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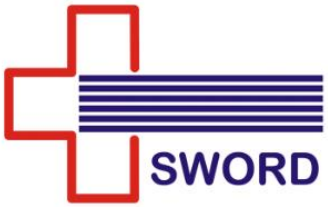

***Title*:**

Quaternary ammonium salts of chitosan. A critical overview on the synthesis and properties generated by quaternization

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Abstract

The quaternary ammonium salts of chitosan are water soluble derivatives which keep the promise for real life applications in a large realm of biomedical fields, such as antimicrobial products, gene therapy, drug delivery, wound healing, tissue engineering and cosmetics. The increased potential for such applications is gained by a beneficial combination

of the intrinsic properties of chitosan with those of the quaternary ammonium units. Since the first report of the synthesis of the quaternary ammonium salts of chitosan, many synthetic routes were developed, each of them with advantages and disadvantages which influence the product's properties. The aim of this review was to systemize these synthetic procedures and the properties of the resulted products, highlighting these advantages and disadvantages, in the desire to help the researchers working in this productive domain to choose the most suitable synthetic pathway when specific properties are targeted.

Keywords: chitosan; quaternary ammonium salts; synthesis; properties

Contents

1. Introduction

The increased awareness regarding the environment, including industrial discharges, disposal of solid wastes, discharges of domestic wastewater, limited resources and not only are of great interest over the past century [1,2]. Consequently, reducing waste, reusing resources and recycling materials can be the three R's that can lead to beneficial changes on our planet. In the field of chemistry, it was promoted the idea of "green chemistry", also called "sustainable chemistry", encouraging the initiatives of natural evolution for preventing pollution [3]. In line with these current requirements, researchers have focused their attention on the development of biodegradable materials, based on natural, renewable resources. Special attention was given to natural polymers that not only are derived from natural renewable resources, but also their decomposition products are environmentally friendly. Among them, polysaccharides are of great interest due to their properties, such as relative low cost, biocompatibility, biodegradability, limited allergic reactions, strong inter- and intra-molecular hydrogen bonds which impart good mechanical properties, ability to assure water permeability, and so on. Besides, the diversity in their structures and the possibility to form a large realm of biopolymer derivatives represent additional important benefits. Such a biopolymer of significant importance is the chitosan (Ch), the fully or partially deacetylated form of the natural chitin polymer, mainly prepared by treatment with strong alkali (Figure 1) [4,5].

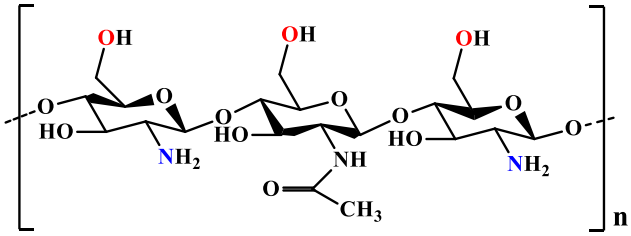
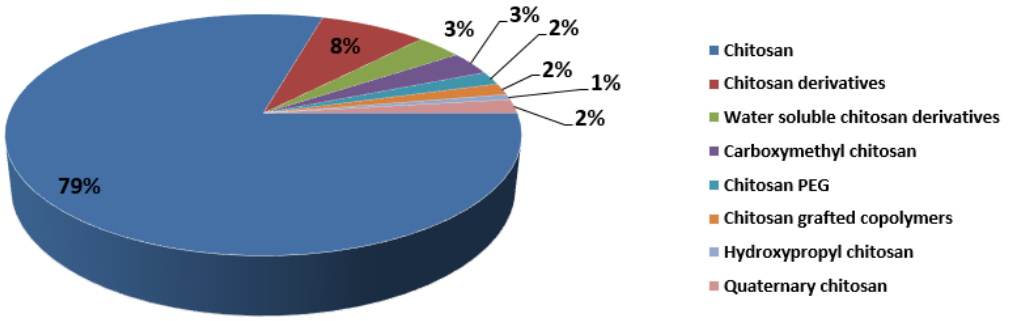


Figure 1. Structure of chitosan

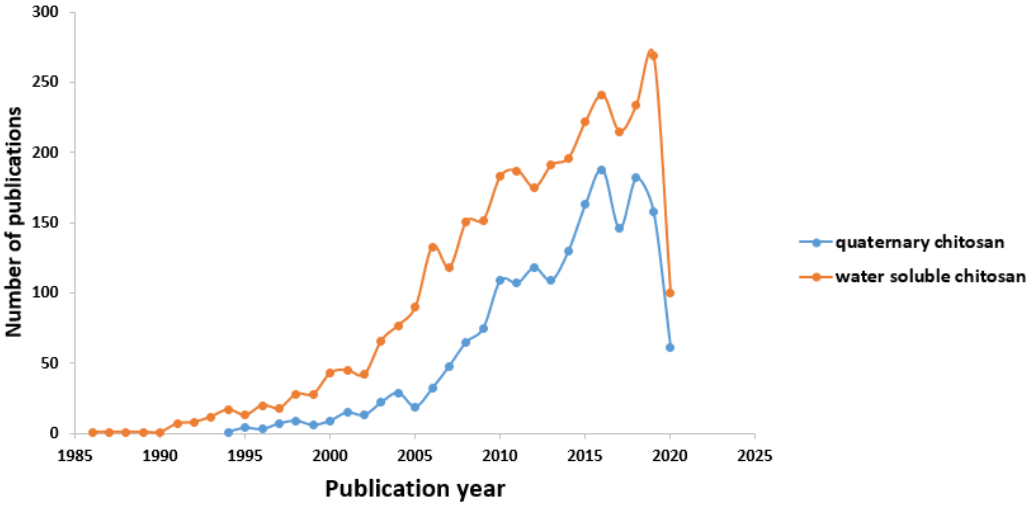
The most abundant source of chitin is the crustacean shell waste, but it is also found in renewable sources including exoskeletons of insects and fungi, as part of their cell wall [6]. The research dedicated to chitosan stressed its outstanding biologic properties which

fascinated the scientists; chitosan is biocompatible, nontoxic (its degradation products are known natural metabolites), hemostatic, antimicrobial, spermicidal, central nervous system depressant, immunoadjuvant, has antitumor effect, improves wound healing/or clot blood, absorbs liquids and forms protective films and coatings, selectively binds acidic liquids lowering serum cholesterol levels and has the property to accelerate bone formation [7-21]. Due to its properties, chitosan, labeled by many natural product suppliers as "too good to be true" is approved by FDA and commercialized as a product/drug which prevents a plenty of diseases, thus insuring a long life [22]. Although chitosan's properties recommend it as a promising polymer for applications of contemporary interest, it has a main drawback, which strongly limits the bio-applications, that is its reduced solubility above pH ~ 6.5. This is related to the strong network of inter- and intra-molecular hydrogen bonds developed among hydroxyl and amine groups, and depends on the molecular weight, deacetylation degree (DD)/acetylation degree (DA) and distribution of the acetyl group, pH, the type of acid used for dissolution and ionic concentration [11]. In order to improve the solubility of chitosan at both neutral and basic pH, the focus point of researchers was its chemical modification. As the chitosan structure includes amine and primary and secondary hydroxyl groups, the main modifications were directed to the grafting of various units at the free amine or primary hydroxyl groups, meant to disturb the H-bond network and thus to improve the chitosan solubility [23-26]. The research attention was directed to specific grafting groups, which can improve or bring new properties to chitosan, when precise applications were targeted [27,28]. Thus, many grafting groups were used along the time: *N*-carboxyalkl [29-31]; *O*-carboxyalkyl [32-35]; *N*,*O*-carboxyalkyl [30-32,34,36-38]; sulfate [39-41]; *N*-, *O*-, *N*,*O*-alkyl [42,43]; polyethylenglycol [44,45]; thiolate [46]; phosphate [47]; quaternary ammonium salts [48,49]. Each derivatization technique brought advantages and disadvantages, and researchers adopted them to prepare valuable products for specific bio-applications. A graphical representation of the number of papers dedicated to chitosan and water soluble derivatives shows that the derivatization did not succeed to bring consistent advantages over chitosan, the main interest going to the chitosan research yet, despite the strong limitations for real life applications implied by its limited solubility. As can be seen in Figure 2 a), at 26th of May 2020, almost 76500 articles about chitosan were published. Second to that, chitosan derivatives were the subject of almost 7600 articles, meaning less than 10%, followed by water soluble chitosan derivatives in general. The second most important class of water soluble chitosan derivatives after carboxymethyl chitosan are the quaternary chitosan derivatives. They are important not only because are water soluble, but also because the quaternary ammonium group brings new

properties which enlarge the application area in the field of bio-medicine with promising results for real life applications.



a)



b)

Figure 2. a) The interest in chitosan/chitosan derivatives over the last 25 years; b) Graphical representation of the interest in quaternary chitosan derivatives compared to water soluble chitosan ones (in line with **Web of Science – Core Collection**)

In this view, the aim of this paper is to analyze the research papers reported on the topic of quaternized chitosan, the main interest going to the understanding of the potential and limitations of the synthetic routes adopted for their obtaining, the structure-properties correlation and the potential of this class for real world applications.

