FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

SYNTHESIS OF PHENYL CARBAMOYLATED GUANIDINE FUNCTIONALIZED CHITOSAN

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2022

To: Dean Michael R. Heithaus
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This thesis, written by Eduardo Hernandez Requejo, and entitled Synthesis of Phenyl Carbamoylated Guanidine Functionalized Chitosan, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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Florida International University, 2022

DEDICATION

I dedicate this dissertation to Maria Teresa, my beloved wife, for her love, support, and encouragement throughout my M.S. journey.

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My sincere gratitude to my mentor Dr. Joong Ho Moon, for his guidance and support. I would also like to extend my sincere thanks to my committee members, Dr. Watson Lees and Dr. Stanislaw Wnuk, for their help during this work.

ABSTRACT OF THE THESIS

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by

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Florida International University, 2022

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Professor Joong Ho Moon, Major Professor

The lack of solubility of the chitosan in organic solvents hinders its potential for chemical modifications. For this reason, it was hypothesized that the insertion of the phenyl carbamoylated guanidine moiety could lead to increased solubility in DMSO and new potential modifications and uses.

This research involved the synthesis of S-methyl phenyl carbamoylated guanidine (SMPCG) starting from the commercially available S-methyl isothiourea. This was followed by the reaction of the SMPCG with chitosan to yield phenyl carbamoylated guanidine functionalized chitosan (PCGCs) with a degree of substitution of 14%.

The chitosan derivative is fully soluble in DMSO and partially in DMF. This modified polymer will be used in future experiments in protein delivery, antimicrobial activity, and siRNA transfection. Future research is also planned to obtain PCGCs with different degrees of substitution.

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LIST OF ABBREVIATIONS AND SYMBOLS

AcOH	Acetic Acid
BOC	tert-Butoxy Carbonyl
BOC_2O	Di(tert-butyl) dicarbonate
CAGR	Compound Annual Growth Rate
$CDCl_3$	Deuterated Chloroform
CP	Conjugated Polymer
CPPs	Cell-Penetrating Peptides
D_2O	Deuterium Oxide
DCM	Dichloromethane
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
DMSO-d6	Dimethyl Sulfoxide deuterated
DNA	Deoxyribonucleic Acid
EA	Ethyl Acetate
FIU	Florida International University
HCl	Hydrochloric acid
KOH	Potassium hydroxide
MW	Molecular Weight
mRNA	messenger RNA
MSA	Methanesulfonic Acid
NMR	Nuclear Magnetic Resonance

PCGCs	Phenyl carbamoylated guanidine chitosan
PEG	Polyethylene Glycol
PEI	Polyethylene Imine
PhNCO	Phenyl Isocyanate
RBF	Round Bottom Flask
RNA	Ribonuclease Acid
RNAi	RNA Interference
ROMP	Ring-Opening Metathesis Polymerization
siRNA	small interfering RNA
SMPCG	S-methyl Phenyl Carbamoylated Guanidine
TFA	Trifluoro Acetic Acid
THF	Tetrahydrofuran

I. INTRODUCTION AND LITERATURE REVIEW

1.1 Chitin and Chitosan

Chitin is a linear polymer of β-1,4-linked N-acetyl-D-glucosamine[1]. It is the second most abundant polysaccharide in nature and is found in insects and crustaceans' exoskeleton and the fungal and yeast cell wall[2]. Chitin has a cellulose-like structure, where the hydroxyl group in the C2 position has been replaced by an acetamido group[3]. Depending on the orientation of polysaccharide chains, chitin displays three polymorphs, denoted as α, β, and γ[4]. A notable characteristic of chitin is its poor biodegradability and a high degree of insolubility in organic and inorganic solvents[5].

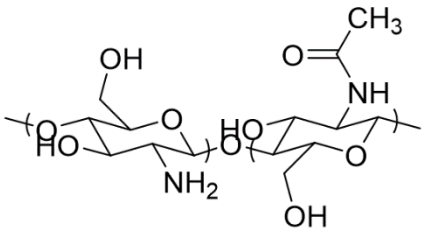

Figure 1. Chitosan.

Discovered by Rouget in 1859 after heating chitin in an alkaline medium[6], chitosan is the most common chitin derivative[7]. Formed by randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl D-glucosamine units[8], chitosan is obtained through the deacetylation of the chitin macromolecule by subjecting it to hot alkali (e.g., NaOH concentrated solutions) treatment[9] (Figure 1). Chitosan has a ridged crystalline structure built through inter-and intra-molecular hydrogen bonds[10]. Owing to the deacetylation process, a unique structural feature of chitosan is the primary amine at the C-2 position of the glucosamine, which confers important functional properties to chitosan[11]. Because of

its linear structure and polysaccharide nature, this biopolymer is biocompatible, non-inflammatory, non-toxic, and biodegradable[12]. The pKa of the primary amine of chitosan is ~6.5, depending on the degree of N-deacetylation[13]. Therefore, the amino groups can be protonated at pH below 5, promoting chitosan solubility in aqueous acidic solutions[14] (Figure 2).

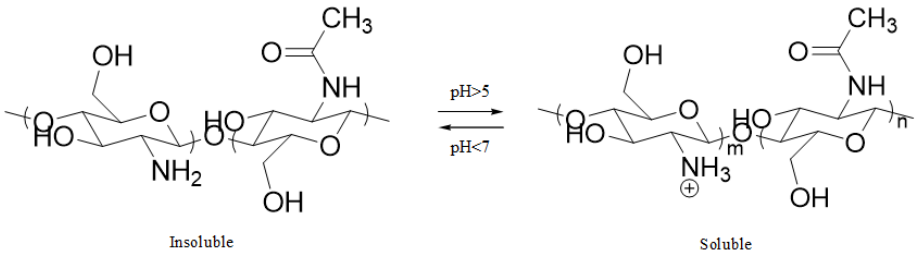

Figure 2. Chitosan solubility in aqueous acidic solutions.

There can be variations in molecular weight, degree of acetylation, and order sequence of the acetylated moieties[11] between different chitosan varieties or chains. Due to this, there is a certain degree of variability in the properties of different chitosan varieties[15].

The final extent of deacetylation is one of the most critical factors affecting the properties of chitosan, as it determines the number of available amino groups in the chitosan chain[16]. Crystallinity, hydrophilicity, degradation, and cell response are all properties affected by deacetylation[17]. Chitosan is characterized by a low acetylation degree, usually below 50%, and molecular weight between 50 and 2000 kDa[18].

However, chitosan also has serious disadvantages, starting with the fact that it is not soluble in water and organic solvents and can only be dissolved by slightly acidic aqueous solutions[19]. This limits notably the range of reaction conditions available for performing chemical transformations involving chitosan[20]. The high dependence of the cationic nature of this polymer on the pH values introduces a degree of unpredictability to its behavior,

especially for protein and nucleic acids carrier uses in biological applications[21]. Under physiological conditions, the pH values will lead to a lowered charge density on the amino groups, with the subsequent reversal of its solubility and cationic properties[22]. Additionally, once the chitosan is transported inside the cells and the endocytosis process has been initiated, the acidic conditions inside the endosome cause the chitosan to become protonated again, which leads to increased electrostatic interaction with the payload and subsequently hinders their release into the cytoplasm[23].

