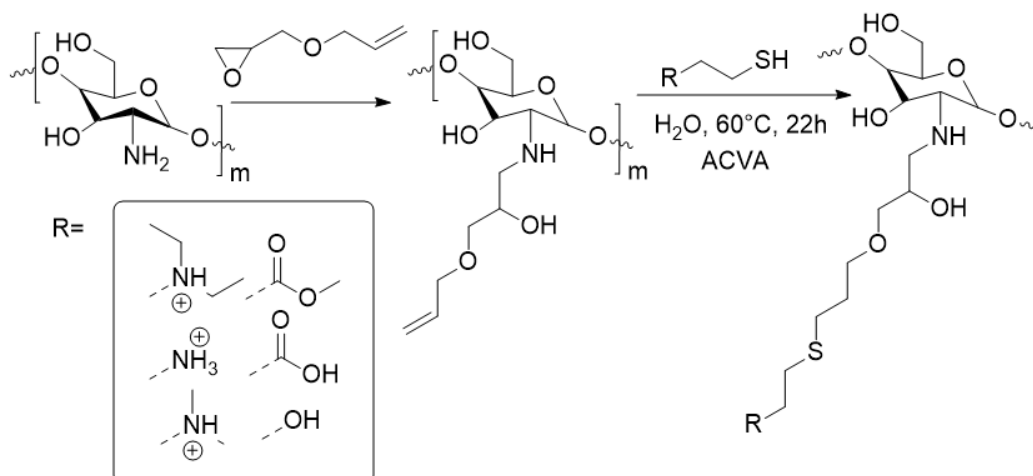
One example of epoxide addition often reported in the literature is the reaction between oligosaccharides and glycidyl methacrylate (GM) leading to the formation of a methacrylated COS by ring opening addition. The reaction conditions were similar in

several studies, and involved mixing different ratios of oligomers and glycidyl methacrylate and heating them at 40 °C up to 12 h under nitrogen atmosphere - or not, depending on the team, and ending with precipitation of the methacrylated COS in cold acetone. Most of the works for this kind of reaction described *O*-methacrylation resulting from a reaction between the primary alcohol of chitosan and glycidyl methacrylate. In reality, *N*-methacrylation is most likely to occur than *O*-methacrylation because amine of the oligomers is more nucleophilic than primary alcohol. In the works presented here, none of them were performed at pH>10 in order to obtain the alkoxide ion. Nevertheless, Cho et al.<sup>53</sup> claimed to observe *O*-methacrylation on 1-3 kg/mol COS. They showed DS of 60% with a 3/1 GM/COS ratio, which is a quite high value. In their case, they then fixed succinyl doxorubicin on chitosan free amine by amidation in presence of EDC and NHS with 18% DS. It is then possible that both *O* and *N*-methacrylation occurred. The methacrylated COS are then cross-linked by photo irradiation using a di-acrylated Pluronic block (Figure 3. *O* and *N*-methacrylation of COS followed by photo crosslinking by diacrylated cross linker which makes it suitable for free doxorubicin additional loading. In the same way, Choi et al.<sup>97</sup> claimed the *O*-methacrylation of 1-3 kg/mol oligomers with 8/1 GM/COS but did not indicate the substitution degree. The methacrylated COS was then crosslinked with di-acrylated pluronic by photo irradiation in presence of Irgacure 2959 to encapsulate a recombinant human epidermal growth factor (rhEGF). The provided <sup>1</sup>H NMR data could not confirm that the *O*-methacrylation occurred. On the contrary, the signal of the proton in position 2, characteristic of the free amine, has changed compared to crude COS. In a second publication<sup>98</sup>, they made 30% acrylated chitosan with 1/2 GM/COS ratio to photo-crosslink with diacrylated pluronic. Radha et al.<sup>99</sup> also claimed *O*-methacrylation in presence of ceric ammonium nitrate (CAN) in water at 80°C. Once again, only IR spectra were provided. In addition, CAN is known to be a radical polymerization initiator, which can lead to the methacrylate polymerization, not described here. To summarize, the grafting of GM onto COS by epoxy ring opening has been done. Nevertheless, the lack of information and poor analysis data on some studies presented above did not allow confirming that the grafting occurred on either the primary amine or the primary alcohol or both. Although some teams claimed that *O*-methacrylation occurred, this was not the more conceivable reaction due to the higher nucleophilic strength of the primary amine and the conditions of pH used. In conclusion, further analysis would have to be conducted on those types of grafting. For example, the alcohol substitution might be checked out by tandem mass spectrometry, amine titration or even <sup>1</sup>H NMR with the anomeric and position 2 proton signal, <sup>13</sup>C NMR and 2D analysis could complete those studies.



**Figure 3. O and N-methacrylation of COS followed by photo crosslinking by diacrylated cross linker**

As seen previously, the reaction of chitosan with an epoxide is most likely to occur on the primary amines. For example, Illy et al.<sup>100</sup> grafted allyl glycidyl ether (AGE) on COS (>2,000 g/mol) for further thiol-ene reaction on the allyl double bond (Scheme 7). The reaction was carried out in water at pH 8 as AGE is water soluble and a DS of 33% was obtained with 2.2 eq of AGE. <sup>1</sup>H NMR spectrum confirmed the addition with new signals corresponding to the introduction of the allyl substituents as well as new peaks for the proton in position 2 of the grafted units. FTIR spectrum also evidenced grafting onto amine by the secondary amine band appearance (1540 cm<sup>-1</sup>) as well as a reduction in intensity for the primary amine band (1590 cm<sup>-1</sup>).