2. Quaternized chitosan derivatives. Generalities

The modification of chitosan by transforming the primary amine groups on C2 into quaternary salts with permanent positive charge was embraced as a strategy for improving the solubility of chitosan concomitant with the enhancement of the antimicrobial, anticoagulant, antioxidant and mucoadhesive properties and also ability to complex negative charged species such as DNA [38,50-54]. A main advantage of the quaternization of chitosan is that it proceeds on the lateral groups, while the main backbone is not affected and thus the intrinsic

physico-chemical and biological properties of chitosan are preserved, while additional properties are gained. The quaternization of chitosan implies on one hand (i) the increase of the positive charge on chitosan, resulting in electrostatic repulsions among the chitosan chains and on the other hand (ii) the grafting of lateral alkyl chains. Both new units should have as effect the disruption of the intra- and intermolecular H-bond networks, unfolding the chitosan chains. This lead to a material with lower stiffness and consequently with improved solubility [55]. It is expected that an increased quaterniazation to lead to an improved water solubility. On the other hand, the resulting polycationic structure should have a positive effect on (i) the interaction with the anionic components present on the surface of the microorganisms, boosting the antimicrobial activity [56-58], (ii) the bonding of the negatively charged DNA, acting as vehicle for its transport to cells, [59] (iii) the opening of tight junctions by electrostatic forces with negative charges on the cell membrane, enhancing the drug transport across epithelia [60]. The alkyl and/or aryl groups generate an increase of the hydrophobic character of the quaternized derivatives, augmenting the interaction with the membrane of the microorganism's cell, which also has a positive impact on the antimicrobial activity [57,61]. Attention should be paid to the length of the alkyl side chain, if they are too long, such as cetyl, it can lead to insolubility [62]. This is the reason why, the quaternization of chitosan achievable by methylation, *N,N,N*-trimethyl chitosan (TMC) was more intense investigated, even if further promising results were also yielded by other alkyl quaternary salts.

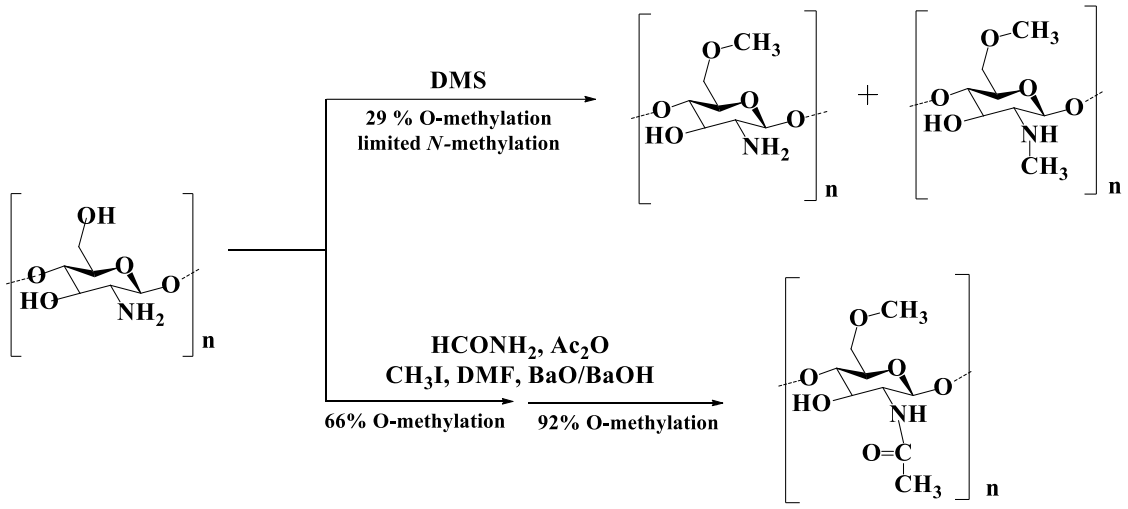
3. Synthetic routes for the synthesis of quaternary salts of chitosan

There are two main routes for introducing the quaternary ammonium salt on chitosan: (i) when the primary amine group of chitosan is transformed into quaternary salt, being directly linked to the chitosan backbone, or (ii) when the quaternary salt is indirectly connected to the chitosan by a lateral spacer.

3.1. Synthetic routes for preparing quaternary salts of chitosan directly at the amine units

The idea of chitosan methylation was initiated by Wolfrom *et al.*, who focused on the investigation of the methylation on a heparin derivative, which has similar structure to chitosan. The methylation followed the Haworth procedure using DMF as solvent, barium oxide/barium hydroxide (BaO/BaOH), and dimethylsulfate (DMS) as methylation agent, when a content of 29% methoxyl units were reached but also *N*-methyl units (Scheme 1). Another procedure which applied an acetylation reaction concomitant with the methylation using methyl iodide (CH_3I) in a basic medium of BaO/BaOH reached a 92% methylation degree, by

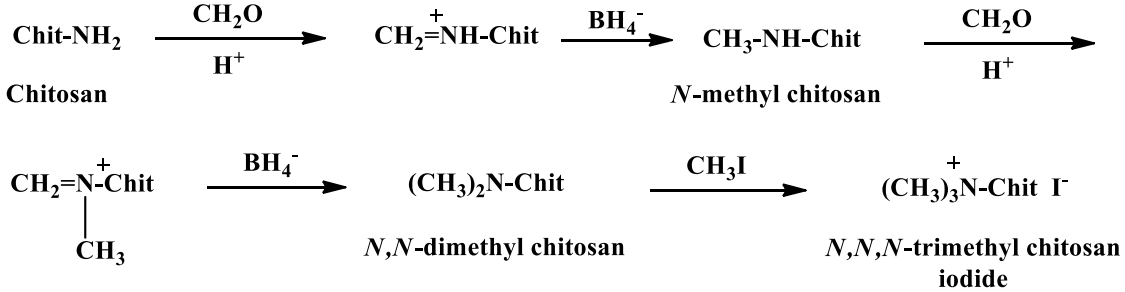
repetitive application of the procedure. The main revelation of this study which was published in 1963, referred to the availability of the free amine sites for methylation along with the primary hydroxyl ones and to the increase of methylation degree by repetitive application of the synthetic procedure. It was also noted the depolymerization of chitosan in basic conditions [63].



Scheme 1. Synthetic pathway of O-methylation, according to Wolfrom [63] (two arrows symbolize the twice application of the synthetic procedure)

3.1.1. Muzzareli method

The first synthesized quaternized chitosan derivative was *N*,*N*,*N*-trimethyl chitosan iodide (TMC). It was obtained by Muzzareli *et al*. by a reaction chain implying *successive alternant steps* of *N*-alkylation with formaldehyde followed by reduction with sodium borohydride, and finally methylation with CH_3I (Scheme 2) [64]. The obtained product was appreciated by authors as having a degree of quaternization (DQ) around 60%, given in fact by 35% quaternized amino groups and 25% *N*-monosubstituted and *N*,*N*-disubstituted ones. Compared to the reference chitosan, the product swelled in water but the targeted water solubility was not reached. It appears that the formation of the *N*-monosubstituted and *N*,*N*-disubstituted secondary by-products drastically affected the water solubility. Moreover, the synthetic procedure is time consuming, implying multiple reaction steps.

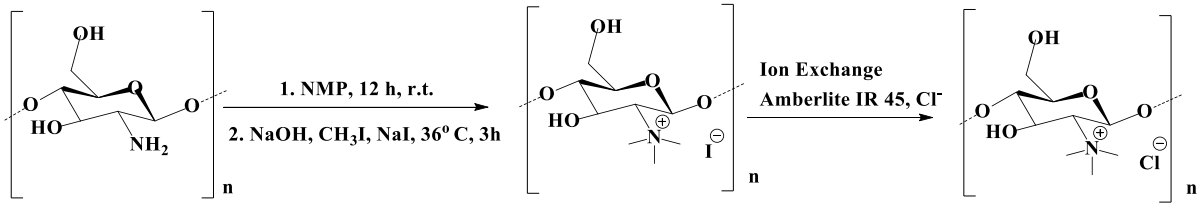


Scheme 2. Synthetic pathway for obtaining *N,N,N* – trimethyl chitosan iodide, according to Muzzareli [64]

Even if the drawbacks of the Muzzareli method limited its applications, it was revealed the potential of the methylation method to reach water soluble quaternized chitosan derivatives, inspiring other researchers, who dedicated their work to achieve this goal. Over time, the procedure of chitosan quaternization was adjusted, mainly starting from the methods applied by Wolfrom and Muzzareli [50,65-70]. They present advantages and disadvantages which will be highlighted as follows.

