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Investigating Chitosan Modified with Triethylammonium Butanamide and Triethylphosphonium Butanamide as Non-Viral Gene Delivery Vectors By Examining Cytotoxicity and Transfection Efficiency

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INVESTIGATING CHITOSAN MODIFIED WITH TRIETHYLAMMONIUM BUTANAMIDE AND TRIETHYLPHOSPHONIUM BUTANAMIDE AS NON-VIRAL GENE DELIVERY VECTORS BY EXAMINING CYTOTOXICITY AND TRANSFECTION EFFICIENCY

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ABSTRACT

Gene therapy is a very challenging field, especially with new emerging genetic disorders. Chitosan (CS), due to chitosan's flexibility, biocompatibility, and biodegradability, has been of interest in the world of gene therapy especially as researchers are gravitating towards non-viral vectors due to the problems caused by viral vectors. Nevertheless, there are still issues regarding solubility, cellular uptake of cargos being transported *in vitro* or *in vivo*, increased cytotoxicity levels, as well as many other things that prevent chitosan from being an efficient gene delivery agent. Here I present five derivatives of chitosan, which were all modified with either triethylphosphonium butanamide (TEPB) or triethylammonium butanamide. In addition to the TEAB and TEPB groups, only two were modified with methoxy-poly (ethylene glycol) or mPEG units. The five derivatives are TEAB1-CS (24%of CS modified with TEAB), TEAB2-CS (40.6% TEAB), TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG), TEPB-mPEG-CS (43% TEPB; 2.6% mPEG), and TEPB-CS (43% TEPB). Cell proliferation and cytotoxicity assays were performed for the derivatives using an XTT assay kit which demonstrated mild to no defects on the growth of the cells. All derivatives were complexed with a TPST1-EGFP plasmid at a ratio of 10:1 and were able to transfect HeLa cervical cancer cells with varying degrees of efficiency. TEAB-mPEG-CS and TEPB-mPEG-CS had low cell viability at 100 μg/mL, however only TEPB-mPEG-CS induce apoptosis in a dose dependent manner. TEAB2-CS and TEPB-CS had low viability from the concentration ranges of 10-100 μg/mL and both induced apoptosis in a dose dependent manner. In particular, TEAB1-CS promoted viability at all treatment concentrations and showed the highest transfection efficiency among all 5 CS derivatives, while TEAB-mPEG-CS, TEPB-mPEG-CS, TEPB-CS, and TEAB2-CS exhibitited similar transfection efficiencies, suggesting that TEAB1-CS would be the most effective for gene transfection.

KEYWORDS: chitosan nanoparticles, triethylammonium-butanamide chloride, triethylphosphonium-butanamide chloride, gene therapy, degree of quaternization, N+/P- ratio, P+/P- ratio, biopolymer cytotoxicity, pDNA transfection

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I dedicate this thesis to God and my parents.

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INTRODUCTION

Biological Characteristics of CS and Its Derivatives

Since its discovery in mid-1800s, chitosan has been used in tissue regeneration, wound healing, cancer therapy, gene therapy, drug delivery, among many other things [1-7]. A table summarizing many recent applications of chitosan and its derivatives is included here (Table 1). Chitosan can be derived from chitin housed on the exoskeleton of insects and crustaceans, such as lobsters, crabs, and shrimp [1, 2, 7]. Conversion of chitin to CS is shown in Figure 1. The degree of acetylation in chitin is around 90%, while chitosan is a fully or partially N-deacetylated derivative with a typical degree of deacetylation of more than 65%. Chitosan is comprised of glucosamine and *N*-acetylated glucosamine units that are linked through β- (1-4) glycosidic linkages (Fig. 1) [8].

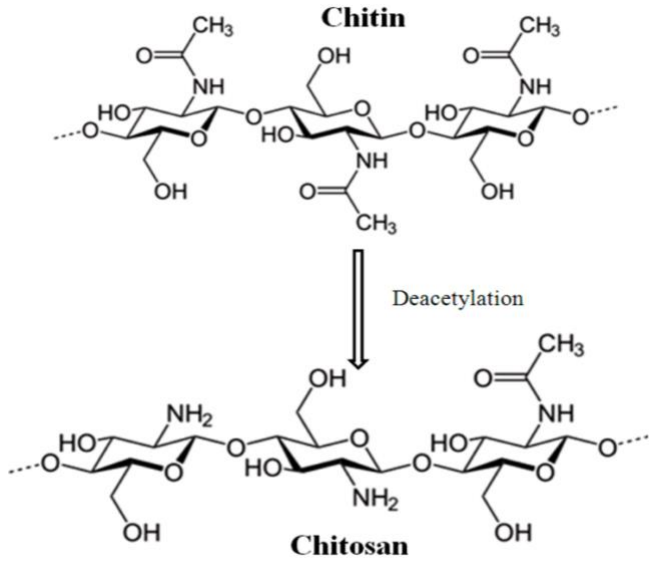

Figure 1. The conversion from chitin to chitosan through chitin deacethylation [1]. Chitin and CS are polysaccharides made up of N-acetyl glucosamine and D-glucosamine functional groups. Chitin has more N-acetyl glucosamine groups than chitosan. CS has three different functional groups, two OH groups and one NH_2 group that can be modified for many biological applications.