1.2 Chitosan Derivatives

Chitosan contains nucleophilic functional groups: a C-2 NH_2 group, a C-3 secondary OH group, and a C-6 primary OH group. Chitosan has been chemically modified at the amino group or at the hydroxyl groups to produce derivatives containing cationic or other hydrophilic and hydrophobic moieties[24]. Because of chitosan's attractive properties, several modifications have been proposed to modify its structure[25] (Table 1).

Table 1. Examples of Chitosan Modifications

Modifications to enhance chitosan solubility	**Modifications to enhance antimicrobial properties**	**Modifications to enhance protein/ nucleic acid delivery**
Mesylate salt formation[26]	Hydroxypropyl chitosan[27]	PEI grafting
N-phthalimide insertion[28]	Biguanidinylation[29]	Guanidinylation[30]
Carboxymethylation[31]	Cyclic substituted chitosan[32]	Hyaluronic acid/ Chitosan multilayer coating
Camphor sulfonic salt formation[26]	N-(2-quarternary ammoniumyl) acyl chitosan derivatives[33]	PEGylation[34]
Etherification[35]	Sulfonamide-chitosan derivative[36]	Attachment of diethylamino ethyl groups[37]

In some cases, these modifications originated from the need to overcome the poor solubility of chitosan[38]. In other cases, the motivation is to enhance a desirable characteristic already present in the chitosan[39] to maximize its functionality as antimicrobial[40] or nucleic acid delivery agent[41]. These modifications typically target the amino or hydroxyl groups to produce derivatives containing cationic or other hydrophilic and hydrophobic moieties[42].

1.3 Chitosan uses

The attractive properties of chitosan and its derivatives have applications in many areas. These include biomaterial and tissue engineering, food and nutrition applications, antioxidant and antimicrobial, and drug and gene delivery. As an example of the growing interest in this biomaterial, in 2019, the global chitosan market size was valued at USD 6.8 billion, with an expected expansion at a revenue-based Compound Annual Growth Rate (CAGR) of 24.7% between 2020 and 2027[43].

1.3.1 Biomaterial and tissue engineering

The applications of chitosan as a biomaterial for tissue engineering have been widely investigated[44]. Chitosan can be processed into different structures, such as films[45], nano-particles[46], hydrogels[47], or fibers[48], which translates into a high degree of versatility for multiple applications[49].

Developing scaffolds based on synthetic or natural polymers in different forms has been a standard method for providing cells with an environment similar to the biological extracellular matrix (ECM)[46]. Used as a scaffold, chitosan can act as physical support for cells[50], allowing them an optimal environment for growing and tissue structuring[51]. The choice of chitosan as a tissue support material is due to the multiple ways its properties can be controlled and engineered[52].

1.3.2 Food packaging

The development of biodegradable polymers for eco-friendly food packaging is one way to minimize the problem of the accumulation of plastics in the environment[53]. Thus, there is a continuous interest in exploring new biomaterials that can serve this purpose.

Chitosan poses good mechanical properties and capacity of selective permeability to O_2 and CO_2[54]. In addition, chitosan also has intrinsic antioxidant and antimicrobial activities against fungi, molds, yeasts, and bacteria[55], which can potentially extend the shelf life by inhibiting the presence and growth of microorganisms and bacteria when in direct contact with fresh or processed foods[56].

Properties like tensile strength and elongation percentage at break and barrier properties are significant in the food packaging industry[53]. Chitosan offers the potential for multiple chemical modifications and polymer blending, allowing for fine-tuning of these and other properties for particular applications[57]. All these factors make chitosan a desirable material for food packaging.

1.3.3 Antioxidant and antimicrobial

Chitosan has unique antimicrobial and antiviral properties, widely described[24,43,58–60]. Several possibilities have been described as the mode of action for chitosan, from binding to bacterial DNA, which leads to inhibition of mRNA, to interaction with surface molecules[61].

The most accepted hypothesis for the origin of this antimicrobial activity is related to the positively charged amino groups in the chitosan chain, which can interact with the

negatively charged surface components of many microorganisms [62]. This would lead to extensive alterations in the surface, resulting in cell death[43].

The antimicrobial activity of CS differs depending on its molecular weight (MW) and the type of attacking microorganism[32]:

- High MW chitosan has a marked antibacterial effect against gram-positive and negative bacteria[61].
- Low MW chitosan exerts a more substantial effect against hyphal growth via a dual effect on spores and fungal cells[63].
- Oligomeric CS has a more potent antimicrobial effect on fungi[64].

1.3.4 Adsorption of metals and dyes

The presence of synthetic dyes and metals in water sources can produce severe health and ecological issues. Therefore, there is a high degree of interest in developing new ways of removing these contaminants from aqueous streams[65]. Chitosan has received attention as a viable adsorbent due to its biodegradability, biocompatibility, and low cost[66]. The presence of several active sites in the form of -OH and $-NH_2$ in the chitosan chain confers this polymer with the ability to interact and adsorb metals[67–69] and dyes[66,70]

1.3.5 Gene delivery

Early efforts to transfect cells using non-viral vectors led to the observation that positively-charged molecules, typically rich in protonating amine groups, could form electrostatic complexes with negatively charged nucleic acids[71] (Figure 3). The resulting nano complexes had transfection capabilities[72]. This transfection capability is currently

described by a mechanism of gene delivery by cationic systems, which includes four steps[73]:

1. Non-specific interaction between cationic particles and cell surface
2. Endocytosis into endocytic vesicles (endosomes)
3. Compaction and release of the genetic material/particle from endosomes
4. Translocation of the released material to the nucleus by membrane receptors and its transgenic expression.

Figure 3. Formation of polyplexes

The use of these cationic materials as gene vectors started with naturally occurring compounds like protamine[74] and spearmine[75]. The available carriers progressively developed into various cationic compounds specifically aimed at gene delivery, like cationic lipids and polymers.