3.1.2. Domard method and its variants

A quaternization method realized in *one step* consists in the reductive methylation of chitosan with methyl iodide in basic conditions. It was reported by Domard in 1986, and was named by the researchers *Domard method* (Scheme 3) [71]. Actually, it is the same technique used by Wolfrom [63], with the difference that DMF was replaced with *N*-methyl-2-pyrrolidone (NMP), BaO/BaOH with NaOH, and DMS with CH_3I. The authors declared that the reaction proceeded in a homogenous medium (with a content of 14% water), but it is unclear how chitosan could have dissolved in a basic medium. All the later reported investigations using this method highlighted that the reaction proceed in a heterogeneous medium. A high quaternization degree (DQ) of 64% was reached after repeating the synthesis thrice. The main disadvantage was the lack of control on the molecular weight of chitosan, a high depolymerization degree being assumed based on the abrupt decreasing of the intrinsic viscosity, despite the low temperature of 36^o C at which the synthesis was performed. The polymers obtained by this method were described by the authors as being water soluble, whatever the pH, for a DQ higher than 25%. Moreover, it was established that an increase of the DQ was reached by applying the synthetic procedure three times, while further synthesis led to a slow decrease of it, concomitant with a viscosity decrease. This was justified by the difficulty to recover the highly substituted polymers with high solubility, but possible methylated products which diminish the solubility should be considered too. The method has gained interest, mostly in the last years, and it was used by many researchers who tried to improve it, in the trial to reach a controlled synthesis of the quaternized chitosan derivatives for bio-applications, such as gene delivery, breast cancer prevention, drug delivery [69,72-79].



Scheme 3. Synthetic pathway for obtaining *N,N,N*–trimethyl chitosan by Domard method [71]

Targeting to reduce the chitosan depolymerization and to have a better control over the parameters influencing the quaternization, Le Dung *et. al* extended the above-mentioned procedure, by increasing the reaction temperature at 60 ^{o}C, and varying the molar ratio between chitosan, methyl iodide and sodium hydroxide, while the reaction time was kept as short as possible, between 30 and 180 minutes [72]. These variations led to a quaternization degree comprised in the range 47-53%, a water soluble product with DQ of 40% being obtained after only 30 minutes. The iodide products were transformed in chloride ones by dissolving into a HCl/ethanol solution. Even so, the depolymerization degree remained the principal issue of this method.

A deeper investigation of this synthesis revealed the difficulty to control the transformation of the primary amino groups into quaternary, ternary or secondary amines [80]. Besides, it was demonstrated that the water solubility can be drastically influenced by the presence of the remnant HCl traces after the exchange reaction of iodide with chloride. Following the synthetic pathway used by Le Dung, Sieval's group [80] pursued this research topic by applying the synthesis twice, followed by the counterion exchange by using NaCl instead of HCl, to lower the impact of acid media on the solubility of the final compound. After the one-step synthesis, it was reached a 35% DQ, lower than the one previously reported, and consequently a poorer water solubility of 2% (w/v). When the procedure was repeated twice, products with a quaternization degree between 40-80% were obtained, with improved water solubility up to 5% (w/v). For a clearer insight on the synthesis, the group performed the synthesis repetition thrice. The DQ increased to 85%, but also complete *O*-methylated products were obtained, which appeared to drastically diminish the solubility. Unfortunately, the molecular weight of the quaternized chitosan was not given, and thus the assessing of its influence upon water solubility was not possible. Also, no information related to the water pH used for the solubility tests was provided.

Concluding, this synthetic strategy towards *N,N,N*-trimethyl chitosan chlorides via reductive methylation with methyl iodide in basic conditions appears to have two main disad-

vantages: the depolymerization in basic conditions and interfering of *O*-methylation, and of *N*-monoalkyl and *N,N*-dialkyl intermediates which seem to diminish the water solubility. An insight on the impact of the reaction conditions reveals the strong alkaline medium as a versatile regent, which on one hand favors the chitosan quaternization, but on the other hand inflicts unwanted side effects, such as the chitosan depolymerization by the cleavage of the glycosidic linkages and the methylation process at the hydroxyl groups of chitosan from the C3 and C6 positions [60,72,80-84]. However, the method is simple and economical, and it still applied by many researchers for preparation of drug delivery systems, indicating that these disadvantages do not minimize their potential for such applications [85-89].

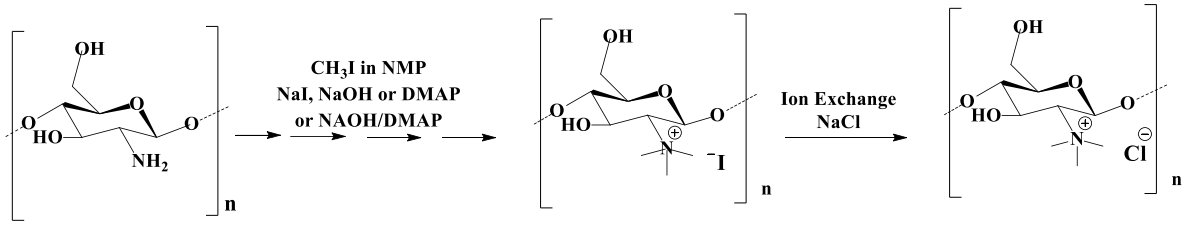
The main question arising is related to the impact of these two unwanted secondary processes on the water solubility of the final quaternized products. It is well known that chitosan solubility increases as its molecular weight decreases, chitosan oligomers having improved water solubility [90]. An insight of the influence of the reaction conditions on the depolymerization was presented by Kotze [91], who measured the molecular weight of the chitosan with different quaternization degrees by size-exclusion chromatography (SEC) and viscosity as well. Although the molecular weight alteration followed a similar trend as the viscosity, decreasing as the quaternization degree increased, it appeared that the viscosity values dropped more abruptly. The drastic diminishing of the viscosity as the quaternization degree increased can be attributed to the decrease of the density of intramolecular and intermolecular H-bonds, caused by the repulsive forces between positive charged nitrogen atoms and the presence of the methyl units on the nitrogen atoms of chitosan [92]. So, it was appreciated that depolymerization is less important than the viscosity reduction indicates.

Similar to the depolymerization, the *O*-methylation and *N*-alkylation became more important once the reaction time is increased by repeatedly applying the synthetic procedure, but it is also influenced by the excess of CH_3I and NaOH reagents or the type of base. Contrary to the depolymerization, it appears to have an antagonistic effect upon the water solubility, and additionally leads to the lack of control on the product structure [83]. In these circumstances, the water solubility of the final quaternized products is given by the balance of three "degrees": quaternization degree, depolymerization degree and degree of *O*-alkyl and *N*-mono- and *N*-dialkyl by-products. The main question arising is: which is the real role of the quaternization on the water solubility? To limit the influence of these unwanted secondary reactions, many attempts were done, mainly by replacing the reagents and/or the solvent with less destructive ones.

3.1.3. Limiting the impact of the inorganic base

3.1.3.1. Replacing inorganic base with organic ones

To overcome the thorny issue of the depolymerization, Hamman *et. al* proposed the replacing of the strong inorganic base NaOH in the Sieval method with an organic one, dimethyl amino pyridine (DMAP) (Scheme 4) [93]. Indeed, the depolymerization did not proceed to the same extent as in the case of NaOH, but the low basic character of the DMAP was directly reflected in a low quaternization degree, even when an excess of methyl iodide was used. Compared to the case of NaOH, the repeated synthesis did not improve the quaternization degree, which barely reached 9.6%. Moreover, the use of a mixture of DMAP with strong NaOH base, only moderately increased the quaternization degree to 34.4%, after repeated synthesis, but drastically altered the molecular weight of chitosan by depolymerization.



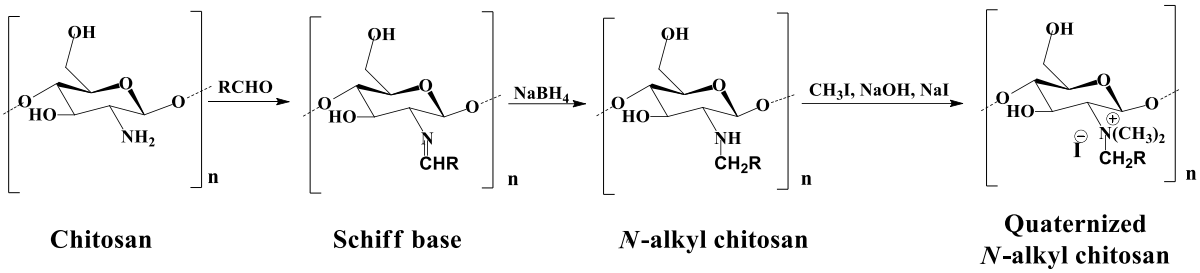
Scheme 4. Route for the synthesis of TMC using organic base or mixture of organic/inorganic bases [93]

(four arrows symbolize the application of the synthetic procedure four times)

Essentially, the two antagonistic processes, the quaternization and the depolymerization, advanced along with the longer contact time of chitosan with the base, so that, the higher quaternization degree was accompanied by a higher depolymerization. The quaternization degree was even slightly lower compared to the case of use of triethylamine (TEA) as an organic base, when the quaternization degree was appreciated 11% [71]. No clear information related to the influence of the quaternization degree on the water solubility were given in these papers (e.g. solubility tests), it can be only supposed they were water soluble following indirect information as the paper titles or the use of the products for further testing, but of course an improvement compared to the pristine chitosan is expected, and the increase of the cationic character is certain. Our opinion is that this method can be applied for the quaternization of the chitosan oligomers which already have better water solubility and antifungal properties, and by this method, these properties can be boosted.