**Scheme 7. Allyl-functionalization of COS and its modification by thiol-ene addition according to Illy et al.<sup>100</sup>**

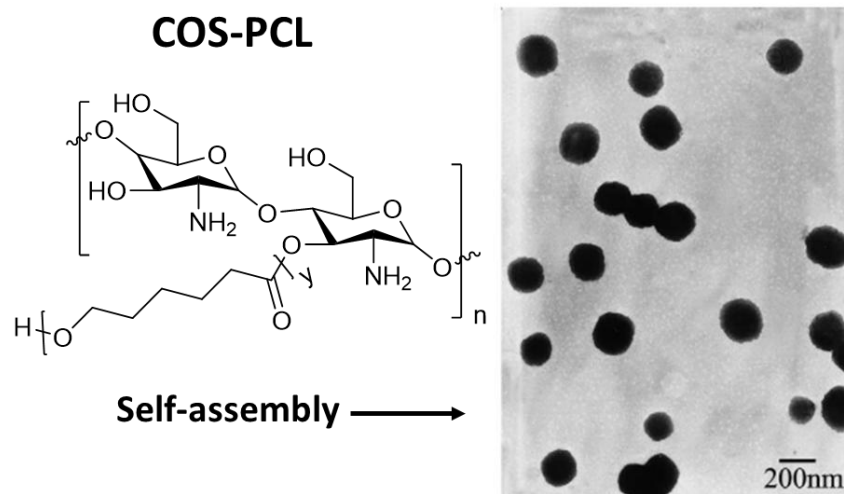
Mei et al.<sup>101</sup> described the grafting of epichlorohydrine on very low molecular weight COS i.e. 550 g/mol, by epoxy amine reaction with 55% DS. Then, the pendant chlorides were cross-linked with tetraethylene pentamine to get hydrogel with promising adsorbent properties. Quienne et al. also described the grafting of furfuryl glycidyl ether on the free amines of 1.6 kg/mol COS with 25% DS. They used the pendant furan to undergo Diels-Alder chemistry with PEG-maleimide structures. Another classical epoxy-amine reaction is the grafting of the glycidyltrimethylammonium chloride. This reaction is generally described on high molecular weight chitosan as it leads to full solubility of modified chitosan in water<sup>102</sup>, but was also described on COS. Huang et al.<sup>94</sup> showed maximum DS of 76% on 6.5 kg/mol COS and used the resulting product to establish the structural factors affecting radical scavenging activity of COS. Chen et al.<sup>103</sup> showed that the antibacterial activity and cytocompatibility of COS functionalized polyurethane membrane were better when COS were quaternized. Both teams performed the reaction in water at neutral pH and showed the *N*-substitution of glucosamine units by NMR and FTIR analysis.

#### II.4. Ring opening polymerization

Polycaprolactone (PCL) is a biodegradable polyester obtained by ring opening polymerization of  $\epsilon$ -caprolactone in presence of a catalyst. Due to its biocompatibility, and being FDA (food and drug administration) approved, it is often used in biomedical devices such as tissue engineering, implants or drug release applications. When grafted onto chitosan, it forms amphiphilic moieties that can self-assemble to encapsulate several drugs. Number of teams have worked on modification of chitosan oligomers by PCL. Wang et al.<sup>104</sup> described the synthesis of COS-PCL in two steps. First, hydroxyl and

amino groups of COS (1,800 g/mol) were selectively protected with chlorotrimethylsilane (TMS) and hexamethyldisilazane (HMDS). At the end, the hydroxyl and amino groups of COS were either trisilylated or disilylated with a majority of unprotected hydroxyl groups in the third position, which they claimed to be due to the relative weak reactivity of this primary alcohol towards silylation. They evaluated by  $^1\text{H}$  NMR that the TMS substitution degree was 76%, meaning that 7 free hydroxyl groups on position 3 per 10 glucosamine/acetyl-glucosamine units remained for initiating the ring opening polymerization (ROP). Those protections also conferred good solubility to the silylated COS in organic solvent such as pyridine, chloroform, tetrahydrofuran (THF) and xylene. Therefore,  $\epsilon$ -caprolactone was polymerized onto chitosan oligomers in presence of stannous octoate by ROP in a chloroform and xylene mixture.  $\epsilon$ -caprolactone was polymerized from the hydroxyl function of COS with different molar ratios, with a maximum DS of 47.3% determined from  $^1\text{H}$  NMR. This step was followed by deprotection in an isopropyl alcohol/ $\text{H}_2\text{O}$ / $\text{HCl}$  mixture to lead to amphiphilic COS-PCL structures. Authors showed that COS-PCL are able to self-assemble in aqueous solution and that the shape of the micelles was dependent on the size of the PCL chain (Figure 4). This synthesis was reproduced several times by other teams. For example, Gao et al.<sup>105</sup> prepared COS-PCL based hollow spheres from a series of metals with water soluble COS (1,800 g/mol). In order to do that, COS-PCL was synthesized as previously except only HMDS was used as protecting group and  $\text{AlEt}_3$  was used for ROP. Besides the reaction solvent was a mixture of chloroform and toluene. They showed different DS of PCL with variation of the number of protecting groups on COS and reached a maximum of one PCL chain by COS. In another work, they also tuned the size of giant COS-PCL vesicles synthesized according to the same protocol, by variation the DS and the length of the PCL chain and showed that only the COS-PCL with both the higher chain length ( $\gamma=178$ ) and the higher DS (10%) can self-assemble into giant vesicles with a diameter higher than 0.5  $\mu\text{m}$ <sup>106</sup>. They showed that the formation of the vesicles was temperature dependent, dissolved above 50  $^\circ\text{C}$  but self-assembled in vesicles again when cooled down. In those previous works, no analysis proved that only the secondary alcohol was not protected and that the ROP occurred on the secondary alcohol. Nevertheless, the quantitative silylation of chitosan was reported before<sup>107</sup> and the assumption that the secondary alcohol is the less nucleophilic one is true. Li et al.<sup>81,82</sup> also used N-phthaloylation of COS to protect the primary amine. Thus, the stain-catalyzed polymerization of  $\epsilon$ -caprolactone was performed directly onto the primary alcohol of COS in pyridine with no need of deprotection. Neither NMR nor DS data were provided. Only FTIR spectra confirmed the grafting of first phthalic anhydride (PHA) and then PCL. The

N-phthaloylation, as previously mentioned (II.2), is known to be a quantitative reaction when working in appropriate ratio of PHA and conditions (water/DMF)<sup>80</sup>. That is why in those conditions (1.7 eq of PHA), the amine group should be completely protected and the O-functionalization on the primary alcohol is the most probable. Indeed, it is a better nucleophile than the secondary alcohol.