The glucosamine of chitosan has primary amine groups with pK_a of 6.5, which allows the biopolymer to only be soluble in acidic solution but not neutral or basic solution [5, 9]. The activity of chitosan is influenced by Degree of Acetylation (DA) due to charge change upon deacetylation. It was reported that chitosan with 25% DA had a lower antibacterial activity than CS with 5% DA [1, 10]. Low acetylation degree constituted for higher positive charges per molecule of chitosan, which is important for stability of the polyplex formed when the polymer complexes with a gene or a drug [5].

Modification of OH and NH_2 Functional Groups on CS

Chemical modification of CS can be used to attain derivatives with preferred properties, and these modifications have proven to be safe for gene therapy usage. For instance, chitosan-DNA complexes integrated into THP-1 leukemia cells did not stimulate the release of pro-inflammatory cytokines [11]. Chitosan has two types of reactive groups that can be modified: 1) free amino groups on deacetylated units and 2) hydroxyl groups on acetylated or deacetylated units such as the -OH groups on C-3 and C-6 carbons (Figure 1) [1, 2, 12]. Chitosan's cationic property from the amino groups is a main advantage, especially in dealing with negative phospholipids or nucleic acids. These NH_2 groups can be protonated to NH_{3+} in an acidic condition and as a result, these protonated NH_{3+} groups are able to form stable polymer complexs with anionic counterparts such as nucleic acids [13, 14]. Along with these primary amino functional groups, chitosan's OH groups offer many biological applications via their chemical alteration [1, 15, 16]. The N-alkylation reaction occurs on the C2-NH_2 group because this group is a stronger nucleophile than the OH group, and thus O-alkylation, which could occur

at C3-OH or C6-OH, is less likely to occur. N-alkylated chitosan derivatives have shown antibacterial and coagulation properties and have thus been used to prepare medical supplies such as medical gauze. This type of derivative has also served as a surfactant for water purification engineering as well [5]. Although O-alkylation is less likely to occur, the hydroxyl group can be carboxylated by reacting with glyoxylic acid or chloroalkanoic acid. The carboxylation reaction, which increases the solubility of chitosan in water, occurs at the C6-OH group, allowing it to be dissolve in a pH greater than 7. Whereas carboxylation at the C3-OH group is more difficult due to steric hinderance [5, 14].

Applications of Chitosan

Chitosan has been incorporated in the production of bandages due to their anti-bacterial properties, as well as enzyme immobilization on solid surfaces and surface coating of enzymes, which allows for reusability, thus promoting cost-effectiveness [17, 18]. Other applications of chitosan include food preservation, as shown in a study that it was used for the promotion of crop growth such as radishes. It has also been used to minimize water loss in field crops, enhance enzyme activity in peanuts, and to prolong the storage of fruits by stimulating cellular defense compounds [1]. However, there have been rare incidences by which chitosan produced undesirable characteristics. For example, a study that was investigating the effects of chitosan-based gene therapy on amniotic fluid (*in vitro*) reported that while CS protected the plasmid DNA from degradation it also formed aggregates in the amniotic fluid, thus meaning that CS can pose a threat during fetal development [19].

Gene Delivery Using Chitosan and Its Derivatives

Gene therapy is the delivery of genes into specific cells for therapeutic benefit and can offer possible lasting treatment for cancer. A challenge for gene therapy is to design a carrier that is effective in protecting the gene of interest from nucleases as well as efficient transfer to targeted cells. Viruses are the most common vectors for gene therapy, although gene therapy using viral vectors is associated with immunogenicity as well as rare cases of disease [20]. However, Due to its versatility, biodegradability, and safety, chitosan has recently been gaining interest as a potential non-viral vector in the field of gene therapy. Additionally, stability in a biological environment is a quality all ideal DNA/RNA delivery vectors should have. Chitosan protect genetic material from being degraded by nucleases through CS-DNA polyplex formation. In formation of the CS-DNA polyplex, the positively charged amine groups on chitosan interact with the negatively charged groups on DNA/RNA in order to form a stable CS-DNA/RNA polymer complex [2, 21, 22].

In 1995, the first ever non-viral gene delivery experiment using chitosan was performed using TMC [23]. TMC is an ammonium quaternized chitosan derivative, first synthesized in 1986, and has been proven to have mucoadhesive properties. This derivative is synthesized by addition of a trimethyl group to chitosan's NH_2 group. The process of synthesizing TMC involves trimethylation of the primary amines of chitosan in alkaline solution. Kean et al. measured the transfection efficiency of pGL3 luciferase plasmid-DNA using TMC and TMOs (trimethylated oligosaccharides) at differing degrees of trimethylation (DTM) in MCF-7 breast cancer and COS-7 monkey kidney fibroblast-like cell lines [24]. The authors found that the transfection was cell dependent. Moreover, the results showed higher transfection efficiencies with TMOs in both cell lines than most of the TMC derivatives. However, in the MCF-7 cell

line, 93% TMC which was the TMC derivative with the greatest degree of trimethylation (93% DTM) had the greatest transfection efficiency [24].