3.1.3.2. Pre-alkylation of chitosan

Targeting to avoid a prolonged contact of the inorganic base with chitosan, and thus to limit its destructive effect, but also to lower the impact of the *O*-methylation, *a two-step* method was proposed**,** implying a *prior alkylation* of the amine units by imination reaction followed by imine reduction [50,65,94]. The advantage of this method should be the high electron density formed at the nitrogen atom after introducing the methyl group, facilitating the formation of quaternary ammonium salt. In fact, this is a variant of the Muzzareli method, consisting in replacing the formaldehyde with other aliphatic aldehydes. The quaternization with methyl iodide of the prior alkylated chitosan theoretically led to quaternary ammonium salts with mixed alkyl chains (Scheme 5).



Scheme 5. Synthetic route for the preparation of *N*-alkyl chitosan derivatives [65]

Unfortunately, no information related to the side effects of this procedure were given, but knowing the peculiarities of the imination reaction of chitosan, some questions arise [95]. The synthesis-oriented scientists willing to apply it should firstly answer to some questions: (i) how efficient is the alkylation by imination followed by reduction in acidic conditions (pH=4.5), considering that imination is a reversible process and the aliphatic aldehydes have low water solubility [95-103]; (ii) which sites will be preferred in the quaternization process with methyl iodide? the non-alkylated or alkylated ones? Moreover, considering that a decrease of the quaternization degree was reported along with the alkyl chain increasing, a large polydispersity can be suspected, caused by both the partial substitution of the alkylated chitosan and the depolymerization. It can be appreciated that the reaction control is quite tricky. This procedure was adopted by other researchers, which extended it by introduction aromatic units *via* imination/reduction step [104]. The proofs of a successful reaction were quite evasive, recommending prudence in applying this method.

In the same line of thought, Hennink proposed a prior dimethylation of chitosan with formic acid and formaldehyde (Eschweiler–Clarke method) to give directly the dimethylated derivative [66]. In this way, the questionable imination process in acidic water was avoided. The dimethylation was found quantitative, allowing the further transformation in quaternary

salt by reacting with an excess of methyl iodide. The authors claimed the total absence of the inorganic base in the quaternization step, but it should be considered that the dimethylated product obtained in the first step was prepared as a gel in the presence of NaOH, and no information related to its efficient washing was provided. The quaternization degree was controlled by the reaction time. Following this procedure, it appeared that the *O*-methylation and depolymerization were avoided, the only unwanted side effect being the presence of the unreacted dimethylated amine group which diminishes the solubility. Moreover, the quaternization degree can be simple controlled by reaction time variation. Good water solubility was yielded for DQ higher than 33%. In order to have a better insight on the advantages of this method, the group compared the results with the ones obtained by applying the Sieval procedure. Interesting, contrary to other reports, they observed that *O*-methylation improved the water solubility of the products; the insolubility appearing to be induced by the partial *N*-methylation. The authors considered the different molecular weight of chitosan as possible source of this different solubility behavior. They assumed that the difference might be due to the fact that low DQ derivatives possess most of the characteristics of chitosan, including the insolubility. Once the *O*-methylation takes place, the inter- and intra-molecular interactions could have been reduced and thus leading to a better solubility, but only for DQ < 24%. Looking to the all methods reported on the synthesis of quaternized derivatives, it seems that this method proposed by Hennink offers the best balance between the use of eco-friendly reagents and the control on the quaternized chitosan product. It was successfully applied for the synthesis of quaternized chitosan derivatives for further use in developing antibacterial products, vaccines and drug delivery formulations [87,105-110].

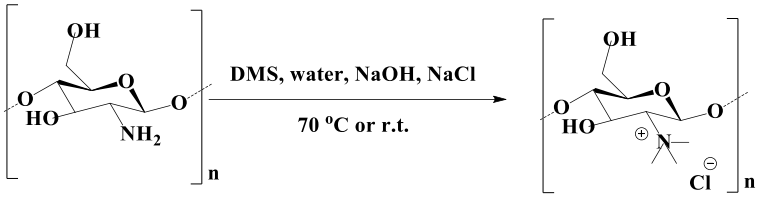
3.1.4. Replacing the solvent

Targeting to limit the *O*-methylation, Masson group proposed to replace the NMP solvent with a mixture of DMF/water in the Domard route. The choice of the solvent was vindicated by the fact that DMF is commonly used for SN2 reactions, and the mixture with water should lower the reactivity of the hydroxyl groups. This variant of the Domard procedure provided indeed uniform products, with a selective formation of the quaternized units at the amine groups, avoiding almost totally the *O*-methylation. Related to the side alkylated products, *N*,*N*-dimethylation was decreasing as the *N*,*N*,*N*-trimethylation was increasing and no *N*-methylation was observed [111,112]. To ensure the *N*-selectivity of the synthesis, the group proposed the repetition of the synthesis four times. The final product had a DQ of 86% and the degree of dimethylation DD reaches 11%, with water solubility up to 80 mg/mL. Howev-

er, no information about the depolymerization was given, it can be supposed that it was not suppressed. This method was further applied by other groups, but not to the same extent as other quaternization methods [48,113,114].

3.1.5. Replacing methyl iodide with dimethyl sulfate

An important disadvantage of the methods applied so far is the use of methyl iodide, which is expensive, volatile and toxic, while halide ions are difficult to remove from solution. This can induce a toxicity degree of the resulting products, with negative impact on the bio-application. Replacing the methyl iodide with dimethyl sulfate (DMS) as methylation agent should bring the advantage of a lower cost and less toxicity, while its high boiling point limits the loss by volatilization. Moreover, DMS can play the role of solvent, too. These arguments are counting for a higher efficiency in comparison to methyl iodide. However, no significant differences were in reality attained, meaning that similar quaternization degrees were reached while the depolymerization and *O*-methylation were not avoided [84,115]. Thinking to the Haworth procedure used by Wolfrom to produce *O*-methylation products using DMS as methylation agent at room temperature, these results were somehow predictable [63]. Compared to Wolfrom procedure, the increase of the reaction temperature to 70 ^{o}C shifted the methylation mainly to the nitrogen atom but didn't suppress the *O*-methylation. Indeed, the reaction performed well with no need for a solvent and a DQ about 52% was achieved after 6 h reaction time, at room temperature (Scheme 6) [115].



Scheme 6. Synthetic route for the obtaining of TMC using DMS as methylation agent [115]

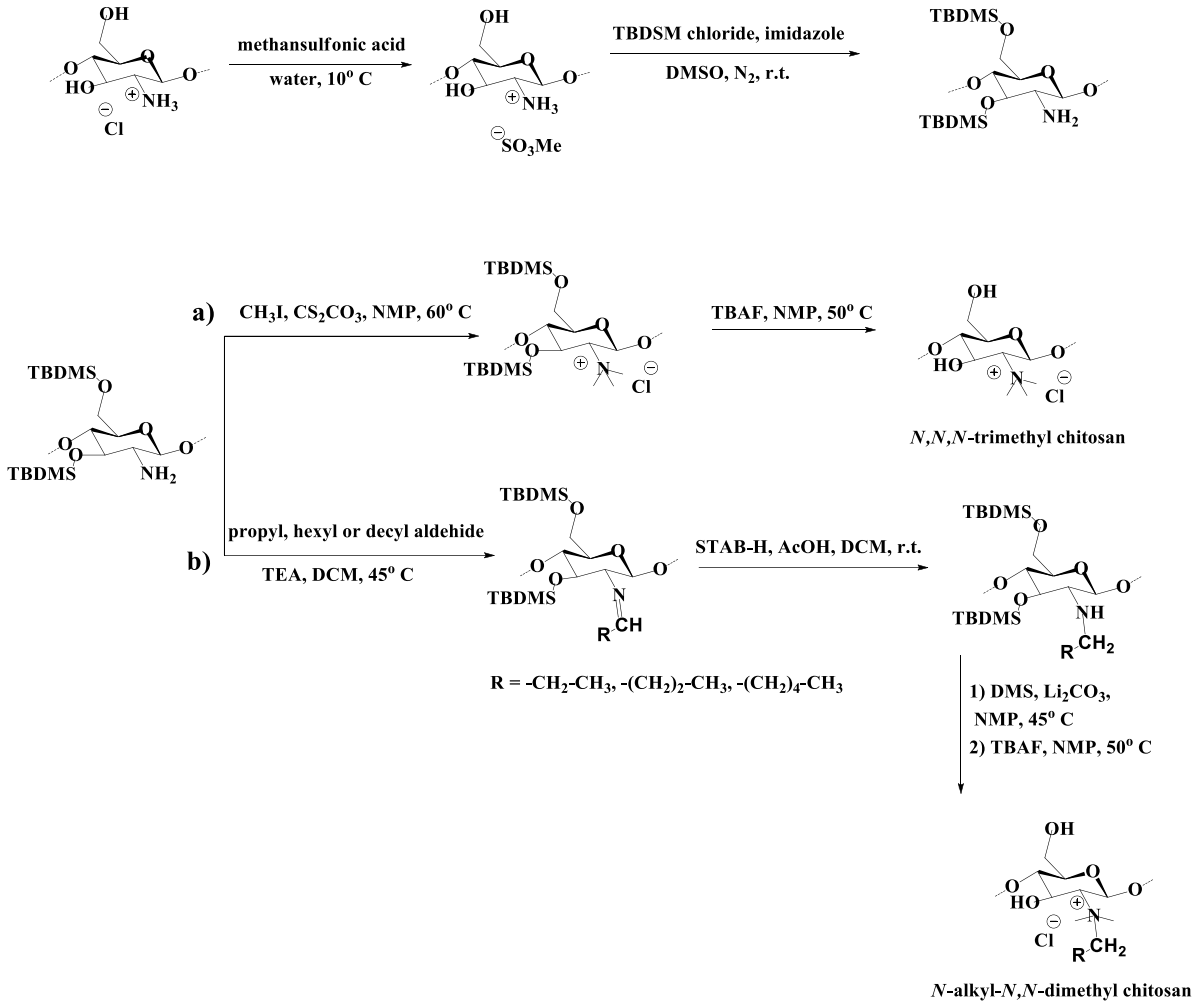
The synthetic route has been applied by other researchers targeting water soluble products for bio-applications, mostly exploiting the antibacterial or anticancer activity of the quaternized derivatives, but also applications involving metal ions removal and dye absorption [116-120].