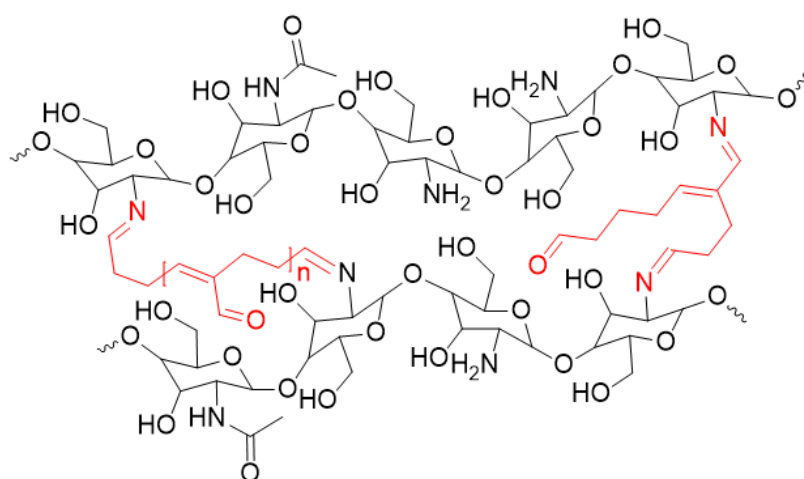
**Figure 4. Structure of COS-PCL and TEM images of the resulting nanoparticles (n=11, y=152; 0.8% (w/v) copolymer)<sup>104</sup>**

In conclusion, the selective protection of COS led to different amphiphilic moieties by grafting either onto amine or alcohol of the sugar units. Nevertheless, we can highlight the lack of structural characterization. Thus, to be more specific on which functional group was grafted, more spectroscopic data such as <sup>13</sup>C or 2D NMR and FTIR analyses could be suggested. In addition, amine titration could be done before and after reaction to check that no change occurred when O-functionalization is claimed.

## II.5. Schiff base

Glutaraldehyde is the most frequently used crosslinker in chitosan network. Both aldehydes of this molecule will react with free amines of chitosan to form imines. Kildeeva et al.<sup>108</sup> showed that the crosslinking mechanism between glutaraldehyde and chitosan is a complex pH dependent process. They highlighted that chitosan catalyzed glutaraldehyde homopolymerization to form irregular products depending on the pH and glutaraldehyde concentration (Figure 5). This phenomenon has to be taken into account when designing new material as it could impact its properties.



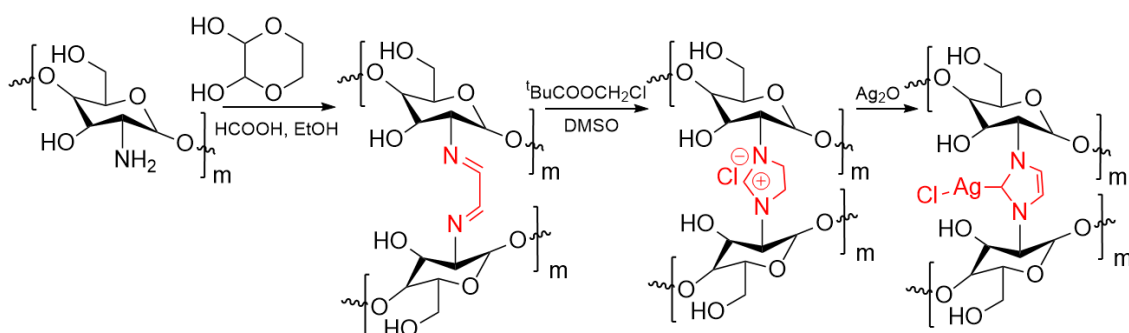


**Figure 5 Possible side chemical structure of chitosan cross-linked with glutaraldehyde**

Many teams used glutaraldehyde and in some cases, COS are mixed with other amino-components and crosslinked to form a new material with additional properties. For example, Ratanavaraporn <sup>109</sup> et al, used different ratios of COS and gelatin (G) with glutaraldehyde as a cross linker to make scaffolds for the osteogenic differentiation of rat bone-marrow-derived mesenchymal stem cells (MSC). Though, they showed that the crosslinking occurs mostly between gelatin units as they flushed away some COS by washing the gel with water. Nevertheless, they managed to reach 70:30 G/COS ratio and obtained promising scaffolds for osteogenic differentiation culture of MSC with better properties than gelatin only scaffolds. Kumari et al. <sup>110, 111</sup>, used glutaraldehyde to fix COS onto a natural protein, bovine serum albumin (BSA) to obtain a promising biopolymer for gene delivery. They used an excess of COS compared to BSA to ensure that COS are cross-linked to BSA and not only BSA to itself. In other cases, components of interest are trapped in the network during the crosslinking of chitosan. For example, oligosaccharide and carboxymethyl oligosaccharide cross-linked with glutaraldehyde can be imprinted with  $\text{Cu}^{2+}$ . This system showed good chelation capacity for depleted uranium <sup>112</sup>. The glutaraldehyde cross-linked chitosan can also have different design such as nanoparticles formed by water in oil micro emulsion. This shape might be a vehicle of interest for different substances, for example to load and carry adenosine triphosphate (ATP) <sup>113</sup>. In some cases, glutaraldehyde is used as a second cross linker as shown by Radha et al. <sup>114</sup> who grafted glycidyl methacrylate onto oligosaccharides and combined the copolymerization of polypropylene glycol initiated by ceric ammonium nitrate and glutaraldehyde to crosslink COS. They established the grafting parameters and showed that this material was suitable for the removal of heavy metals from waste water.

Glutaraldehyde is a good cross linker in general as the imine formation between its two aldehyde functions and the free amines of chitosan is favorable. Nevertheless, the imine formation is reversible in certain conditions (pH and temperature) and the mechanism is both pH and concentration dependent. Nevertheless, glutaraldehyde is toxic, ecotoxic and corrosive.

Necol et al.<sup>115</sup> proposed another way to crosslink oligochitosan through Schiff base formation leading to heterocyclic carbenes (Scheme 8). The synthesis of the diimine involved the addition of 2,3-dihydroxy-1,4-dioxane in acidulated ethanol activated by sonication. This was followed by the addition of chloromethyl pivalate, which led to the formation of the imidazolium ring. The final step consisted in the addition of Ag<sub>2</sub>O. In this reaction, the Ag-C bond is formed by introduction of silver between the chloride anion and the imidazolium ring. They showed that the reaction was more efficient on 2 kg/mol COS than on glucosamine with higher yields and silver bonding. They explained this observation with the better solubility of COS in alcohol and the hydrogen bonding which are in favor of the imidazolium ring formation.