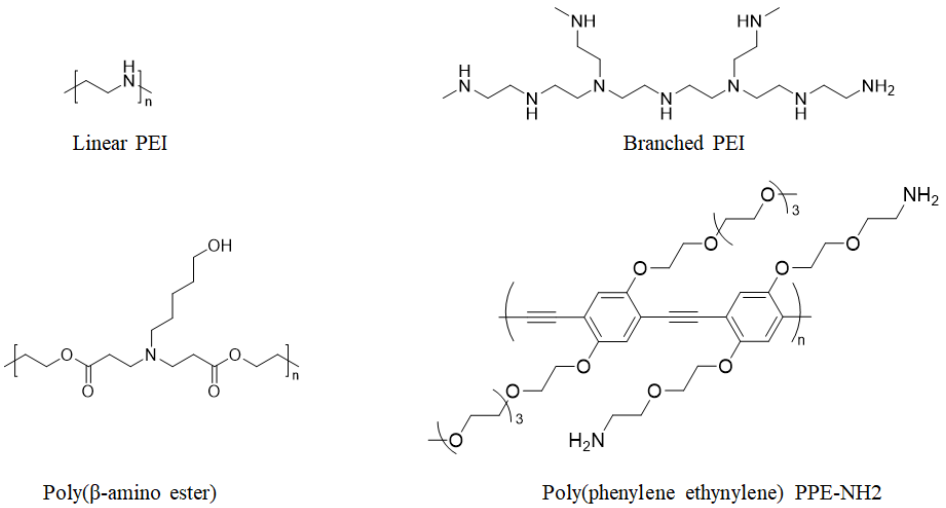

Figure 4. Examples of cationic polymers

Cationic polymers (Figure 4) are an attractive choice for non-viral nucleic acid vectors due to their repeating unit architecture, which grants a large concentration of positive charges per molecule, allowing for increased complexation efficiency while forming polyplexes with the genetic material[76]. These polyplexes have shown the ability to protect the nucleic acids from degradation and allow their successful delivery into the cell cytoplasm[77]. Of these synthetic compounds, the conjugated polymers[78] and the carbamoylated guanidine modified ROMP polymers [79] synthesized by Dr. Moon's research group at FIU are successful examples. Another example is the PEI, which has become the golden standard of non-viral vectors due to its high transfection capability but suffers the downside of being very toxic and non-biodegradable.

Chitosan has also been utilized as a cationic polymeric vector[80]. At acidic pH values, the primary amines in the chitosan backbone become positively charged, enabling chitosan to bind to negatively charged DNA/siRNA via electrostatic interactions, leading to the spontaneous formation of nano-sized complexes (polyplexes)[81]. Given the substantial number of positive amines present in the chitosan due to its polymeric nature, there will be a high complexation efficiency, which allows for a high gene payload[82]. Furthermore, chitosan is slightly charged at neutral or basic pH but still able to bind to the nucleic acids through secondary (non-electrostatic) interactions, such as hydrogen bonding and hydrophobic interactions[83].

1.4 The carbamoylated guanidine moiety

In 2019, Dr. Moon's research group at FIU synthesized a carbamoylated guanidine functionalized CP as a non-viral vector[79]. As a result of this work, it was shown that adding carbamoylated guanidine moieties (Figure 5) to polymers confers a decisive advantage in

terms of transfection capabilities, gene payload, and biocompatibility over PEG and PEI derivatives, especially when transfecting rigid nucleic acids as siRNA. This work aimed explicitly at the transfection of human airway epithelium cells, representing a more significant challenge due to a dense layer of negatively charged mucus[79].

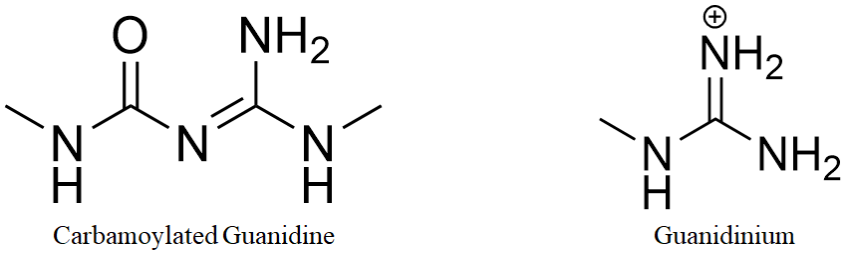

Figure 5. The carbamoylated guanidine and guanidinium groups

The carbamoylated guanidine moiety has a positive delocalized shielded charge on the guanidine substructure, which can establish bidentate interactions with negatively charged compounds, as shown in Figure 6. The presence of the urea substructure extents hydrogen bonding, which translates into stronger interactions with anions (an attractive characteristic for gene vector applications). It also offers the possibility of modification at the positive charge, which can improve biophysical properties.

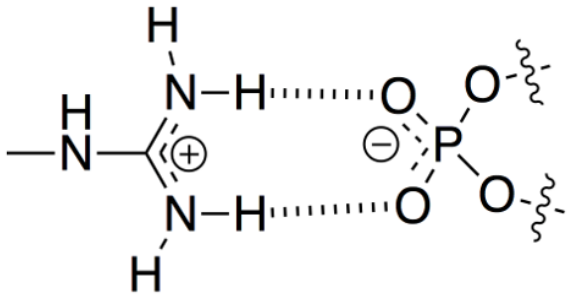

Figure 6. Guanidine bidentate interaction with phosphate

1.5 Research Purpose

Considering the above, this research intends to study the feasibility of obtaining a phenyl carbamoylated guanidine chitosan derivative. It is hypothesized that this modified chitosan will be soluble in DMSO, which will expand its applicability, especially in biological applications, and increase the range of further chemical modifications. This research plan involves synthesizing this target molecule starting from the unmodified chitosan.

II. EXPERIMENTAL METHODS

2.1 Materials and Instrumentation

2.1.1 Materials.

Chitosan (CS), purified powder (MW ~ 15000, 85% deacetylated), was purchased from Polysciences, Inc. Potassium hydroxide (KOH), Triethylamine (TEA), hexane, ethyl acetate (EA), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetone and tetrahydrofuran (THF) were acquired from Fischer Scientific. Mercuric chloride ($HgCl_2$), dichloromethane (DCM), methanesulfonic acid (MSA), and phenyl isocyanate (PhNCO) were purchased from Millipore Sigma. S-methylisothiourea hemisulfate was purchased from Tokyo Chemical Industry (TCI). Sodium hydrogen bicarbonate ($NaHCO_3$) was purchased from Alfa Aesar. Di(tert-butyl) dicarbonate (BOC_2O) was purchased from Chem Impex International Inc. Deuterated dimethyl sulfoxide (DMSO-d6), deuterated acetic acid, deuterium oxide (D_2O), and deuterated chloroform ($CDCl_3$) were acquired from Cambridge Isotope Laboratories.

The DMF and the DMSO were dried over micro sieves acquired from Fischer Chemicals in the laboratory before use.

2.1.2 Nuclear magnetic resonance (NMR).

1H NMR spectroscopy studies were performed using a Bruker 400 MHz NMR spectrometer for data collection and Topspin software for data analysis. All 1H NMR studies were conducted at 25^{o} C in $CDCl_3$, DMSO-d6, or D_2O / Acetic acid-d4 1% v/v. $CDCl_3$ was used for the mono-BOC S-methyl isothiourea and the BOC S-methyl phenyl carbamoylated guanidine, while the DMSO-d6 was utilized for the phenyl carbamoylated

guanidine functionalized chitosan. D_2O / Acetic acid-d4 1% v/v was used as a solvent for acquiring the spectra of the unmodified chitosan.

All samples prepared for 1H NMR analysis contained 10 mg of product and 700 µL of solvent. The solution was then placed into a Branson 2510 ultrasonic bath for 20 min to ensure that the samples were completely dissolved in the solvent and then analyzed. Chemical shifts are expressed in parts per million (δ) using a residual solvent proton as the internal standard.