3.1.6. Prior protection of the hydroxyl groups of the chitosan backbone

A potential strategy to avoid the *O*-methylation process consists in the quaternization of a prior protected chitosan intermediate. It was revealed that protected chitosan intermediates grafted with bulky units on C3 and C6 hydroxyl groups can partially disrupt the H-bond

network, leading to intermediates with enhanced solubility in organic solvents. This allowed performing the quaternization reaction in a more homogeneous phase [121]. The most effective protection was achieved using *tert*-butyldimethylsilyl (TBDMS) groups, due to the good stability in basic or moderate acidic media and facile deprotection under strong acid conditions [122]. The quaternization of the prior protected chitosan (silylated chitosan) was performed by a modified Domard method, using methyl iodide as methylation agent, but replacing the NaOH base with a weaker base, such as Cs_2CO_3. The last step was the deprotection of the hydroxyl groups, using tetrabutylammonium fluoride (TBAF) solution in NMP (Scheme 7a). The authors reported that, for the first time, it was achieved a fully quaternized *N*,*N*,*N*-trimethylchitosan, without any evidence of *O*- or *N*,*N*-dimethylated product. The product was claimed to be water soluble, but there is no clear information regarding the pH of the solvation media or the concentration. The idea of a prior protection was further developed using a combination of *tert*-butyloxycarbonyl (BOC) and TBDMS protection stage [123].

In line with this strategy, Benediktdottir *et al.* proposed to use the prior protection in order to obtain derivatives with different substituents, mostly *N*-alkyl-*N*,*N*-dimethyl chitosan [124]. To do this, they applied a prior imination/reduction step to the prior protected chitosan, to obtain firstly *N*-alkyl chitosan, using for the imination aliphatic aldehydes with 3-5 C atoms and the reduction was achieved with sodium triacetoxyborodydrate (STAB-H) and acetic acid (AcOH), different with the procedures previously described (*Scheme 7b*). The *N*-alkyl protected chitosan was quaternized with DMS in the presence of a weaker base such as Cs_2CO_3 and Li_2CO_3. Compared to other authors, the negative effect of the reversible imination reaction in acidic water was avoided by performing the imination reaction in an organic solvent dichloromethane (DCM) in which the silylated chitosan intermediate was soluble. Although this may seem an effective strategy, it was reported that the protection-deprotection step can lead to a severe degradation of the chitosan backbone by acid hydrolysis of the *O*-glycosidic bond and the total removing of TBDMS is difficult [124]. So, this method can be a choice, when the polymerization degree doesn't affect the targeted application, or, on the contrary. With some slight variations, it was applied for the preparation of antibacterial products [123,125,126].



Scheme 7. Synthetic pathway for the preparation of a) TMC and b) *N*-alkyl-*N*,*N*,dimethyl chitosan [124]

3.1.7. The effect of halogen counterion on the quaternized products

Starting from idea that the heavy halogen iodine induces a decrease of solubility and a toxicity degree [127], it was adopted as a strategy that the resulted iodine quaternary ammonium salts to be transformed in chloride or bromide by an exchange of the counterion, usually between iodine and chloride [128]. The exchange is also favored by the higher stability of Cl^- in comparison to I^-, and the products have better water solubility [71]. The counterion exchange was performed at the beginning using HCl, but it was proved that can lead to a misinterpretation of the solubility of the final compound in water, due to the possibility of acid traces in water that can influence the pH of the solution. In order to overcome these, Sieval [80] used NaCl instead of HCl for an accurate solubility test of the quaternary ammonium salt of chitosan. Also, if NaCl is added during the synthesis, it results directly in chlorides derivatives [120]. Although chitosan and its different salty forms, including acetate, citrate and lactate have been the center of interest of many researchers, TMC with these counter-ions has

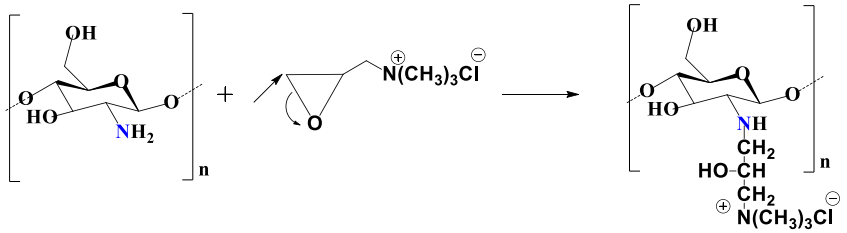
been barely explored. Britto *et al.* analyzed TMC in acetate form due to the better resolution and possibility of detection in NMR, but its properties remained unclear, only the DQ using MAS ^{13}C NMR was better interpreted [84].

3.2. Indirect binding of the quaternary salt to chitosan. Side chain quaternary ammonium chitosan derivatives

The most used method for the synthesis of the quaternized chitosan derivatives was the per-methylation reaction. However, a method which can be applied with good results refers to the indirect grafting of the quaternized ammonium salt, by reacting chitosan with a derivative containing it. The most used ones are given below.

3.2.1. Indirect binding by reacting with glycidyltrimethylammonium chloride

Reacting chitosan with glycidyltrimethylammonium chloride (GTMAC) in a *heterogeneous system in water*, at 60 °C, water soluble *N*[(2-hydroxy-3-trimethylammonium)propyl] chitosan chloride (HTCC) was obtained (Scheme 8) [129]. The authors claimed that, even though both amine and hydroxyl groups are susceptible for nucleophile substitution with the epoxide ring, the chemical modification of chitosan with GTMAC in water resulted only in *N*-substitution. By varying the molar ratio between the glucosamine unit of chitosan and GTMAC from 1/1 up to 1/6, the substitution degree progressively increased from 38 to 95, an almost complete *N*-substitution being reached for a six-fold excess of GTMAC. No information about a possible chitosan depolymerization was provided, but a latest article noticed the chitosan depolymerization, resulting in the decreasing the molecular weight at half, measured by gel-permeation chromatography (GPC) [130].



Scheme 8. Synthetic route for the reaction of chitosan with GTMAC [129]

The method is simple and the DQ can be controlled by adding different portions of GTMAC or by variation of the temperature, from room temperature to 90 °C [131-140]. Using the *microwave power*, the reaction time was consistently diminished, but the authors also observed a deeper depolymerization, the molecular weight of chitosan decreasing at almost a quarter [141]. It was found that the substitution degree of the products can be controlled by

varying the temperature, power and time, but in the context of such a deep depolymerization under the microwave effect, it is questionable if this is a true advantage [142].