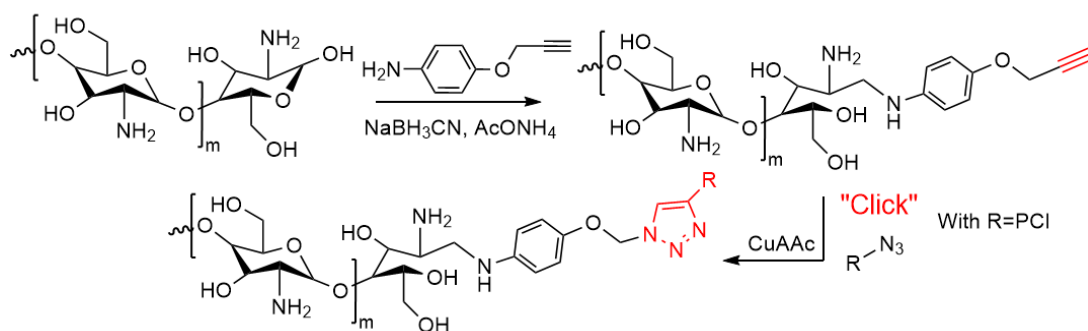
**Scheme 8. Synthesis of chitooligosaccharide silver complex through imidazolium ring formation**<sup>115</sup>

Other aldehydes were described in the literature. For example, Ma et al.<sup>116</sup> grafted vanillin through Schiff base formation onto carboxymethyl oligochitosan (CMCOS) with DP from 3 to 8. The reaction was carried out in ethanol and acetic acid solution for 10 h. They obtained a whole range of DS from 24 to 41% with 1:1 and 1:3 vanillin/CMCOS ratios respectively. Wang et al.<sup>117</sup> also grafted vanillin on COS from 3 to 5 kg/mol with different molar ratios in a water/ethanol mixture. They showed that when the DS is too high, about 47%, the vanillin grafted COS is no longer soluble in water. They also highlighted the pH dependency of the imine formation when no reducing agent was used. Indeed, the benzoic imine was proved to be stable when pH>7.4. At lower pH, the reversibility occurred and COS were no longer grafted with vanillin. This showed great potential for drug delivery. Indeed, the vanillin grafted COS can self-assemble and

encapsulate cancer drug and then be disrupted and dissolved at the contact of cancer cells due to pH changes. Among Schiff base formation on COS, Ren et al.<sup>118</sup> grafted phenolic acid by enzymatic modification. Phenolic acid was first oxidized in presence of laccase and then reacted on free amines of COS (Scheme 10 b)). Elshaarawy et al.<sup>119</sup> functionalized COS with previously synthesized vanillyl-imidazolium ionic liquids with 1:1 molar ratio in a 2% acetic acid ethanol solution and obtained a DS of 45% calculated by mass difference between the original COS and the product. They claimed that the reaction was favored as those optimized condition can led to almost total conversion of COS, indeed, with 27% DA only 18% of free amines remained. This synthesis led to the preparation of oligochitosan-based silver nanoparticles, following a green in-situ protocol, which may find applications in biomedical textiles articles. With the same idea, Sofy et al.<sup>120</sup> grafted 3-(4-acetyl-3-hydroxyphenoxy)propyl triphenyl-phosphonium salts with 28% DS.

Meng et al.<sup>75</sup> used a different pathway to graft spermine onto stearic acid grafted 18 kg/mol COS. They first oxidized the COS backbone using sodium periodate ( $\text{NaIO}_4$ ) leading to the formation of two aldehyde groups among the polymer chain. The oxidization degree was 12.4%. This oxidation was followed by the addition of spermine and sodium borohydride ( $\text{NaBH}_4$ ) to reduce the previously formed imine. Finally, 7.6% of the aldehyde were linked with spermine. The choice of  $\text{NaBH}_4$  as a reducing agent allowed not only to reduce the imine but also the resulting aldehyde to avoid any further side reaction. Nonetheless, the action of  $\text{NaIO}_4$  led to the breakage of the polymer backbone as reduction of the molecular weights was observed.

Among other types of chemistry, Guerry et al.<sup>121</sup> developed an aniline mediated reductive amination. In this reaction, aniline can react with the chitosan reductive end to form an imine. They first performed a model reaction on tetra-N-acetyl-chitopentaose and propargylamine dissolved in ammonium acetate buffer in presence of sodium cyanoborohydride ( $\text{NaBH}_3\text{CN}$ ). They showed that the addition of aniline and the in situ generation of the aniline iminium enhanced the formation of the propargylamine iminium through transamination. When transposed to 2 kg/mol COS, the aniline-catalyzed reductive amination showed some limitations and poor yields. Nonetheless, the direct use of a propargyl-aniline derivative led to significant yield (70%). The alkyne terminated COS were then clicked to azide terminated PCL structure for synthesis of amphiphilic structures<sup>122</sup> (Scheme 9). This chemistry may open the way to the synthesis of various structures and copolymers through click chemistry on the COS reducing end.



**Scheme 9. Reductive amination of COS followed by « click » reaction by CuAAc coupling**

Generally, aldehydes are very reactive toward the free amine of COS. This can easily lead to network formation but also to the introduction of new functionalities on COS. The Schiff base reversibility can undergo different applications such as drug delivery or selective protection for further reactions as described below (II.9)<sup>123, 124, 125, 126</sup>.

## II.6. Michael addition

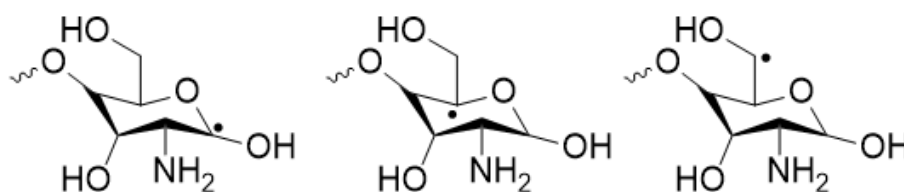
The Michael addition is the nucleophilic addition of a carbanion or another nucleophile to an  $\alpha,\beta$ -unsaturated carbonyl compound containing an electron withdrawing group. Michael addition with chitosan is rather an aza-Michael reaction involving the primary amine of chitosan as a Michael donor, and an electron deficient alkene molecule as a Michael acceptor. For example, Chen et al.<sup>103</sup> managed to graft COS onto polydopamine (PDOPA) polyurethane (PU) membranes simply by immersing the PU-PDOPA membranes in COS solutions with various concentrations. The aza-Michael reactions occurred between the aromatic ring of PDOPA and the primary amine of COS at room temperature. Ren et al. also described the aza-Michael addition of phenolic acid on 1 kg/mol COS in enzymatic conditions followed by its in situ polymerization. Nevertheless, they did not provide substitution degrees.