Nevertheless, TMC showed higher cytotoxicity compared to the unmodified chitosan. Usually, caveats to TMC are related to its molecular weight (MW); a MW of 400 kDa displayed high cytotoxicity, while 5 and 25 kDa TMC exhibited little to no toxicity.[1],[45, 46] Research has shown that the cytotoxicity is due to the positive charge of TMC which might be interacting with the negatively charge cell membrane which could lead to cell membrane damage [1, 24, 25].

There are very few reports on the cellular uptake mechanisms of CS-DNA/RNA polyplexes. To understand the cellular uptake of CS-pDNA polyplex, Hashimoto et al. synthesized mannosylated chitosan (Man-C) with 5% and 21% degree of substitutions for use as gene carriers into mouse peritoneal macrophages and COS7 cells, in order to understand the cellular uptake of CS-pDNA polyplex [26]. The authors discovered that transfection with both 5% and 21% Man-C were better than unmodified chitosan in the macrophages, but in the COS7 cells, the carrier efficiency was greater with the 5% Man-C than 21% Man-C derivative [22, 26]. The Man-C derivative with 21% substitution might have been causing extracellular damage due to 21% Man-C being more hydrophilic than the unmodified and 5% chitosans.

Additionally, a factor that affects gene therapy is the CS-nucleic acid N+/P- ratio. N+/P- ratio is the ratio of positively charged amino group on chitosan to negatively charged phosphate groups on nucleic acids [2]. N+/P- ratio influences the stability of the polyplex formation, the transfection efficiency, and polymer-cell interactions [2]. A study comparing transfection efficiencies of 6-amino-6-deoxy-chitosan (6ACT) at N+/P- ratio of 2.5 and chitosan-DNA at N+/P- ratio of 5 and found that the 6ACT derivative was the better gene carrier. 6ACT-DNA and CS-DNA complexes were also compared to polyethyleneimine (PEI) which had a higher N+/P-

ratio, and the results showed that PEI had better transfection efficiencies than both complexes [27]. High N+/P- ratio can lead to strongly formed polyplex through electrostatic interactions. However, if the bond between the DNA and the polymer is too strong, this can prevent the nucleic acid from being released once it arrives at its site of action, thus hindering the release of the gene from the polymer complex [2, 28]. Additionally, an extremely low N+/P- ratio can cause formation of aggregates which affect cell internalization, thus resulting in poor transfection [22].

Another category of chitosan derivative used for gene delivery is thiolated chitosan. Thiolated chitosan derivatives were discovered in the early 2000s, especially novel derivative in which chitosan is conjugated to 4-thiobutylamidine (TBA), chitosan-TBA. Due to their cell permeability and mucoadhesive properties, these types of derivatives show enhanced polymer-DNA complex stability, and excellent gene delivery both *in vivo* and *in vitro* [29]. During reducing conditions, breakage of the disulfide bonds lead to dissociation of the DNA being delivered, whereas during oxidation, the disulfide bond formation in the thiolated chitosan is favored, which leads to tight binding of DNA and a stable solid polyplex.

Finally, gene delivery must be safe for use in gene therapy. However there have been rare incidences by which chitosan produced undesirable characteristics. A study that was investigating the effects of chitosan-based gene therapy on amniotic fluid (*in vitro*) reported that while CS protected the plasmid DNA from degradation it also formed aggregates in the amniotic fluid [19]. CS can be chemically modified to attain derivatives with preferred properties, and these modifications have been proven to be safe for gene therapy usage. The key is producing the optimal modification. A study reported that chitosan-DNA complexes integrated into THP-1

leukemia cells did not stimulate the release of pro-inflammatory cytokines. Nevertheless, TMC showed higher cytotoxicity compared to the unmodified chitosan.

Usually, caveats to TMC are related to its molecular weight (MW); a MW of 400 kDa displayed high cytotoxicity, while 5 and 25 kDa TMC exhibited little to no toxicity [1, 30, 31]. Research has shown that the cytotoxicity is due to the positive charge of TMC which might be interacting with the negatively charged cell membrane, which could lead to cell membrane damage. Thus, TMC with higher MW would have more positive charge than TMC with lower MW and could cause more damage to the cell membrane [1, 24, 25]. *In vitro* hemolysis experiments were performed using chitosan-TBA to evaluate its safety on red blood cells. The derivative displayed a low hemolytic effect on the red blood cells which might have been attributed to the change of the primary amine moieties into secondary amine groups after thiol modification with TBA [1, 29]. Although some toxicity is observed with some chitosan derivatives, some modification, or the degree of modification appear to ameliorate the toxic effects and can be used to make safe and effective gene delivery tools.

Other Applications of Chitosan

Drug Delivery Using CS and Its Derivatives. Although chitosan has anticoagulant properties and was involved in wound healing, it wasn't until the late 1990s that the chitosan was used as a drug delivery carrier. This is mainly due to chitosan's solubility issues, which prevents it from delivering the drug to biological systems [32]. Moreover, due to unmodified chitosan's pK_a of 6.5 it is not a stable drug carrier. Drug delivery agents must be stable at physiological pH. In order to overcome this problem, there have been many attempts to modify chitosan by derivatizing the OH or NH_2 groups on the polymer. Common types of modifications to make

chitosan more biocompatible include quaternization, sulfonation, carboxymethylation, N- and O-hydroxyalkylation [13]. Examples of hydrophilic modifications to chitosan listed in this review include quaternization using ammonium groups and N-modification with the succinyl group. One study demonstrated the usage of N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC) as a drug delivery agent for ribavirin, and the results showed an initial burst release of the drug at increasing degree of substitution (Table 1) [3, 5, 15, 16]. As modified CS derivatives are internalized via endocytosis, they are able to deliver therapeutic drugs into cells without endangering these biologically active cargos [17, 32-34].