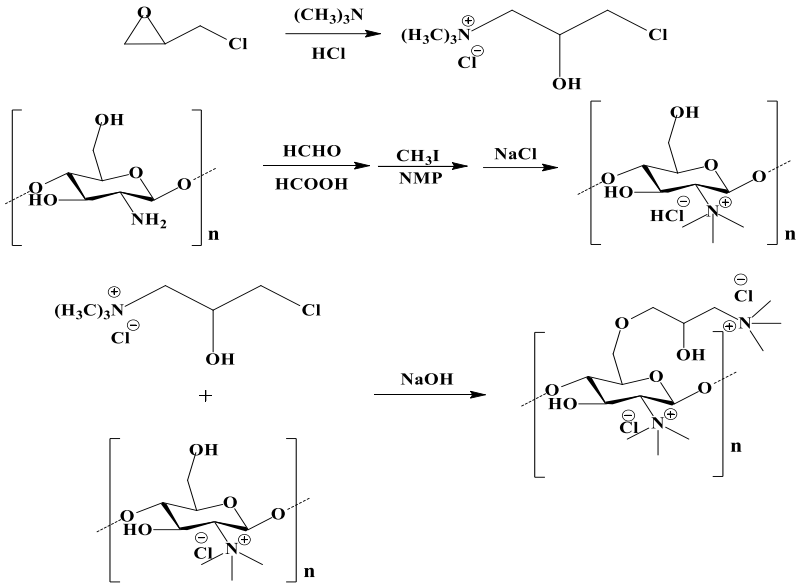
Starting from the premises that GTMAC can suffer some side reactions under neutral or alkaline conditions, i.e. it can be easily hydrolyzed and transformed into 2,3-dihydroxypropyl trimethyl ammonium chloride and can give intermolecular polymerization in water or ethanol, diminishing the grafting efficiency of chitosan, Ruihua proposed to use a *homogeneous acidic media*. For this aim, perchloric acid was chosen, considering that it does not react with GTMAC due to the low nucleophilicity, favoring thus the quaternization towards higher substitution degree [143]. Indeed, a high substitution degree of the chitosan of 86.9% was reached for a molar ratio of glucosamine/GTMAC of 1/8. The method was adopted by many researchers, replacing the perchloric acid with the more ecofriendly acetic acid solution [144-148]. Comparing the substitution degree reported for similar molar ratios of glucosamine and GTMAC, it appeared that the heterogeneous medium is more suitable than the acidic one for higher substitution degree. However, deeper investigations of this synthetic pathway demonstrated that the pH drastically impacts the reaction site of chitosan, an acidic medium favoring a more selective *N*-alkylation, while in a basic medium the reaction perform indiscriminately at *N*- and *O*- sites [149-150]. The simple explanation is given by the increased electrophilicity of the protonated NH_2 groups. In such a way, the substitution degree also increased. *N*-alkylation appeared to be also favored by replacing the water medium with an ionic liquid, e.g. 1-allyl-3-methylimidazole chloride [151]. The use of an *ionic liquid medium* combined with the use of an acid pretreated chitosan leads to a substitution degree exceeding 100%, indicating that *O*-substitution can also occur even in acidic media. The method was also successfully applied for the synthesis of amphiphilic chitosan, by applying the quaternization after a prior reaction with hydrophilic species, i.e. galactosyl, branched-PEI [152].

3.2.2. Indirect substitution by selective reaction at primary hydroxyl group

From an applicative perspective, when not only the improved solubility is targeted, but also an increased polycationic character, it is desirable that the indirect quaternization to be performed at the oxygen site. This will preserve the amine sites at C2, increasing the polycationic nature of the quaternized chitosan. To reach this goal, the amine groups of chitosan were prior protected by imination to *N*-benzylidene chitosan under vacuum [153]. After the preparation of the *O*-quaternary ammonium-*N*-benzylidene chitosan by classical reaction with GTMAC, the targeted *O*-quaternary chitosan was obtained by deprotection in an acidic medi-

um followed by purification by reprecipitation into acetone. This is an excellent method when chitosan with increased polycationic character is targeted, and the method was applied especially for preparation of drug delivery systems or protein loaded nanoparticles [154-156].

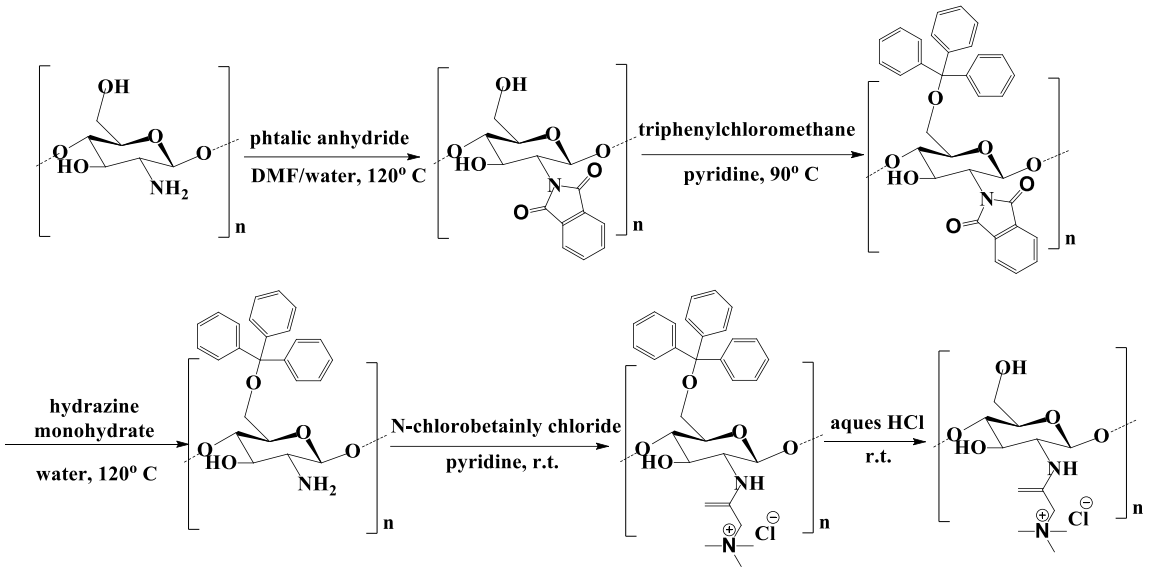
In the same line of thoughts, the *O*-quaternization was applied to the previous synthetized TMC derivatives (Scheme 9) [157]. The *O*-quaternization after the prior protection of amine site by imination was also performed by replacing GTMAC with other species containing quaternary ammonium salts [158].



Scheme 9. Synthesis of *N,O*-quaternary ammonium salts [157]

3.2.3. Indirect substitution by selective reaction at primary amine group

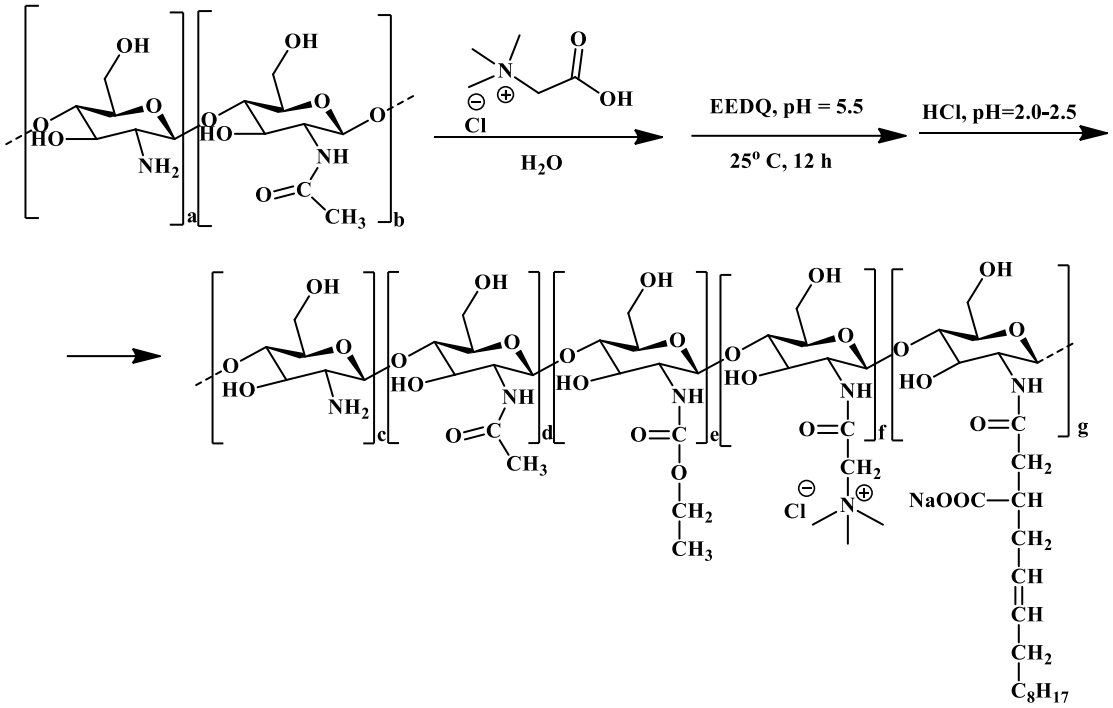
With the aim to avoid the polydispersity given by the random substitution of the amine units into *N*-alkyl units, Holappa proposed a regioselective quaternization by means of a reaction of the prior protected chitosan with betaine, at the primary hydroxyl units [159]. The protection step was achieved with phthalic anhydride to give first *N*-phthaloylchitosan, followed by a reaction with triphenylchloromethane in pyridine which, by reacting with hydrazine monohydrate formed chitosan with triphenylmethyl group at the hydroxyl function from C2. The quaternization was achieved by reacting with *N*-chlorobetainyl chloride in pyridine and the final step was the deprotection (Scheme 10).



Scheme 10. Synthetic pathway for the preparation of chitosan *N*-betaines [159]

The disadvantage of such of method is the high depolymerization degree recorded for higher quaternization degrees. This was attributed to the long synthetic protection-deprotection procedure (five steps). Interesting, applying the same method to chitosan and some amphiphilic derivatives, without a protection step, the depolymerization appeared to not occur, indicating this protection route as a destructive stage [160]. The reaction was performed with betaine, in the presence of a coupling agent, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), a stable and cheap reagent (Scheme 11). In this last case, the authors reported a side reaction consisting in the formation of *N*-(ethoxycarbonyl)chitosan, but this process was not considered an inconvenience because *N*-ethoxycarbonylation of chitosan might have a positive effect on the formation of nanoparticles in view of further application for drug delivery.