Quienne et al. grafted furfuryl glycidyl ether on COS, as described above, to perform Diels-Alder reaction with monomaleimide moiety. They evidenced that aza-Michael reaction could occur between the maleimide double bond and the free amine of COS. This reaction was shown to be solvent and pH dependent. Indeed, when working in DMSO, the double bond of the maleimide reacted with the primary amine through Michael addition. On the contrary, when working in 1 or 2% acetic acid solutions, the primary amines were protonated and not nucleophilic enough to react with the double bond and the Diels-Alder reaction occurred. Similarly, when acetic acid was added in DMSO, the reaction did not occur anymore.

Finally, the use of COS allows working at neutral/basic pH but also in other solvents such as DMSO. This previous experiment showed that it can open the way to reactions that were not possible with chitosan when working in acidic conditions.

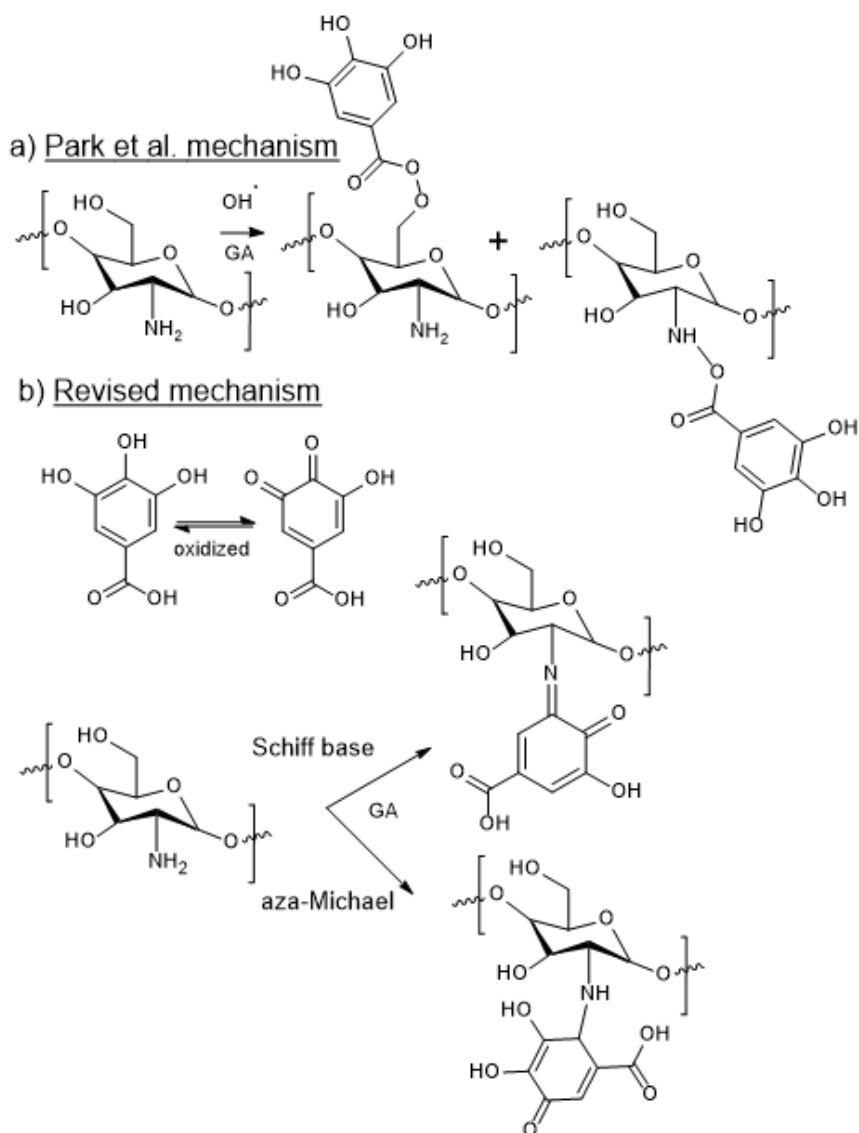
## II.7. Radical process

Park et al.<sup>127</sup> reported grafting of gallic acid (GA) onto COS through radical process. Radicals were first obtained from ascorbic acid oxidation with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)<sup>128</sup>. Those radicals were then able to react with COS. They claimed that the radical positions from COS were on both the primary amine and the primary alcohol. Nonetheless, Gryczka et al.<sup>129</sup>, who described the degradation of chitosan from 690,000 g/mol to 20,900 g/mol using electron beam, advocated very poor selectivity toward reactive radicals (like OH radicals). They used electron paramagnetic resonance spectroscopy (EPR) to explain the radical formation mechanism and scission of the polymer chain, and also claimed that all the CH and CH<sub>2</sub> sites in the pyranose ring were involved. Their version of free radical localization on chitosan is presented below (Figure 6).



**Figure 6. Localization of free radicals on chitosan units according to Gryczka et al.**<sup>129</sup>

The radical formation on COS was followed by GA addition which is composed of an aromatic ring. However, GA was already described before in the literature as a scavenger for both OH and OOH radicals<sup>130</sup>. In addition, Park et al. described the scavenging properties of their GA-grafted COS. Thus, the radical formation onto COS in presence of GA is not likely to occur and even less either on the primary amine or alcohol. Nonetheless, the <sup>1</sup>H NMR spectrum combined with UV analysis, showed that the GA grafting occurred. Addition of amines on oxidized GA through Schiff base formation, acid base reaction or aza-Michael addition was already described before<sup>131</sup> and the reaction is very much alike from the work of Ren et al.<sup>118</sup> on phenolic acid. This is why a most probable structure for the GA-grafted COS was proposed (Scheme 10).