Table 1. Examples of chitosan, its derivatives, and their applications.

Chitosan Derivative	**Application**	**Target /Cargo**	**Discovery Timeline**	**References**
Low molecular weight chitosan (LMWC)	Antibacterial/antioxidant	Superoxides, DPPH (2,2-diphenyl-1-picrylhydrazyl), and H_2O_2 radicals	1993	[1]
Chitosan; Chitosan-soy; Chitosan-soy-tetra ethyl ortho silicate (Cht/soy/TEOS)	Wound healing	Stimulate macrophage and attract neutrophil to site of wound	1994	[1] [30] [32]
N-succinyl-chitosan (Suc-Chi)	Drug delivery	Treatment for arthritis	1994	[1] [46]
Trimethyl chitosan (TMC)	Gene delivery	pDNA	1995	[45] [46]

N-benzyl sulfonate chitosan	Adsorbent	Removal of heavy metals and pollutants	1996	[1] [34]

Table 1 continued. Examples of chitosan, its derivatives, and their applications.

Chitosan Derivative	**Application**	**Target /Cargo**	**Discovery Timeline**	**References**
Collagen chitosan-based matrices	Tissue engineering	Attachment to bovine adrenal chromaffin cells	1998	[26] [27] [28]
Dextran sulfate (DS)-chitosan	Tissue regeneration, cell proliferation	Human smooth muscle cell and endothelial cells	2000	[23] [26] [29]
PEG-Chitosan	Gene delivery	Survivin-siRNA	2001	[12]
Glycol-Chitosan (Glycol-CS)	Gene delivery	Multi drug resistant 1 (MDR1)-SiRNA	2001	[12]
Chitosan-coated polylactic acid	Gene delivery	Sequestosome I siRNA	2001	[81]
Palmitoyl chitosan	Drug delivery	Subdermal and oral drug delivery	2005	[18] [39] [41]
Mannosylated chitosan (Man-C)	Gene delivery	pDNA	2006	[49] [50]
Chitosan-*N*-acetylcysteine (Ch-NAC)	Drug and gene delivery	FD4 parcellular marker, acyclovir	2007-2008	[49]
PEGylated-TMC (PTMC)	Gene delivery	pDNA	2008	[45]
Chitosan-betainates (CsB)	Gene delievery	DNA	2009	[82]

N, O-carboxymethyl chitosan (N, O-CMC)	Drug delivery	Chemotherapeutic drug curcumin	2010	[1] [40] [42]
Trimethyl-chitosan-cysteine (TMC-Cys)	Gene delivery	pEGFP	2010	[49]

Table 1 continued. Examples of chitosan, its derivatives, and their applications.

Chitosan Derivative	**Application**	**Target /Cargo**	**Discovery Timeline**	**References**
Chitosan-hydroxybenxotriazole (Chitosan-HOBT)	Gene delivery	siRNA delivery and gene silencing	2010	[35]
Chitosan-thioglycolic acid (Ch-TGA)	Gene delivery	pDNA	2011	[49]
N-sulfurfuryl chitosan	Anticoagulant, adsorbent	Non-thrombogenic properties	2012	[1] [39]
N, *O*-sulfonated chitosan	Anticoagulant	Strong anticoagulant	2012	[1] [39]
N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HACC)	Drug delivery/gene delivery	Anti-viral drug ribavirin	2014	[3] [5] [16] [17][35]
2-acrylamido-2-methylpropane sulphonic acid modified LMWC	Gene delivery	pDNA	2015	[83]
Chitosan-lauric acid	Antibacterial drug delivery	Antibacterial drug ciprofloxacin against S. aureus and E. coli	2016	[14] [51]

Double *N*-quaternized chitosan (DQCS)	Antibacterial/antioxidant	Scavenging ability against superoxides, DPPH, and hydroxyl radicals	2018	[4]

Table 1 continued. Examples of chitosan, its derivatives, and their applications.

Chitosan Derivative	**Application**	**Target /Cargo**	**Discovery Timeline**	**References**
Single *N*-quaternized chitosan (QCS)	Antibacterial/antioxidant	Scavenging ability against superoxides, DPPH, and hydroxyl radicals	2018	[4]

Modified chitosans such as TMC show promise results in the intranasal administration of certain drugs and proteins, due to their cationic propertiesthat allow for complexation with the drug of interest. Intranasal admistration allows for direct access to the brain, which has many advantages such as rapid onset of action and fewer adverse effects [35]. Turabee et al. developed a hydrogel made from pluronic F127 (PF127) in order to treat the malignant glioblastoma cell line U87MG, using a murine model. They discovered that after addition of TMC to the PF127 hydrogel, delivery and release of the anticancer drug docetaxel (DTX) was much better compared DTX alone or DTX encapsulated with PF127, *in vitro*. The authors were able to show that PF127-TMC hydrogel was capable of tumor suppression, *in vivo*, with the delivery of DTX [13, 36].