One of the modifications applied to this method aimed to achieve *N,N,N*-trimethyl-*O*-alkyl chitosans, a series of amphiphilic chitosan derivatives that have high effectiveness in drug delivery, more prone for nanoparticle preparation. The method implies four steps: the *N*-protection with phthalic anhydride followed by *O*-alkylation with different alkyl bromides, deprotection of amine group and the *N*-quaternization [161,162].



Scheme 11. Synthetic pathway for the quaternization with EEDQ; Before quaternization, a+b=1 (DD=95%), after quaternization, c+d+e+f+g=1 [160]

3.2.4. Indirect substitution by reacting with other species containing quaternary ammonium salts

The procedure of indirect quaternization was applied using other species containing quaternary ammonium salts, such as *N*-(3-chloro-2-hydroxypropyl)trimethylammonium chloride or (2,3-epoxypropyl) trimethylammonium chloride [150]. The reaction proceeds mainly at the primary amine groups of chitosan in homogeneous medium of acetic acid aqueous solution.

Another technique for grafting quaternized groups on the chitosan backbone is by copolymerization with 2-(acryloyloxy)ethyl]trimethylammonium chloride solution, when ammonium persulfate is used as an initiator, reaching series of quaternary chitosan (Figure 12) [163,164].

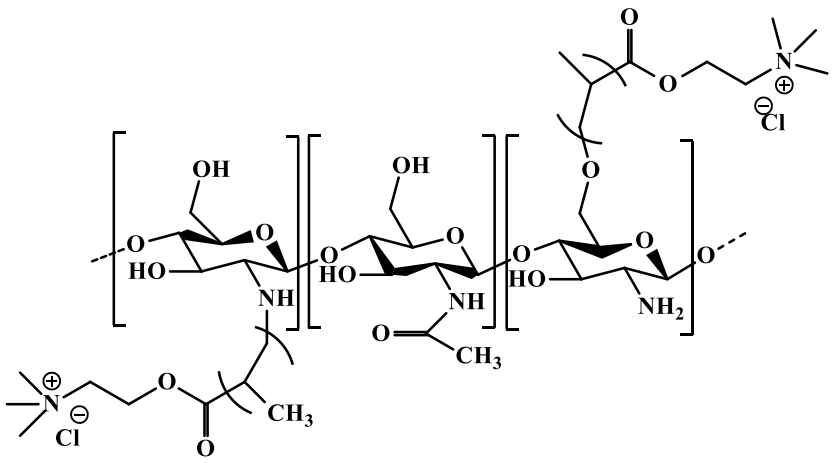


Figure 12. Chemical structure of quaternary chitosan by Chen [163]

4. Advantages and disadvantages of the methods. A comparison

As can be seen, the methods described in the section 3 are applied for preparation of the quaternary salt derivatives, without a clear correlation with the targeted applications. We tried to tabulate the synthetic conditions of some principal methods, with their advantages and disadvantages, in order to help the scientists to choose the right synthetic pathway, function of the targeted properties (Table 1).

Table 1. A comparison between the most important quaternization strategies

Reaction conditions	R	DA (%)	DQ (%)	DD (%)	DS O (%)	Solubility	Ref.
MeI, NaI, NaOH in NMP							
One step synthesis: 36±1 ^{o}C, 3h 4 times repetition	Me	5	25 64	(-)	(-)	WS (DQ > 25)	[71]
One step synthesis: 60 °C, 60 min.	Me	12	53	(-)	0	WS	[72]
Multi-step synthesis 1st: 60 °C, 60min; 2nd: 60 °C, 30 min; Supplementary step: 60 °C, 60 min.	Me	7	>70	Yes	yes	WS 5% (w/v)	[80]
Multi-step synthesis 1st: 60 °C, 60min; 2nd: 60 °C, 30 min; Supplementary step: 60 °C, 60 min; 3rd : 60 °C, 60min.	Me	7	>85	yes	100	Poor WS	
Multi-step synthesis 1st: 60 °C, 45 min; 2nd: 60 °C, 30min; 3rd: 60°C, 45 min; 4th:	Me	7	22.1 36.3 48.0	yes	yes	(-)	[91]

60°C, 30min.			59.2				
Multi-step synthesis 1st: 60 °C, 1.5 h; 2nd: 60 °C, 1.5 h Supplementary step - 60 °C, 60 min	Me	3	23.7	(-)	25.9 O3 18.1 O6	(-)	[83]
DMS, NaOH, NaCl, r.t., 6h	Me	4	52.5	yes	yes	(-)	[115]
MeI, NaI, NaOH in ***DMF/H_2O*** r.t., 48 h, 4 times repetition	Me	5	86	11	(-)	80 mg/mL	[112]
1st: **Protection** with di-TBDMS; 2nd: Cs_2CO_3, MeI in NMP, 24 h, 50 °C, 3 times repetition with addition of MeI; 3rd: **Deprotection** with 1 M TBAF in NMP, 50 °C, 72 h	Me	3	97	0	0	Soluble in water and organic solvents	[124]
a) CHO_2H, CH_2O (70 °C, 118h); b) NMP, MeI (40 °C, 70h).	Me	7	61	(-)	0	WS at pH 7, r.t.	[66]
Indirect synthesis							
Substituted radical: Betaine (Be)							
1st: **Protection** with phthalic anhydride, 120 °C, 8 h; 2nd: Synthesis of ***N*-Phthaloyl-6-*O*-triphenylmethylchitosan**, in pyridine, 90 °C, 24 h; 3rd: **Deprotection of N** with hydrazine monohydrate, in water, 100 °C, 15 h; 4th: **Quaternization** with *N*-chlorobetainyl chloride in different ratios, at r.t., 72 h; 5th: **Deprotection of O** with HCl 1 M, r.t., 4h.	Be	15	DS(%) 40, 80, 90 for 1, 2, and 4 eq. of N-chlorobetainyl chloride	0	67 mg/mL	[159]
Substituted radical: 2-hydroxypropyl-3-trimethylammonium (*)							
Chitosan dispersed in distilled water; addition of GTMAC, stirring at 60° C, 15h.	*	14.5	DS(%) 95 for a six fold excess of GTMAC	0	(-)		[129]
1st: **N-protection** with benzoyl hydride; 2nd: ***O*-quaternary aminonium-*N*-benzylidene chitosan** Isopropyl alcohol and GTMAC	*	8	(-)	33.3 after 16 h	WS		[153]

addition to *N*-benzylidene chitosan, 70 °C, 16 h; 3rd: **Deprotection** with HCl alcohol solution r.t., 24.						
1st: **TMC synthesis** according to Verheul 2008; 2nd: Synthesis of– **CHPTMAC** trimethylammonium aqueous solution, HCl and anhydrous ethanol, pH 8; addition of epichlorohydrin – stirred 1 h at 10 °C and 1 h at 35 °C; purification; 3rd: **Quaternization** TMC in water, pH 11 with NaOH, 1 h stirring. CHPTMAC added dropwise for 1 h, and reacted for 5, 10 or 20 h; pH 7 with HCl, dialyzed and lyophilized.	*	4.4	TMC: 68.9	TMC: 26.7	Final compound: 22.4 - 5 h 33.1 - 10 h 42.7 - 20 h	WS	[157]
Chitosan dispersed in deionized water, complete solubilization after addition of perchloric acid; Aqueous GTMAC addition at 60 °C in three aliquots at intervals of 0.5 h; the reaction was performed at 80 °C, 8h.	*	10	DS(%) 86.9		(-)	Soluble over a wide pH range	[143]
Substituted radical: 2-[(Acryloyloxy)ethyl]trimethylammonium - AETMAC(**)							
Chitosan dissolved in 2% aqueous acetic acid at 80 °C; AETMAC and ammonium sulfate added at 80 °C for 3 h; Purification.	**	15-25	yes		yes	(-)	[163]

R: substituted radical, **DA** (%): Degree of acetylation, **DQ** (%): Degree of quaternization, **DD** (%): Degree of dimethylation, **DS O** (%): Degree of *O*-substitution, (-): not provided by authors, WS – Water soluble.

5. The main properties induced by quaternization and suitable applications

The motivation beyond the tremendous synthetic effort towards a controlled synthesis of quaternized chitosan derivatives was to yield a class of water soluble biopolymers with increased polycationic character, suitable for bio-applications. The main requirements for such applications are the biocompatibility and biodegradability, which are intrinsic chitosan properties. However, the main question is: how the quaternization affects these properties? Further, what specific properties are induced by the presence of the quaternary ammonium? In

the next paragraphs we try to find an answer to these questions, highlighting the delicate balance of properties which should be fulfilled for targeted applications.