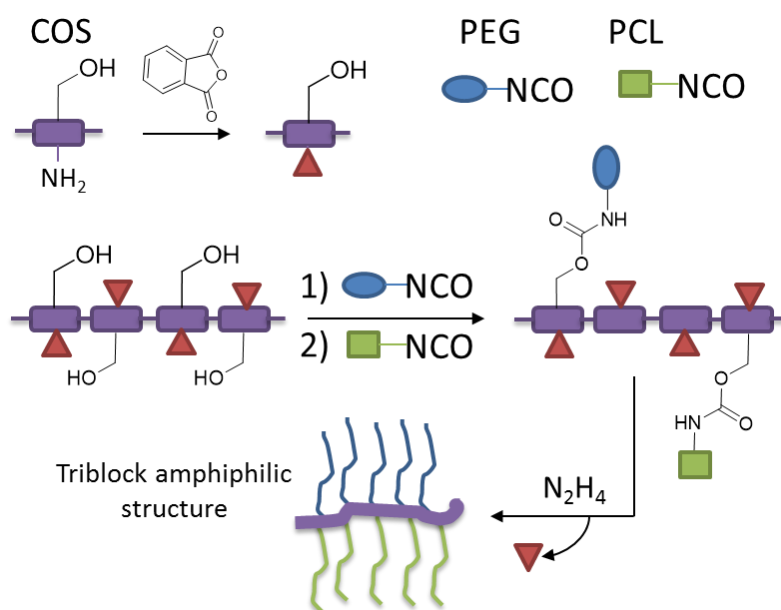
**Scheme 10. a) Grafting of GA on COS according to Park et al.<sup>127</sup> b) Revised mechanism proposed by the authors**

## II.8. Isocyanate

The functional group with the formula  $R-N=C=O$  defining isocyanates can undergo a broad range of chemical reactions. In the case of chitosan, they are most likely to react with the primary amine but can also react with the primary alcohol in certain conditions.

For example, Xu et al.<sup>132</sup> achieved biodegradable chitooligosaccharide-based polyurethane (PU) with COS and polycaprolactone/diurethane isocyanate as cross linker. The prepolymer isocyanate reacted with the primary amine of COS to form ureido bond as  $NH_2$  groups showed higher reactivity towards isocyanate than primary alcohol. The use of COS as a chain extender afforded better thermostability, hemocompatibility and

surface hydrophilicity to the PU film than reference. The remaining free amino groups might still be available for further modification and to extend the application field. Ren et al.<sup>118</sup> also used a diisocyanate cross linker, previously synthesized from isophorone diisocyanates (IPDI) and polytetramethylene ether glycol (PTMG), to form waterborne polyurethanes. In another approach, Liu et al.<sup>83</sup> synthesized new tri-block copolymers from COS, PCL and PEG (polyethylene glycol) that can self-assemble to encapsulate anti-cancer drug, doxorubicin. They used a different pathway than the previous teams<sup>81, 59</sup>. The first step consisted in the N-phthaloylation of COS using phthalic anhydride as described before (II.2). This resulted not only in the protection of the primary amine but also changed the solubility of the oligomers that were no more soluble in water but still soluble in organic solvents such as DMF or DMSO. Previously synthesized PCL and PEG both terminated with isocyanate function (NCO) were grafted onto free hydroxyl groups of COS in DMSO. COS were deprotected by hydrazine hydrate and the products were purified by dialysis to obtain triblock copolymers (PCP).



**Scheme 11. Synthesis of PCP copolymers through N-phthaloylation of COS followed by urethane formation**

The low efficiency of this reaction was evidenced by size exclusion chromatography (SEC) with only two PEG-NCO and PCL-NCO chains respectively grafted on 2 kg/mol COS, probably due to steric hindrance and hydrogen bonding from COS but also the length of PEG and PCL chains (~2 kg/mol) that were introduced at only 1 eq NCO/OH. The resulted copolymers were self-assembled in PBS and loaded with doxorubicin. The remaining free amine of the triblock might also be cross-linked by genipin to increase the drug release period.

In summary, isocyanates are able to react with both primary amine and alcohol of COS. They can be used as efficient cross-linkers as the N=C=O function is very reactive. Nevertheless, a lot of isocyanates are toxic and the desire to replace them in any kind of applications is recently gaining an increasing interest in both industrial and academic communities.

## II.9. Nucleophilic substitution

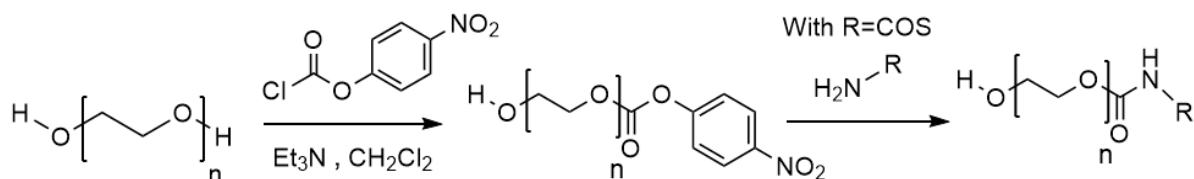
As with many reactions, nucleophilic substitution on COS can occur on both amine and alcohol although the primary amine is more reactive.

To favor the O-substitution, some teams protected the amine before the addition of the electrophile substrate. Yue et al.<sup>123, 124</sup> for example, used benzaldehyde protection by Schiff base formation to graft cinnamyl alcohol and geraniol on the primary alcohol of COS. The protection was performed in basic conditions, which is in accordance with the imine stability described before (II.5), it also led to a solubility change. At first, they transformed both cinnamyl alcohol and geraniol into cinnamyl bromide and geraniol bromide, respectively. The protected N-benzylidene COS dissolved in DMF was then added dropwisely to both modified alcohols dissolved in DMF. The primary alcohol can react on the electrophilic site of both products resulting in nucleophilic substitution of the bromide. At the end, amine deprotection was performed by dissolution of the resulting product in an HCl/ethanol solution for 24h followed by precipitation of the O-grafted COS by addition of NaHCO<sub>3</sub>. They obtained 24 to 37% DS for geraniol and 6 to 18% DS for cinnamyl alcohol. Following the same pathway, Liu et al.<sup>126</sup> grafted kojic acid chloride onto 1 kg/mol COS. After protecting the amine, they performed the reaction in DMSO and precipitated the O-substituted COS in acetone. DS for benzaldehyde substitution was not provided. Indeed, if residual amines are available, they might react with the electrophile and N-substitution might occur. As a matter of fact, Liu et al. also described the N-substitution of piperazine modified chlorokojic acid when no amine protection is used<sup>133</sup> and in the previous work presented above, they claimed to obtain DS>100%<sup>126</sup>. The spectroscopic data can only prove the grafting but are not precise enough to confirm if only O, N or both substitutions occurred.