Moreover, in 1994, a patent was requested to use N-succinyl-chitosan or Suc-Chi (originally developed in the late 1990s for wound dressing) as a treatment for arthritis. Suc-Chi

exhibits low toxicity, is biocompatible, and can be retained for a long period of time in the body. It has been used as a drug carrier and when conjugated with the chemotherapeutic drug mitomycin C, it presented antitumor activity against many tumors (Table 1) [1]. Furthermore, chitosan loaded with dopamine was proven to minimize cytotoxicity and used to facilitate transport the dopamine across the blood brain barrier for Parkinson's disease [22, 37, 38].

Research has shown that delivery of cargo such as hydrophobic drugs are problematic due to their hydrophobic nature. Due to chitosan's insolubility in hydrophobic solvents, its ability to transport hydrophobic drugs is restricted. In order to resolve this limitation, researchers have modified chitosan with hydrophobic groups such as pyridine, among many other groups, to improve the 'encapsulation efficiency' of the hydrophobic cargo [13, 39]. Examples of hydrophobic modifications include palmitoyl units and the carboxymethyl group. When chitosan was substituted with palmitoyl units (degree of substitution 40-50%), its best drug release characteristics were displayed, which proved that palmitoyl chitosan could be used in subdermal and oral drug delivery applications (Table 1) [40-42]. The chitosan derivative, N, O-carboxymethyl chitosan (N,O-CMC) nanoparticles was used to carry hydrophobic chemotherapeutic drugs such curcumin (Table 1) [1, 43, 44]. Nevertheless, such hydrophobic modification of chitosan polymer tend to have low reproducibility, proving that chitosan is not as successful in delivering hydrophobic drugs [45]. Despite this, chitosan still serves as one of the most effective drug delivery agents.

Chitosan-Coated Materials and Complexes. Chitosan and its derivatives have also been used as encapsulating agents for proteins and glycans such as bovine serum albumin, hemoglobin, and dextran due to their high affinity for the cell membrane [41]. Moreover, when chitosan was used to coat PLGA (poly(lactic-co-glycolic acid)) microparticles containing the

tetanus toxoid, CS improved the stability of the drug and PLGA by preventing degradation by lysozyme. Furthermore, this CS-PLGA coating enhances the nasal transport of the drug due to chitosan's mucosal adhesion or mucoadhesive properties. Another study demonstrated that conjugation of chitosan to poly(acrylic acid) (PAA), an anionic polyelectrolyte, was useful in the delivery of gastric antibiotic drugs due to the chitosan-poly (acrylic acid (Ch-PAA) derivative being stable under acidic conditions [41].

It is widely accepted that modified chitosans have chemical properties superior to unmodified chitosan. However, unmodified chitosan has various favorable characteristics that make them effective gene delivery agents such as biocompatibility, low cytotoxicity, biodegradability, and stability when forming complexes [2]. Unmodified chitosan that was used for the removal of organic matter such as algae, proved to be more efficient than t coagulating inorganic compounds such as ($Al_2(SO_4)_3$, $KAl(SO_4)_2$, $Ca(OH)_2$). Chitosan was albe to remove about 95% of algae from algae-containing waters [1]. Regardless of these characteristics, unmodified chitosan has certain constraints, a main one being solubility. Chitosan is insoluble in physiological pH, thus, it is necessary for modifications that make it easier to incorporate the CS-coated materials and complexes into cells.

Tissue Engineering Applications Using CS Derivatives. Chitosan's ability to be refined into porous material is an excellent characteristic that is useful for tissue engineering applications because it can be made into scaffold grafts for tissue engineering [30, 31, 46]. These scaffold grafts stimulates the regeneration of certain types of tissues such as bone tissue, among many others. Tissue engineering using chitosan-based matrices in transplantation procedures of bovine adrenal chromaffin cells was discovered in 1998 (Table 1) [47-49]. Since then chitosan and its derivatives have been used in many tissue regeneration due to their low to

non-existent tissue toxicity, biodegradability, as well as peritoneal adhesion prevention [34]. Among such experiments is one study that reported that modification of chitosan with multiple proteins such as collagen, gelatin, and albumin enhanced its biocompatibility. The results demonstrated that a matrix made up of collagen modified chitosan attached more readily to the cells than the other proteins (Table 1) [47]. Additionally, chitosan's hydrophilic surface promotes cell proliferation and adhesion much better than several synthetic polymers. Chupa et. al reported the use of both heparin-CS and dextran sulfate (DS)-chitosan complexes to stimulate cell proliferation and tissue regeneration of human endothelial cells and smooth muscle cells, *in vivo* [30, 47, 50]. These type of experiments thus prove the potential of CS to be used in scaffolds that enhance and promote cell and eventually tissue regeneration.