5.1. Biocompatibility

As the main modification of chitosan is the grafting of positively charged quaternary ammonium groups, they should be responsible for any modification of the properties, but the presence of the secondary by-products such as *N*-alkyl, *N*,*N*-dialkyl or *O*-alkyl units, the length of the alkyl units and the site of the positively charged nitrogen on the chitosan chain should not be neglected. Moreover, the influence of such units is more complex, considering that they affect the supramolecular architecture of the final product as well, directing different spatial orientations of the groups and consequently different properties. Besides, the molecular weight and deacetylation degree of the chitosan backbone play an important role too. All these structural and supramolecular factors affect the biocompatibility of the quaternized chitosan, and explain the controversial data reported by different investigations. Thus, some authors reported that DQ does not influence the toxicity of TMC, being non-toxic even for high values [165-167] while others related that a DQ of 40% exhibits a cytotoxic response that increases once the molecular weight and the DQ is increasing, those differences being justified by the variance of DD [73,168]. Some researchers reported that a DQ of 18% induces a good biocompatibility [133], while different articles describe better biocompatibility for lower (DQ=9%) and higher DQ (DQ=98%), and worse biocompatibility for intermediate ones (DQ=40%, 58%) [138]. On contrary, others found good biocompatibility only for low DQ and low concentrations [169]. It was found that the partial *O*-methylation of TMC induces a better biocompatibility attributed to the beneficial effects on the enzymatic biodegradability [66]. Deeper investigations of the influence of DD/DQ ratio on the biocompatibility revealed that once the DD/DQ ratio exceeded 1, the cytotoxicity decreases and a non-toxic polymer can be achieved once the DD/DQ is about 3:1. It was assumed that the cell cytotoxicity is a consequence of the positive charge of TMC, and the steric effect induced by the methyl groups of dimethylamino shielded part of the positive charge of quaternary groups, hindering their interaction with the anionic parts of the cell membrane [170]. There are studies evidencing that chitosan quaternization is accompanied by the appearance of a cytotoxicity degree, mainly due to polymer aggregation on the surface of the cell, impairing the important functions of the membrane [158]. Nevertheless, a proper comparison of these data is difficult, because different cell lines and different concentrations were used for biocompatibility investigations, in function of the targeted application or cell availability, e.g. there are investigations which

show different cell viability on different cell lines [138, 171]. The diversity of these data suggests that the optimum DQ and appropriate dose of quaternized chitosan should be investigated for each sample depending by the targeted investigations.

5.2. Permeation of the epithelial tissues. Drug delivery applications

The increased positive charge on the quaternized chitosan improves the permeation of the epithelia cells, demonstrating a strong, reversible effect of the opening tight junctions. Moreover, it improves the mucoadhesive properties. This makes them excellent absorption enhancers for hydrophobic drugs across the epithelial tissues, such as intestinal epithelia, buccal mucosa, cornea, derma. This effect directed quaternized chitosan derivatives towards drug delivery, wound dressing and cosmetics applications, but also recent studies have focused on their usage as adjuvant for vaccines, providing encouraging results [172,173]. An important aspect for these applications is given by the fact that quaternized chitosan can be easily manufactured as hydrogels, films, fibers, nanoparticles or vesicles, enlarging the realm of species which can be transported [174]. For their bio-application, the quaternization degree should be carefully considered as it is related to the solubility and the biocompatibility.

The absorption-enhancing property of the quaternized chitosan is influenced by the DQ, as a result of the number of positive charges that are available for interactions with negatively charged sites on the epithelial membrane. Again, the first question that arises is: What DQ is the appropriate one for permeation enhancement? Studies on Caco-2 cell line on [^{14}C]-mannitol transport in neutral pH indicate that a high charge density is required to have a positive impact on the paracellular permeability, corresponding to a DQ of 39% or higher [175]. Adding to that, TMC having DQ of 61% proved to have a positive effect on enhancing nasal and rectal absorption of insulin in rats at neutral pH, where chitosan is not effective [74]. TMC having a DQ of 36.4% combined with fucoidan into nanoparticles was reported to modulate the barrier function of the Caco-2 intestinal epithelial cell monolayer thus enhancing the paracellular transport of insulin across the intestinal barrier [176]. The group of Hamman considered that when DQ reaches 60%, the steric effects of methyl groups and the flexibility of the molecule could have a negative impact on the absorption property, and therefore they have tested derivatives with DQ in the range 22-49%. It was found that in neutral environment (pH 7.4), TMC with DQ 49% reached the highest reduction in trans-epithelial electrical resistance (TEER) and did not increase with increasing the DQ [177]. Related to the influence of the molecular weight of chitosan on the permeability of the corresponding quaternized chitosan, Di Colo *et. al* did not find clear differences [178]. The group synthetized TMC using chitosan

of high Mw and low Mw, with a DQ between 3% and 90% and tested for the capacity to increase the permeability of ofloxacin over corneal epithelium. It was observed that TMC with intermediate DQ 35-45%, at concentration 0.001% w/v, had a positive impact on the permeability enhancement, without being influenced by the Mw, when the analysis was performed *in vitro*, on rabbit corneal epithelial cells on polyester membranes. Also, an increase in the DQ did not result in an increase in efficiency. Nevertheless, *in vivo* tests on rabbits evidenced a difference between high Mw and low Mw, first one providing higher minimum inhibitory concentration. The TMC was used as matrix for the prolonged release of a large range of drugs (blue dextran, 5-fluorouracil) [163,179]. Table 2 summarizes the exposed data regarding the relationship between the DQ and its influence on the permeability, for an easier understanding of positive charge impact.

Table 2. Overview on the permeation capacity of TMC

DQ (%)	Remarks	Ref.
> 39	Positive effect on the intestinal permeation of a [^{14}C]-mannitol; increases with the increase in DQ and concentration	[175]
61	Effective in increasing the absorption of insulin after nasal and rectal administration	[74]
36.4	In combination with fucoidan in self-assembled nanoparticles, enhances the paracellular transport of insulin across the intestinal barrier	[176]
49	High permeation enhancing of [^{14}C]-mannitol	[177]
35-45	*In vitro:* significant permeability enhancement of ofloxacin over corneal epithelium, independent of polymer M_W *In vivo:* higher Mw showed a stronger effect on the permeability enhancement	[178]

5.3. DNA transfection

Due to the increased positive charge, the quaternized chitosan is a good candidate for the design of natural based non-viral vectors for gene delivery, as it can easily interact with negative charged DNA to form polyelectrolyte complexis (polyplexix). Comparative investigations highlighted the significant improvement of the gene transfection of the TMC oligomers compared to the pristine chitosan, for polyplexis formed with RSV-α3 luciferase plasmid used in transfection of COS-1 and Caco-2 cell lines [180]. Investigation of the influence

of the DQ on the transfection efficiency revealed an optimum value of 44%, for higher values being observed cytotoxic effects. However, compared to the polyethyenimine (PEI), a traditional building block for non-viral vectors, the quaternary oligomeric derivatives proved less toxic effect [73]. Moreover, the indirect binding of the quaternary salt to the chitosan backbone proved higher transfection efficiency and lower cytotoxic effect than PEI, even for high molecular weight of chitosan, up to 250 kDa [181]. The superior ability to bind and transfect DNA was highlighted also for the quaternary chitosan derivatives compared to chitosan substituted with other building blocks, such as *N,N*-dimethylethylamine [182]. Polyplexis of DNA with poly-(ε-caprolactone) stabilized with TMC displayed significant cytotoxicity for DQ higher of 66% and low or moderate one for low or intermediate DQ values (4% ,10%, 18%) [183].

5.4. Antimicrobial activity

The most important and studied property of the quaternized chitosan derivatives is the antimicrobial activity. Compared to the pristine chitosan, by quaternization are reached both a better solubility at physiologic pH and an enhanced antimicrobial activity. Adding to that, the antibacterial activity should be amplified by the hydrophobicity induced by the various chains used for the preparation of the quaternary salts [94,128, 184].

TMC proved activity on both Gram positive (*Staphylococcus aureus, Enterococcus faecalis*) and Gram negative (*Escherichia coli, Pseudomonas aeruginosa*) species of bacteria. It appeared that quaternary derivatives with short alkyl chain present high activity against *S. aureus*, while introducing a more hydrophobic chain, such as hexyl, stimulates the activity against *E. coli* and *E. faecalis*. On the other hand, there was no direct relationship between the activity against *P. aeruginosa* and the chain length [125]. Also, quaternized chitosan derivatives showed improved activity against other strains, such as *Botrytis cinerea* [185], *F. oxysporum f sp. cucumerium*, *F. oxysponim f sp. niveum* [186], *E. faecalis* [187]. Relative to the fungicidal effect, shorter alkyl chains (C-4) proved more potent effect activity against *Candida albicans* strains, while those containing longer alkyl chains (C-8 and C-12) were much more effective against *Candida* biofilms formed on solid surfaces [188]. The position of the quaternary ammonium does not appear to influence the occurrence of antimicrobial activity. HTCC derivatives also showed enhanced activity against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Acinetobacter baumanni* and also drug-resistant bacteria including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium* and β-lactam-resistant *Klebsiella pneumoniae* [189-193].

6. Conclusions

Quaternized chitosan derivatives are a class of water soluble chitosan biopolymers with enhanced potential for applications such as antimicrobial products, drug delivery formulations, gene transfection. The multitude of synthetic pathways used for their synthesis leads to a variety of products, differing not only by the quaternization degree but also by the site of grafting the quaternary ammonium salt, the presence of side-reaction units such as *O*-methylated, *N*-methyl or *N*,*N*-dimethyl units, and also different combinations of the radicals linked to the amine. All these impact the properties of the final products. As the quaternary salts of chitosan already proved their potential for natural based products, it is expected to significantly influence the further developing of a future generation of ecological materials.

Acknowledgements

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Data Availability

Data not available. The submitted manuscript is a review paper.

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