As another example of nucleophilic substitution, Zhang et al.<sup>134</sup> first grafted methylchloroformate on free amine of COS with a DS of 88% followed by chloroacetyl chloride grafting onto the primary alcohol to form 2-urea-COS. Both reactions were carried out in excess of reactant to ensure high DS. The product was then reacted with N-phenyl-N'-pyridylurea that can be efficiently added by nucleophilic substitution of the chloride beard by the chloroacetylchloride. Ramalingam et al.<sup>63</sup> described the synthesis



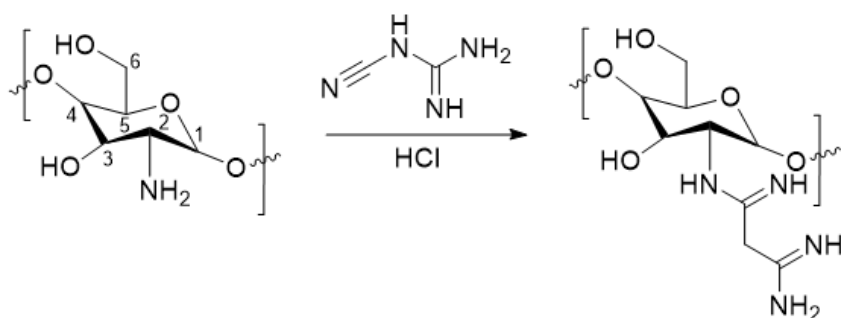
of 15 kg/mol N-trimethyl-chitosan with 22.5% DS by nucleophilic substitution of iodomethane. This reaction is well known for the formation of quaternary amine that improves the solubility range of chitosan in water. Song et al.<sup>64</sup> used 4-nitrophenyl chloroformate (NPC) (Scheme 12) **Erreur ! Source du renvoi introuvable.**, one of the most common reagents for the activation of alcohols, thiols and amines<sup>135</sup>, to modify PEG. The addition of COS on this reactant led to nucleophilic substitution of nitrophenol and amide formation with 90 % DS.



**Scheme 12. Activation of PEG with NPC and addition on COS**

### II.10. Guanidination

Wang<sup>136</sup> grafted dicyandiamine also called 2-cyanoguanidine (CG), which is a nitrile derived from guanidine, on COS. They studied the microwave assisted reaction between the primary amine of COS and the nitrile function carried out by CG and established the best conditions to be a microwave power 400 W for 15 min at pH 1 with a 1:2 COS/CG ratio (Scheme 13).



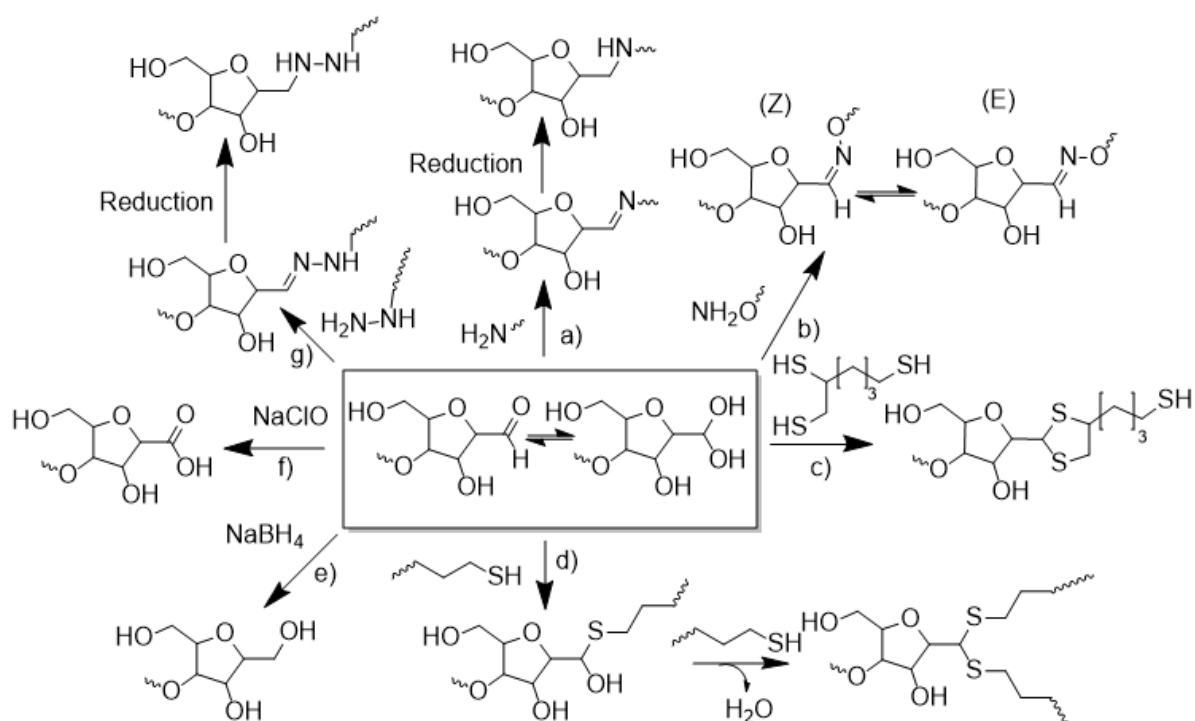
**Scheme 13. Guanidination of COS**

In the following study<sup>137</sup>, they used the previously determined conditions to graft CG with 18% DS calculated from elemental analysis comparison. The pendant amine thus introduced was then used in combination with the primary amines of COS, to graft the product on a previously activated membrane bearing acyl chloride groups. Microwave action seemed to be a nice way for improving grafting degree while working in acidic water.