2.2 Solubilization of chitosan in acetic acid 1% v/v solution

First, 2 ml of acetic acid (AcOH) glacial were pipetted into a 100 mL volumetric flask, followed by the addition of water until the graduation mark. The solution was then transferred to a 250 mL round bottom flask (RBF) and cooled down to 4^0C in a laboratory fridge for one h. 2 g of chitosan were weighted and slowly added to the cold solution with gently stirring. The mixture was cooled to 4°C in the laboratory fridge for 1 h. The cold mixture was then vigorously stirred, and the chitosan solubilized utterly, causing the solution to become denser, with a final concentration of 20 mg/ mL of chitosan. The final solution was stored in the fridge at 4^0C.

The solution was pipetted into the reaction vial for subsequent uses until the desired amount of chitosan was achieved (e.g., for 100 mg of chitosan, 5 mL of the solution were added to the vial). This allowed faster reaction setup times by eliminating the need to solubilize the chitosan every time a reaction was undertaken.

2.3 Synthesis of mono-BOC protected S-methyl isothiourea

The procedure was based on the protocol described previously by Liang *et al.*[84]. A mixture of 60 mL of H_2O and 60 mL of THF was prepared in a 200 mL graduated beaker and then added to a 250 mL RBF. After this, 12 g of S-methylisothiourea hemisulfate and 7.23 g of $NaHCO_3$ were dissolved in the mixture, followed by the addition of 14 g of di(tert-butyl) dicarbonate while slowly stirring. A rubber septum was used to cover the RBF mouth, with a needle inserted to avoid pressure buildup. The reaction was stirred at room temperature for 24 hours.

After 24 hours, the reaction was stopped. The mixture was concentrated under reduced pressure using a Buchi R-114 rotary evaporator. The residue was then partitioned in 50 mL of Ethyl Acetate three times. The three organic fractions were collected, mixed, washed with brine twice, dried over magnesium sulfate ($MgSO_4$), and filtered. The solvent was evaporated under reduced pressure using the rotary evaporator, leaving a white residue. The solid was then collected and dissolved in 25 mL of hexane, stirring while heating with a heat gun. The solution was cooled down and kept at 0 °C overnight. The solvent was decanted and filtered, and the remaining solid was vacuum dried overnight to afford 9.28 g of product as white crystals.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.51 (9H, s), δ 2.46 (3H, s), δ 7.62 (2H, s).

2.4 Synthesis of mono-BOC protected S-methyl phenyl carbamoylated guanidine

A reaction path similar to the one described by Hsu *et al.*[85] for the preparation of amidinoureas was followed for preparing the S-methyl phenyl carbamoylated guanidine (SMPCG). A 40 mL glass vial was flame dried and then purged with N_2, followed by the addition of 9.28 of dried recrystallized mono-BOC protected S-methyl isothiourea. 6 mL

of anhydrous DCM was then added dropwise while slowly stirring to dissolve the mono-BOC protected S-methyl isothiourea. Lastly, 8.72 g (8 mL) of phenyl isocyanate were added dropwise while stirring, and the reaction was then kept at 60 °C for 48 h.

After 48 h, the reaction was stopped. The solvent was evaporated under reduced pressure using a Buchi R-114 rotary evaporator, leaving the crude mix as a white residue. The solid was collected and vacuum dried overnight to afford 11.66 g of crude. The crude mixture was purified using a Yamazen Parallel Frac FR-260 flash chromatography device. The fractions of the crude mix were first dissolved in DCM and introduced in a Yamazen silica gel injection column (M size, 20x75 mm) and then separated using a mixture of hexane/ ethyl acetate 80:20 ratio on a Yamazen normal phase silica gel column (L size, 3.0x16.5 cm, particle size 30 um). The fractions of interest were collected based on the peaks and time of elution. The solvent was evaporated under reduced pressure using the rotary evaporator, leaving a white residue. This solid was collected and dried overnight to afford 6.1 g of pure product.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.50 (9H, s), δ 2.39 (3H, s), δ 7.10 (1H, t, $^{3}J_{H-H}$ = 7.4 Hz), δ 7.33 (2H, t, $^{3}J_{H-H}$ = 7.9 Hz), δ 7.50 (2H, d, $^{3}J_{H-H}$ = 7.6 Hz), δ 12.19 (1H, s).

2.5 Synthesis of deprotected S-methyl phenyl carbamoylated guanidine

The removal of the BOC group was carried out using a modification of the procedure described by Greene[86]. Initially, 2 g of mono-BOC protected SMPCG were dissolved in 6 ml of DCM, followed by the addition of 3.68 g (2.5 mL) of trifluoracetic acid (TFA) dropwise while stirring. The solution was then kept at 35^{0}C for 14 h. The solution was then concentrated under vacuum, followed by the addition of ethyl ether in excess. The mix was stirred for 20 min. Once the precipitation was observed, the vial was centrifugated at 4000

rpm for 6 min. The solvent was removed by decantation, and the white residue was vacuum dried overnight to afford the product as a white solid.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 2.56 (3H, s), δ 7.04 (1H, t, $^{3}J_{H-H}$ = 7.4 Hz), δ 7.30 (2H, t, $^{3}J_{H-H}$ = 7.9 Hz), δ 7.55 (2H, d, $^{3}J_{H-H}$ = 7.6 Hz), δ 9.82 (1H, s).

2.6 Synthesis of phenyl carbamoylated guanidine functionalized chitosan

Starting from the procedure described by Ch. Yuan and R. Williams[87] for the synthesis of amidinoureas, the following method was used to prepare the phenyl carbamoylated guanidine functionalized chitosan (PCGCs). Initially, 1.92 g of SMPCG and 0.84 g of HgCl2 (activator) were added to a 40 mL glass vial containing 2 mL of dry DMF and sonicated for 10 min to achieve complete solubilization. After this, 0.10 g of unmodified chitosan was added to the solution, followed by 10 min of sonication. Lastly, 0.31 g (0.44 mL) of TEA were added dropwise. The reaction was kept at 80^{0}C for 72 h while stirring. After 72 h, the vial was removed from the hotplate, and the reaction mixture was filtered twice. The resulting homogeneous solution was added to a glass vial containing 4 mL of acetone and then centrifuged at 2500 rpm for 3 min. At the end of the centrifugation cycle, the solvent was removed by decantation, and 2 mL of dry DMSO was added to the solid residue. The mix was sonicated for 20 min to ensure complete dissolution and then filtered. The resulting homogeneous brown solution was added to a glass vial containing 2.5 mL of acetone. This vial was then centrifugated at 2500 rpm for 3 min. At the end of the centrifugation, the solvent was removed by decantation, and 1 mL of dry DMSO was added to the solid residue. The mix was sonicated for 10 min to ensure complete dissolution and then filtered. The resulting homogeneous solution was added to a glass vial containing 2 mL of acetone. After a third and final centrifugation cycle at 2500 rpm for 3 min, the solvent

was removed by decantation. The solid residue was vacuum dried overnight to afford 60 mg of PCGCs.

PCGCs:

^{1}H-NMR (DMSO-d6, 400 MHz) δ 1.98 (1H, s), δ 3.10 (4H, d, $^{3}J_{H-H}$ = 6.4 Hz), δ 3.44 (2H, m), δ 4.35 (1H, s), δ 6.96 (1H, t, $^{3}J_{H-H}$ = 7.3 Hz), δ 7.27 (2H, t, $^{3}J_{H-H}$ = 7.9 Hz), δ 7.44 (2H, d, $^{3}J_{H-H}$ = 7.6 Hz), δ 8.88 (1H, s).