Wound Healing and Anticoagulant Properties of CS and Its Derivatives. Wound healing using chitosan was first discovered in late 1980s-early 1990s [13, 51]. Studies have shown that chitosan improves skin wound healing and facilitates re-epithelization. There have been numerous articles investigating chitosan and its derivatives' influence on peritoneal adhesion formation. These studies demonstrated that as the degree of acetylation on chitosan biofilms increased, cell proliferation and adhesion decreased [52]. Chitosan also reduces inflammation at the wound site, and promotes dermal regeneration. Additionally, the biopolymer has the capability to accelerate wound healing because it stimulates macrophages and attracts neutrophils [1, 53, 54].

Research has shown that derivatives of CS substituted with sulfonate groups have high anticoagulant activity due to their similarity with heparin, a common anticoagulant medication. These sulfonated derivatives have no side-effects and are less expensive compared to heparin and have in fact have replaced heparin in many pharmaceutical and clinical settings [1].

Examples of such derivatives are N-Sulfofurfuryl chitosan, O-sulfonated chitosan, and N,O-sulfonated chitosan. Amphoteric water-soluble chitosan derivatives such as N-sulfofurfuryl chitosan and N-benzyl sulfonate chitosan, possess non-thrombogenic properties and can be used for blood-coagulating applications. They have also been used in wastewater applications.[1] N,O-sulfonated chitosan also showed strong anticoagulant activities by inhibiting thrombin activity, as did O-sulfonated chitosan, which was reported to have excellent inflammation inhibition activities (Table 1) [1]. These sulfonated chitosan derivatives are also used to remove organic pollutants and heavy metals, such as Cd^{2+} , Zn^{2+} , Ni^{2+} , Pb^{2+} , Cu^{2+} , Fe^{3+} , and Cr^{3+} from industrial sewages and proved to remove these pollutants and heavy metals much better than unmodified chitosan could (Table 1) [1, 55].

Antioxidant and Antimicrobial Properties of CS and CS Derivatives. In 1992, chitosan was discovered to have antioxidant and antimicrobial properties in Japan by researchers who were trying to investigate its effect on macrophage activation and antimicrobial activity [56]. Derivatives of chitosan, especially low molecular weight chitosan (LMWC), have displayed strong scavenging activity towards superoxides such as H_2O_2 and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals [1, 56]. A study measuring the antioxidant activity of unmodified chitosan and two derivatives, double N-quarternized chitosan (DQCS) and single N-quarternized chitosan (QCS), demonstrated that DQCS had the best scavenging ability compared to both unmodified CS and QCS in the presence of DPPH, hydroxyl radicals and superoxide radicals [4].

Another way chitosan demonstrates its antioxidant properties is that it protects from hypertrophy of adrenal glands (induced by LPS), prevents from shrinkage of the thymus, changes of hormones, glycolysis and glycogenolysis activation, and lipid peroxidation in liver

cells [1]. Sinha et al. investigated the antimicrobial activities of chitosan crosslinked with fatty acids (lauric, stearic, and capric saturated fatty acids) as well as drug delivery of ciporoflaxacin against *S. aureus* and *E. coli*. They discovered that the chitosan-lauric acid derivative and the chitosan-lauric acid-ciporoflaxacin complex had inhibitory effects against the two microbes [57]. Additionally, when unmodified chitosan was complexed with lipopolysaccharide (LPS), it inhibited cytokine production in macrophages (RAW 264.7) [1, 13, 57]. The CS-LPS complex also led to more enhanced phagocytic activity of the macrophages than when the macrophages were stimulated with LPS alone.

Limitations of Chitosan as a Non-Viral Vector and the Future of Chitosan

The derivatives aformentioned have demonstrated the various applications and versatility of chitosan in many different fields. The potential for chitosan to serve as as a gene delivery agent is based on its variability in the N+/P- ratio of the polyplex. However, modifications on chitosan such as addition of thiol groups, quarternization on the amino groups, addition of hydrophobic groups, addition of sulfono group, etc. Limitations of chitosan that impact drug and gene delivery are due to pK_a degree of acetylation and MW. High MW chitosan can lead to unstable CS-DNA polyplex formation as well as problems with cellular uptake and release of the nucleic acid into the cytoplasm [1, 21, 58, 59]. Although, DA and MW can become advantageous in chitosan becoming a vector for drug and gene delivery, e.g. through modification, other limitations include an increase in cytotoxicity as well as a reduction in gene binding capacity when the biopolymer has been modified with a high degree of substitution [59].

Many of these derivatives have good characteristics such as high gene delivery capacity, protection of cargo from lysozyme degradation, low cytotoxicity and solubility. Nevertheless,

cytotoxicity could be caused by steric effects in *N*-substituted (quaternization of the amino groups on chitosan) derivatives such as TMC which can be seen in many of the chitosan derivatives [24, 25]. This could serve as a precaution to researchers as they investigate certain degree of substitutions that would make chitosan a more efficient gene delivery vector. In order for these derivatives to be efficient gene carriers, they must demonstrate excellence in these characteristics. With this information, research should be geared towards using chitosan and its derivatives in treating of genetic and autoimmune disorders, as more awareness about these diseases is being brought to light.

Moreover, factors such as degree of deacetylation, ionic strength of the solution, and the molecular weight influence the solubility of chitosan and its ability to be an efficient delivery agent [16, 37]. Another limitation of chitosan include the inability to release therapeutic cargo intracellularly after endocytosis when complexed with DNA, thus leading to a less efficient delivery of DNA, *in vitro* [28, 60, 61]. Endo-lysosomal release of these genetic materials into the cell is just as important as internalization in the cell [6, 60, 62, 63]. As these CS nanoparticles are internalized via endocytosis, they can deliver drugs into the cytoplasm without endangering these biologically active cargos.