### III. Grafting on aldehyde

The depolymerization of chitosan leads to the formation of a chain end aldehyde, 2,5-Anhydro- D- mannofuranose (AMF), when using sodium nitrite. This method has been widely described for being efficient and reproducible to obtain COS. Yet, this reaction leads to the formation of side reaction when the aldehyde reacts with the free amines of chitosan to form imines, thus leading to branched structures and in acidic condition, to the liberation of 5-hydroxymethyl-2-furfural (HMF) with additional breakage of the glycosidic bond <sup>46</sup>. This is why some teams tried to block the aldehyde moieties. For example, Salim et al.<sup>138</sup> used sodium chlorite to oxidize the aldehyde into an acid (Scheme 14 f)). While avoiding the side reactions, the chain end function remained reactive and further reaction on the carboxylic acid could be undergone. They reproduced this reaction on oligomers of DP 10, 20 and 30 with high yield (80 %). Bezrodnykh <sup>139</sup> et al compared the action of sodium borohydride and hydroxylamine on AMF chain end COS. NaBH<sub>4</sub> allowed reducing aldehyde in alcohol but also led to the reduction of the formed imines, which entailed an increase in the molecular weight as branched oligomers are formed (Scheme 14 e)). Hydroxylamine led to the formation of chitosan oximes, an irreversible structure that allowed quenching the aldehyde chain end (Scheme 14 b)). In another approach, some teams used the formed aldehyde to functionalize the oligomers. For example Salim et al.<sup>140</sup> also made 4-(hexyloxy)aniline-linked COS with excellent mass yield (92 %) through Schiff base formation (Scheme 14 a)) followed by reduction of the formed imine by sodium cyanoborohydride (NaBH<sub>3</sub>CN). This mild reducing agent is far less reducing than NaBH<sub>4</sub> and is therefore especially used to convert imines into amines. Moussa et al.<sup>141</sup> synthesized octanoichydrazide linked oligomers via reductive amination (Scheme 14 g)). Following the same method, they also functionalized oligomers of different molecular weight with various amino compounds to obtain high potential COS-based conjugates <sup>142</sup>. Aniline, O-hydroxylamine and hydrazide derivatives were chosen for their lower pKa values (3-6) compared to free amine of COS (6.5-7). In that case, intramolecular imine formation were avoided and only amination and oximation occurred. Those reactions showed quantitative functionalization of COS with no degradation of the starting oligomer chain and introduction of various clickable functional groups. These promising results opened the way for the preparation of new diblock copolymers, as illustrated in their work by the synthesis of COS-b-polyethylene glycol. Coudurier et al. <sup>143</sup> recently described the grafting of dioxamine on the resulting aldehyde. They managed to avoid crosslinking between units by using an excess of amines and a

dropwise addition of the oligomer solution. They highlighted the formation of (E) and (Z) isomer during oximation by  $^1\text{H}$  NMR ((E)-oxime 7.6 ppm and (Z)-oxime 7.0 ppm). Pickenhahn et al.<sup>144</sup> showed thioacetylation using mercaptoethanol and mercaptopropionic acid on a model study, first on AMF molecule and then on 2 kg/mol COS-AMF. For this reaction, they played on the equilibrium between the aldehyde and gem-diol form of AMF in solution (Scheme 14 d)). This can lead to the formation of hemithioacetal and thioacetal when working in thiol excess. They estimated the excess needed was at list 10 eq of thiol by AMF units and showed that freeze drying is in favor of thioacetal formation. Thioacetal was shown to be the most stable form compared to hemiacetal. As a direct application, they reacted a 2 kg/mol thiol bearing PEG on the aldehyde of a 10 kg/mol COS and resulted in 61% of thioacetylation. They also developed a novel approach allowing a more efficient one-step activation of the aldehyde by thioacetylation (Scheme 14 c))<sup>145</sup>. This process relies on the use of a trivalent linker bearing a thiol-hook and a thiol-tail. At first the thiol-hook reacted with the aldehyde through hemithioacetal intermediate followed by water elimination to result in thioacetal formation. They showed that time and trivalent thiol amount increment could displaced the equilibrium toward the thioacetal moiety. At the end, the oligochitosan was bearing a thiol-tail that is then able to react with any thiol-reactive entities.



**Scheme 14** Scheme summing up the possible reaction on 2,5 anhydro-D-mannose end chain oligomers : a) Schiff base formation followed by reduction ( $\text{NaBH}_3\text{CN}$ ,  $\text{NaBH}_4$ )<sup>140</sup> b) Oximation with formation of isomer (E) and (Z)<sup>139, 143</sup> c) Thioacetylation with trivalent linker<sup>145</sup>

**d) Thioacetylation** <sup>144</sup> **e) Reduction** <sup>139</sup> **f) Oxidation in carboxylic acid** <sup>146</sup> **g) Addition of hydrazine followed by reduction** <sup>141, 142</sup>

The use of aldehyde chain end is new and not yet widely used. Nevertheless, it is a promising way for synthesizing diblock copolymers. It was shown before that low molecular weight oligomers are soluble in DMSO. This solvent can lead to the use of hydrophobic moieties thus leading to amphiphilic structures. In addition, the NMR characterizations are easier due to new signals of the aldehyde around 8 ppm that are not overlapping the one from the rest of the oligomer chain.

#### **IV. Conclusion**

In this review, we demonstrated that many reactions are possible on chitosan oligomers. In fact, like chitosan, COS bears two reactive functions, amine and hydroxyl, that can undergo any kind of reaction. Nevertheless, the low molecular weight of COS lead to lower viscosity in aqueous solutions, larger pH range solubility and open the way to work in other organic solvents. All those parameters can lead to the introduction of new functions on those biomolecules in high yields. In addition, certain depolymerization routes lead to the formation of aldehyde chain end COS that are suitable for block copolymer synthesis and amphiphilic structures. Those structures can find applications in a lot of fields especially in pharmaceutical and biomedical devices area. In this study, we also demonstrated that there is an obvious lack on structural information when functionalizing COS. Indeed, DS are not always provided and sometimes miscalculated. This is why, even if the chemistry on chitosan and its derivatives is well developed, further research on yield improvement and structural characterization is most welcomed.

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