2.7 BOC deprotection of phenyl carbamoylated guanidine functionalized chitosan

For the deprotection of the phenyl carbamoylated guanidine functionalized chitosan (PCGCs), 0.02 g of BOC protected PCGCs were dispersed in 1 ml of DMF, followed by the addition of 0.027 g (1.5 mL) of HCl 0.5 M dropwise while stirring. The reaction was kept at 40^{0}C for 24 h while stirring.

After 24 h, the vial was removed from the plate, and the reaction mixture was filtered twice. The mixture was added to a glass vial containing 3 mL of acetone and then centrifuged at 2500 rpm for 3 min. At the end of the centrifugation cycle, the solvent was removed by decantation, and 1 mL of dry DMSO was added to the solid residue. The mixture was sonicated for 10 min, filtered, and added to a glass vial containing 2.5 mL of acetone. This vial was then centrifugated at 2500 rpm for 3 min. At the end of the centrifugation, the solvent was removed by decantation, and 1 mL of dry DMSO was added to the solid residue. The mixture was sonicated for 10 min, filtered, and added to a glass vial containing 2 mL of acetone. After a third and final centrifugation cycle at 2500 rpm for 3 min, the solvent was removed by decantation. The solid residue was vacuum dried overnight to afford 13 mg of dry solid.

NMR analysis did not confirm the success of this reaction.

III. RESULTS AND DISCUSSION

3.1 Synthesis strategies

The strategy for synthesizing the carbamoylated guanidine chitosan derivative involved exploiting the nucleophilicity of the amine groups to attack the SMPCG (figure 7), using $HgCl_2$ as an activator and TEA as the base to form the desired product. This reaction would be aided by the excellent leaving group character of the S-methyl. It was hypothesized that conversion via a single reaction would lead to a higher yield when compared to the alternative path of successive reactions to modify the amine moiety using guanidinylation first, followed by BOC protection and reaction with a primary aromatic amine, like aniline.

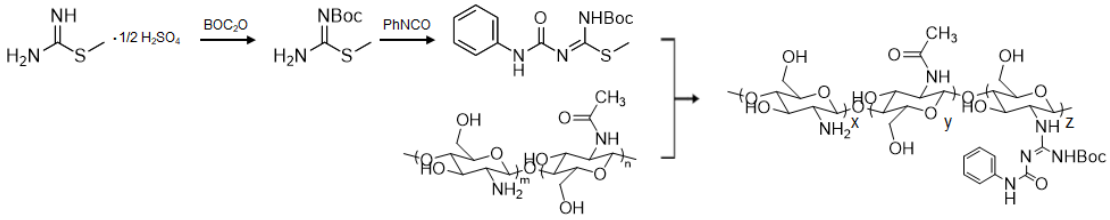

Figure 7. Scheme for the synthesis of the PCGCs.

3.2 Solving the chitosan solubility problem

It was foreseen that the lack of solubility of the chitosan in organic solvents would be an obstacle to this synthesis. Therefore, this issue had to be solved first to ensure a good conversion percentage of the chitosan amines into the desired carbamoylated guanidine.

To remediate this problem, two routes were tried first:

1- Dissolve the chitosan in an acetic acid 1% v/v in water solution, and then diluting the chitosan solution in DMSO or DMF.
2- Synthesis of a chitosan mesylate salt soluble in DMSO.

A chitosan solution in acetic acid was prepared and then diluted in DMSO or DMF for the first route. Before the reaction, the pH was stabilized to 8-9 by adding KOH to ensure that the chitosan amines were deprotonated. Nevertheless, none of the reactions tried using this method gave the desired product, and thus, it was decided to abandon this path. It was hypothesized that the loss of solubility of the chitosan under basic conditions might have contributed to the failure of these reactions.

The formation of DMSO soluble chitosan salts had been previously described by Sashiwa *et al.*[26]. The mesylate salt is obtained by reacting the chitosan with MSA using water as a solvent (figure 8).

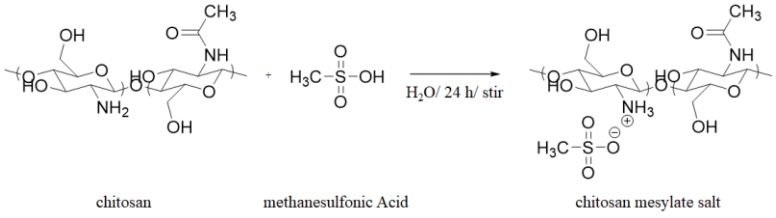

Figure 8. Chitosan mesylate salt formation

Interestingly, freeze-drying is essential in achieving solubility in DMSO[26]. Therefore, it was decided to utilize this method to dry the chitosan salt before attempting dissolution in DMSO. Accordingly, the mesylate route provided a DMSO soluble chitosan salt upon

sonication treatment for 20 min. Nevertheless, using the chitosan mesylate salt did not lead to the expected product.

One cause for the failure of these reactions is that the formation of the mesylate salt relies on the ionic interactions of the protonate amines in the chitosan chain with the mesylate anions. During the reaction of the chitosan with the SMPCG under basic conditions, these ionic interactions can undergo two paths, both of which negatively impact the possibility of reaction success:

1- A portion of the amine groups remain protonated during the reaction, thus ensuring the continuation of these ionic interactions and the solubility in DMSO but inhibiting the nucleophilic character needed to react with the carbodiimide intermediate (see section 3.4 for details in the proposed reaction mechanism).

2- The addition of the base leads to deprotonation of the vast majority of the amine groups of the chitosan, in which case the nucleophilicity is enhanced at the expense of a loss of solubility in DMSO, which leads to a decrease in the overall interactions between the chitosan and the SMPCG.

Considering the above, it was decided to attempt the reaction by simply dispersing the chitosan in DMF, in conjunction with the 1:10 ratio of SMPCG and activator. The use of sonication allowed for the generation of homogeneously dispersed mixtures. The base was added last to avoid premature aggregation of the reagents in the vial before the reaction started. Using this method, the target molecule was finally achieved.

3.3 Synthesis of the S-methyl phenyl carbamoylated guanidine

The synthesis of the SMPCG was designed considering the methods described previously[88–90]. The use of the methylisothiourea as the starting point was chosen based on reagent price and availability, as well as the fact that the S-methyl is an excellent leaving group, which contributes to the successful completion of the first step of the proposed reaction mechanism (see section 3.4 below for details) for obtaining the target compound.

For the production of the S-methyl phenyl carbamoylated guanidine in the desired configuration, the phenyl isocyanate can only be allowed to react with the primary amine of the S-methylisothiourea. Subsequently, to avoid the possibility of side reactions, the secondary amine of the starting reagent needs to be protected.