Although chitosan has been used in various applications, there are very few gene therapy-based studies using chitosan modified with ammonium or phosphonium salts such as triethylphosphonium butanamide (TEPB) and triethylammonium butanamide (TEAB). Phosphonium-containing polymers were reported to have fewer toxic effects on HeLa cells following transfection with gWiz-Luc plasmid. The study reported cell viability higher than 70% with phosphonium derivatives and less than 60% viability with their ammonium analogues [64]. This is due to the charge distribution on adjacent carbon groups attached to the phosphonium

cation. Due to nitrogen's high electronegativity compared to carbon, the positive charge of carbon is distributed on adjacent carbons, which results in a negative charge on the nitrogen atom. Some of these positive charges on the adjacent carbons affect the cell membrane by interacting with the negative components of the cell membrane. This interaction can then endanger the cell and cause toxicity. Whereas, phosphorus has the opposite charge distribution due to it being more electropositive than carbon, thus resulting it a negative charge on the adjacent carbons and a overall positive charge on the phosphorus. The sizes of the cationic polymers as well as charge densities influence the binding affinity of this biopolymers. Meaning that such phosphonium polyelectrolytes are more cationic than the ammonium-containing derivatives thus allowing for more effective binding of DNA [64]. Therefore, the aim of the present work is to determine if these chitosan derivatives can be used as efficient gene delivery vectors [8, 59].

HYPOTHESIS

In this project I tested the transfection efficiency of two types of chitosan derivatives, chitosan containing triethylphosphonium butanamide (TEPB) groups and chitosan containing triethylammonium butanamide (TEAB) groups in the HeLa cervical cancer cells. As mentioned above, phosphonium polyelectrolytes have fewer cytotoxic effects on HeLa cells than ammonium analogues. In addition, PEGylation of biopolymers has been reported to inhibit apoptotic effects because the PEG units reduce cytochrome c, which promotes apoptosis, and also improve mitochondrial function [65]. Foremost, I hypothesized that the two TEPB derived CS polymers would have limited to no cellular toxicity compared to the three CS derivatives containing TEAB. This part of the project was tested using a XTT assay cell viability kit.

Following, an apoptosis kit was used to further quantify the effects of the derivatives. I hypothesized that the TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) derivative would not induce apoptosis, in comparison to the other four derivatives used (TEAB1-CS (24% TEAB), TEAB2-CS (40.6% TEAB), TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG), and TEPB-CS (43% TEPB)). Third, I hypothesized that both TEPB-CS (43% TEPB) and TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) derivatives would be the most efficient gene delivery vehicles due to the high percentage of triethylphosphonium butanamide, which has been stated to improve transfection efficiency. Moreover, I speculated that TEAB2-CS (40.6% TEAB) would be the least effective vehicle for gene delivery due to TEAB2-CS having a high percentage of TEAB (40.6%). Conversely, here I present that TEAB1-CS (24% TEAB), due to its low degree of quaternization (DQ), was the most efficient gene delivery vector among all tested CS derivatives.

MATERIALS AND METHODS

Preparation of Stock Solutions of Chitosan (CS) Derivatives

Triethylphosphonium butanamide -chitosan (TEPB-CS), Triethylammonium butanamide -chitosan (TEAB1-CS and TEAB2-CS), Triethylammonium butanamide methoxy-poly (ethylene glycol)-chitosan (TEAB-mPEG-CS), and Triethylphosphonium butanamide-mPEG-CS derivatives (TEPB-mPEG-CS) were prepared with sterile 1X PBS (pH, 7.4) to make stock solutions. TEAB1-CS (24% is the degree of quaternization with TEAB to CS) was dissolved 1X PBS for final concentration of 2.78 mg/mL. TEAB2-CS (40.6% is the degree of quaternization with TEAB to CS) was dissolved in 1X PBS for a total concentration of 10 mg/mL. TEPB-CS (43% is the degree of quaternization with TEPB to CS) was dissolved in 1X PBS for a total concentration of 5 mg/mL. TEAB-mPEG-CS (40.6% is the degree of quaternization with TEAB to CS; 3.1% of mPEG added to TEAB) was dissolved in 1X PBS for a total concentration of 3.33 mg/mL. TEPB-mPEG-CS (43% is the degree of quaternization with TEPB to CS; 2.6% of mPEG added to TEPB) was dissolved in 1X PBS for a concentration of 5 mg/mL.

Cell Culture

HeLa S3 cervical cancer cells plated on a 75 cm^2 flask were sustained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 12% fetal bovine serum (FBS) and antibiotics streptomycin and penicillin at 37^oC in a humidified incubator with 5% CO_2. After reaching confluency, the flask was then washed twice with 10 mL 1X PBS, followed by the addition of 3 mL of Trypsin-EDTA mixture to assist in the detachment of the cells from the flask. The cell culture was further incubated at 37^oC for 20-25 minutes for detachment from the

plate, followed by neutralization and separation using 10 mL of DMEM. The cells were counted using a hemocytometer to determine the appropriate amount needed for each assay listed below.