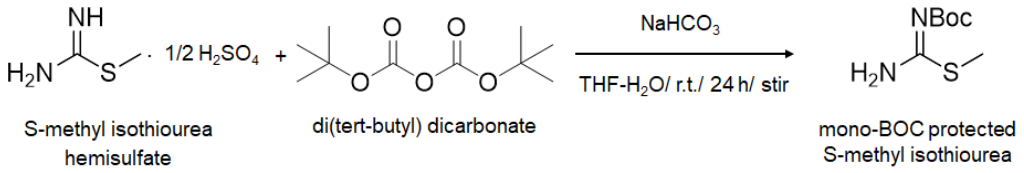

Figure 9. BOC protection of the S-methyl isothiourea.

The BOC group is traditionally used in the protection of amines[86]. In this case, the BOC group also has the added benefit of being an electron-withdrawing moiety, which helps to ensure electron deficit at the carbodiimide carbon (see section 3.4 below for details). Therefore, it was decided to employ BOC protection of the secondary amine.

Mild conditions were used for this reaction to ensure the prevalence of the mono BOC-protected product. As described by Hsu *et al.*[85], the use of $NaHCO_3$, a weak base, in

conjunction with diluted concentrations of BOC_2O in THF-H_2O excess (Figure 9), leads to the production of the mono-BOC protected product with a 99% yield. Purification was possible via partition in EA/ H_2O, followed by recrystallization in hexane.

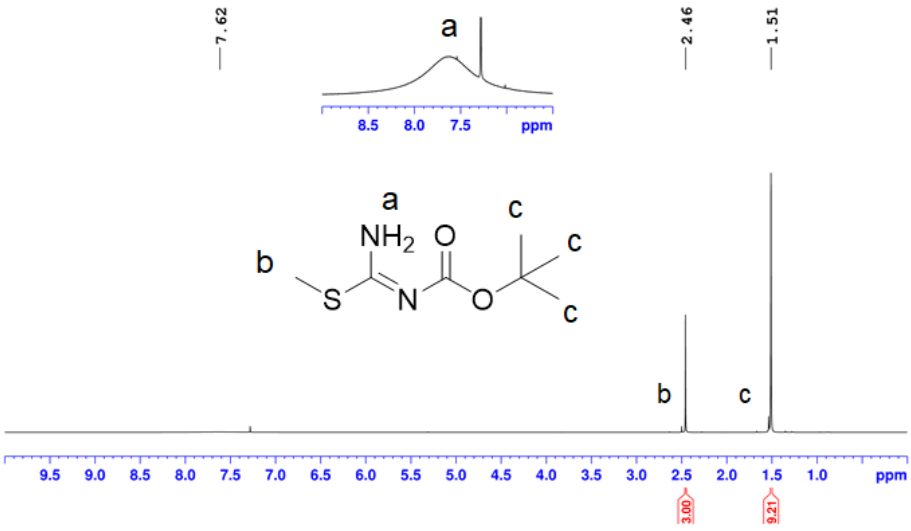

Figure 10. ^1H NMR (400 MHz, $CDCl_3$) spectrum of mono-BOC protected S-methylisothiourea.

Once the mono BOC-protected product was purified, the second step involved the synthesis of the SMPCG via the reaction with the PhNCO (Figure 11). The use of DCM ensured an easy solvent removal by heating under vacuum. The crude mixture was purified by chromatography, using a mix of hexane and EA in an 80:20 ratio.

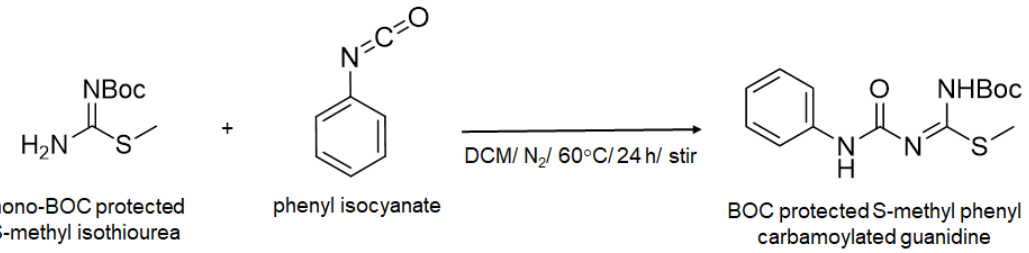

Figure 11. Synthesis of the BOC protected S-methyl phenyl carbamoylated guanidine.

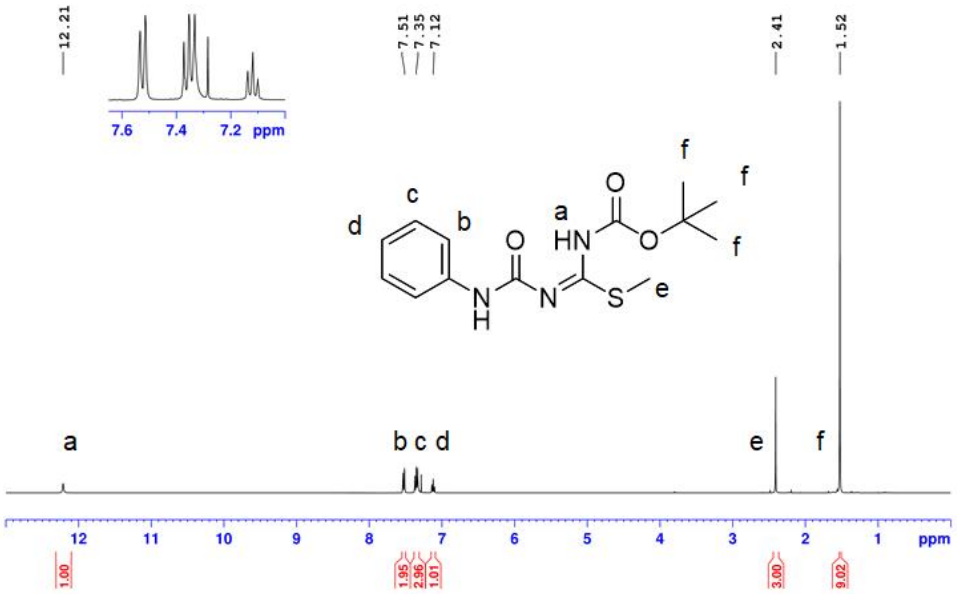

Figure 12. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of BOC protected S-methyl phenyl carbamoylated guanidine.

3.4 Reaction of the chitosan with the mono BOC S-methyl phenyl carbamoylated guanidine

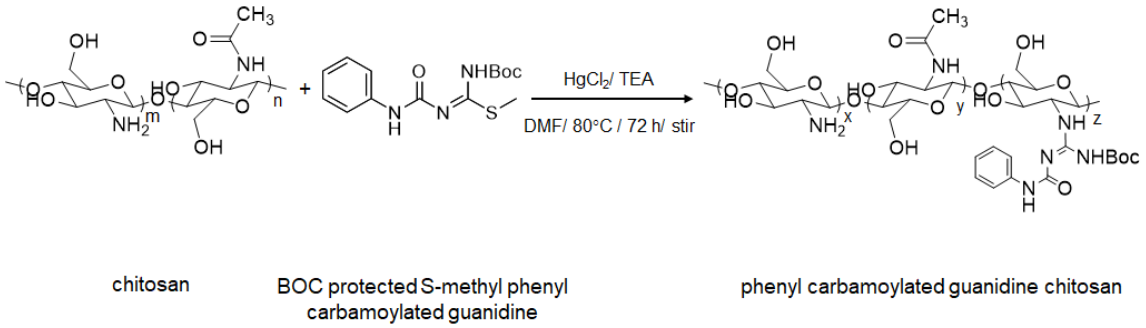

Figure 13. Reaction of the chitosan with the mono BOC S-methyl phenyl carbamoylated guanidine.

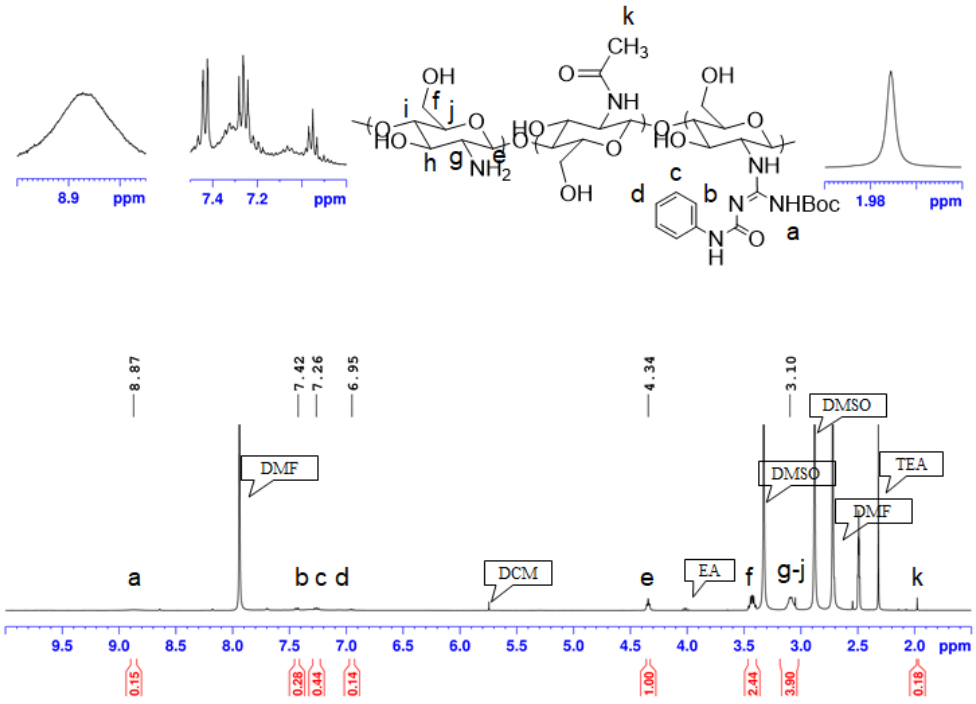

Figure 14. ^{1}H NMR (400 MHz, DMSO-d6) spectrum of phenyl carbamoylated guanidine chitosan.

As noted by Kim *et al.*[90], this reaction (Figure 13) undergoes the following route (Figure 15):

1- Elimination of the S-methyl promoted by $HgCl_2$ and subsequent complexation of the anionic sulfur with the mercury.

2- Formation of a carbodiimide intermediate with an electron-deficient center.

3- Attack of the primary amine group of the chitosan on the electron-deficient center at the carbodiimide carbon.

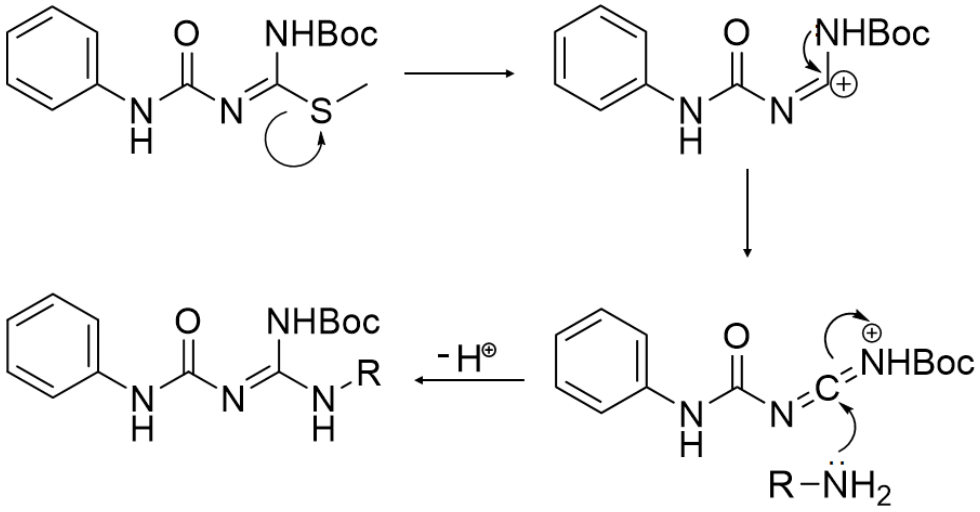

Figure 15. Proposed reaction mechanism for the chitosan modification.

This mechanism is supported by spectroscopic evidence for the formation of the carbodiimide intermediate[90] the high affinity of the Hg^{2+} to form complexes with anionic sulfur compounds[91].

It has been described that the presence of the BOC group facilitates the occurrence of the third step of this reaction[88]. Owing to its electron-withdrawing nature, the BOC group

increases the electron deficiency at the carbodiimide carbon center, which aids in the nucleophilic addition of the primary amine moiety.

Subsequently, the BOC group serves two roles in this reaction:

1- Ensure that only the primary amine on the S-methylisothiourea reacts with the phenyl isocyanate to yield the SMPCG.

2- Facilitate the attack of the primary amine of the chitosan with the carbodiimide intermediate to produce the target molecule.

Additionally, using a base guarantee that the amine groups in the chitosan remain deprotonated during the reaction, enhancing its nucleophilicity and facilitating the attack on the carbodiimide intermediate.

During this research, several reactions were set up with and without using $HgCl_2$. None of the reactions in which the initiator was omitted were successful, which confirms the critical role of this reagent in the first step of the reaction, both as an activator and in avoiding the regeneration of the SMPCG via the reaction of the S-methyl anion with the carbodiimide.

In a separate set of reactions, the BOC group was removed in the SMPCG, and the deprotected compound was used in a set of reactions, none of which yielded the desired product. This reaffirms the theory that the presence of an electron-withdrawing group facilitates the attack on the carbodiimide intermediate by increasing the electron deficiency of its carbon center.

Regarding the solubility of the modified chitosan, it is soluble in DMSO as originally hypothesized and partially soluble in DMF. This product is insoluble in water, acetone, ethanol, and $CDCl_3$.

3.5 NMR analysis of the product and determination of the degree of substitution

As shown in Figure 16 and Tables 2 and 3), the NMR results of the PCGCs were compared against the spectra of unmodified chitosan (see Appendix for full ^1H NMR spectrum and peaks interpretation of the unmodified chitosan).

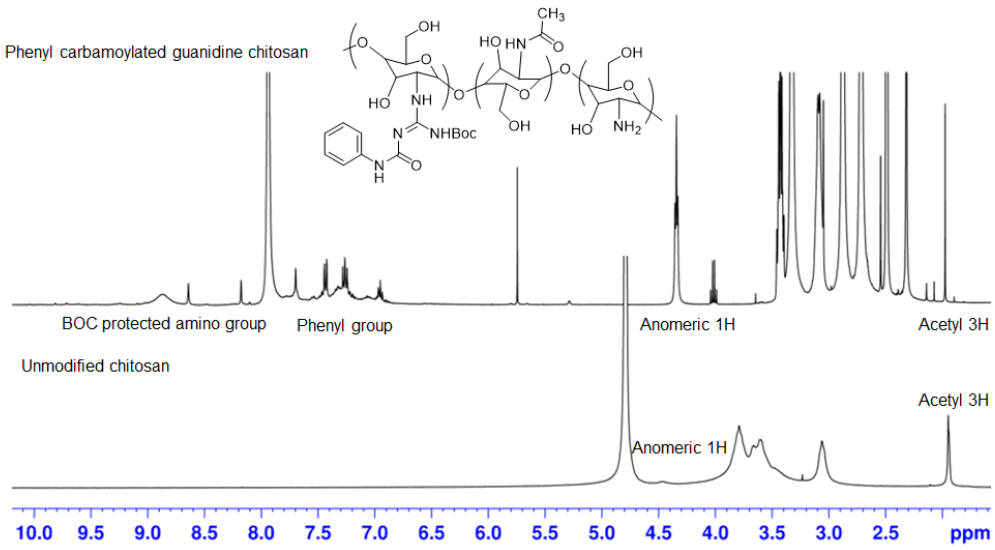

Figure 16. ^1H NMR spectra comparison of unmodified chitosan (400 MHz, D2O/ Acetic acid-d4 1%) and phenyl carbamoylated guanidine chitosan (400 MHz, DMSO-d6).

The degree of substitution indicates how many primary amine groups of the unmodified chitosan reacted with the BOC SMPCG to form the PCGCs. As such, it is an essential indicator of the extension of the reaction.

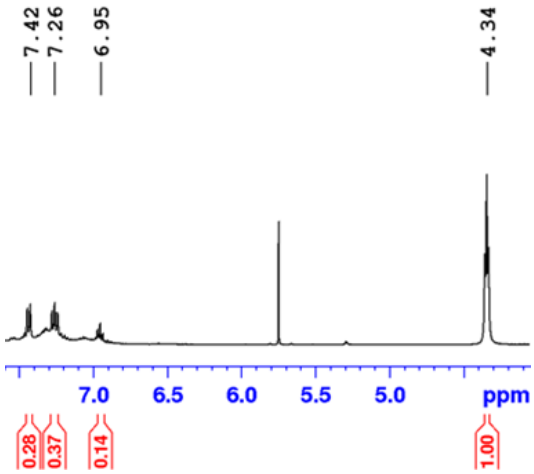

Figure 17. Illustration of the ^{1}H NMR determination of the degree of substitution of the PCGCs.

For the PCGCs, the percentage of substitution was established by establishing the integration ratio of the anomeric hydrogen in the chitosan chain and the single phenyl hydrogen in the phenyl carbamoylated guanidine substituent (Figure 17). The single anomeric hydrogen is present in all the chitosan units, so it is a good indicator of the size of the polymer chain. Likewise, the phenylic hydrogen is present in all the phenyl carbamoylated moieties, so it is a reliable pointer for the presence of said substituent.

The peak area was 1.00 for the anomeric hydrogen and 0.14 for the phenylic hydrogen. The integration ratio demonstrates a substitution of 14%, which means that 14% of the amines initially present in the unmodified chitosan chain have reacted with the BOC SMPCG.

3.6 BOC deprotection of the phenyl carbamoylated guanidine functionalized chitosan

For the deprotection of the PCGCs, the usual route employing TFA and DCM, as described by Greene[86], was denied due to the lack of solubility of the product in DCM. The use of

DMSO as solvent was not considered due to the possibility of side reactions, so an alternative path was used, with HCl replacing the TFA as the acid of choice, and employing DMF as solvent to ensure at least partial solubility of the BOC PCGCs (Figure 18).

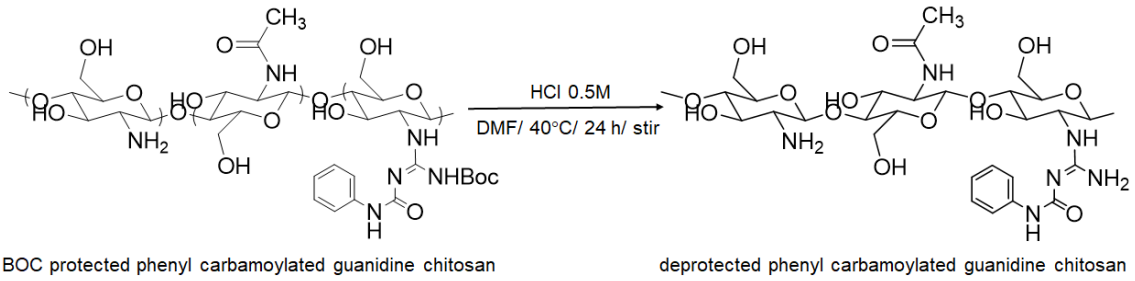

Figure 18. Deprotection of the PCGCs.

This reaction was tried a number of times, without success. Even though the reaction included heating the mixture to 40^0C, the NMR did not confirm the obtention of the deprotected PCGCs in any of the iterations.

It is hypothesized that a factor contributed to the lack of success of this reaction was the molarity of the HCl. The concentration of the acid mentioned in literature[86] is 3M, but the acid available at the moment of the reaction had a concentration of 0.5M. To remediate this, an excess of acid was used in combination with heat, but ultimately it did not lead to the desired results.

IV. CONCLUSION

During this research, reaction conditions for synthesizing PCGCs have been established. These conditions include circumventing the lack of solubility of chitosan in organic solvents by using sonication to achieve a high degree of dispersion in the reagents mixture.

It has also been found that the reaction does not occur in the absence of $HgCl_2$ or BOC protection of the SMPCG. These findings are consistent with the proposed mechanism for the production of PCGCs, which include the $HgCl_2$ as an activator and an agent avoiding the regeneration of the SMPCG via the reaction of the S-methyl anion with the carbodiimide. The mechanism also includes the BOC group in the role of increasing the electron deficiency at the carbodiimide carbon center, which facilitates the attack of the primary amines of the chitosan.

The PCGCs product was characterized by NMR spectroscopy, and its spectrum was compared with the unmodified chitosan and the SMPCG starting product spectra. The degree of substitution was established to be 14 % using analysis of the NMR spectra.

In virtue of its solubility in DMSO and the presence of the phenyl carbamoylated guanidine moiety, the chitosan derivative obtained during this research will be used in future experiments in protein delivery, and antimicrobial activity, and siRNA transfection. Future research is also planned to establish the conditions to obtain PCGCs with different degrees of substitution to evaluate the optimal value for transfection, protein delivery, and antimicrobial activity.

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APPENDIX

^{1}H NMR (400 MHz, D_2O/ Acetic acid-d_4 1%) spectrum of unmodified chitosan.