Cell Viability

Two sets of cell viability experiments were performed using an XTT assay from Biotium. The first set was to compare the TEABs-CS, while the second set was to compare the TEPBs-CS. HeLa cervical cancer cells served as recipients to test cell viability via the XTT assay. These experiments were repeated twice to obtain the same results and each experiment was performed in triplicate set. The HeLa cells were seeded on a 96 well plate with each well containing 10,000 cells. The plate was incubated at 37$_{o}$C with 5% CO_2 for 24 hours in order to adhere to the wells. The following 24 hours consisted of treatment with the chemicals. Treatments for TEAB1-CS (24% TEAB), TEAB2-CS (40.6% TEAB), TEPB-CS (43% TEPB), TEPB-mPEG-CS (43% TEPB; 2.6% mPEG), and TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) were performed with serial dilutions from 0-100 μg/mL. After treatment, the cells were incubated for another 24 hours in the same conditions listed earlier and measured using XTT. XTT Activation Reagent, known as N-methyl dibenzopyrazine methyl sulfate or PMS, was mixed with XTT solution for a ratio of 1:200, and 25 μL of this mixture was added to each well. The EL808 96-well spectrophotometer from Biotek was used to perform this assay, at an absorbance value set at A450-A630nm, using the Gen5 3.03 software program to plot the cell viability at hour 7 (Fig. 2B&C). IC_{50} curves below (Fig. 2D) were obtained using the IC_{50} calculator from AAT Bioquest. IC_{50} calculation was made with curves fitted according to following equation: $Y= [\text{Min. value} + (\text{Max value-Min. value})]/[(1 + (x/IC_{50})^{\text{Hill coefficient}}]$. The minimum value was set at zero. Cell viability was calculated following background substraction

from hour zero on standard curve (Fig. 2E&F). The O.D. for non-treated control served as standards for cells trea The following equation was used to calculate cell viability for the cells treated with the derivatives: [$O.D_{NTC} - O.D_{CS}/ O.D_{NTC}$] x100%.

Apoptosis

To examine the potential apoptotic effects of TEAB- and TEPB- containing chitosan derivatives on the HeLa cells, an apoptosis assay was performed [66]. For 24 hours in the conditions stated above, 50,000 HeLa cells were seeded on a 24-well plate. Treatments for TEABs-CS and TEPBs-CS were performed on the same 24-well plates, with serial dilutions ranging from 0-100 μg/mL. The treatments were performed in quintuplicate sets, and cells were grown using DMEM media supplemented with 12% fetal bovine serum (FBS) and antibiotics streptomycin and penicillin. The treated and non-treated cells were then measured using Annexin V-APC and propidium iodide (PI). On the day of quantitation, the cells were washed twice with 1 mL of 1X PBS, trypsinized with 0.25 mL of trypsin, and then neutralized with 0.30 mL of DMEM supplemented with FBS and antibiotics streptomycin and penicillin. The mixture was centrifugated at 400 x G force for 10 minutes, followed by the addition of 5 μL of Annexin V-APC and 5 μL of PI. The mixture was incubated for fifteen minutes and visualized using an *Attune NxT* flow cytometer, and each cell was quantitated using the BL3 channel for PI and and RL1 channel for Annexin-V APC.

Plasmid DNA (pDNA) Preparation

TPST1-EGFP plasmid was purchased from Addgene (Cat # 66617). The GFP-fused TPST1 marks the trans-Golgi network membrane [67]. TPST1-EGFP plasmid carrying bacterial

strain was grown on an LB agar plate containing Kanamycin (50 μg/mL) and a single colony transferred to TB liquid media containing 50 μg/mL Kanamycin. The plasmid DNA was purified using a QIAprep® Spin mini-prep kit purchased from Qiagen. The DNA was measured using a Qubit™ dsDNA BR assay kit from Invitrogen by Thermo Fisher Scientific for a total concentration of 186 ng/μL. Agarose gel electrophoresis using 1% agarose gel containing 0.5 μg/mL of ethidium bromide (EtBr) was used to visualize the size of the pDNA. The gel was visualized using the Azure c300 chemiluminescent western blot imaging system from Azure biosystems (Figure 8C).

DNA Binding Assay to Determine N+/P- and P+/P- Ratio

Agarose gel electrophoresis using 1% agaorose gel containing 0.5 μg/mL of ethidium bromide (EtBr) was used to complex TEPB-CS (43% TEPB) with the TPST1-EGFP plasmid at N+/P- ratios of 8:1, 4:1, 2:1, 1:1, 1:2 (Figure 8A). This same method of gel electrophoresis was used to complex TEAB1-CS (24% TEAB) with the pDNA to determine the P+/P- ratio at which stable polymer-DNA complex formation at the same ratios mention above (Figure 8B).

Transfection Assay

Lipofectamine LTX from Thermo-Fisher Scientific and Takara Bio's Xfect were served as positive controls for this assay. To quantitate the efficiency of the first twenty-four hours of gene delivery, 100,000 HeLa cells were seeded using DMEM media unto a 24-well plate, in order to obtain 70-90% confluency by the time of transfection, in the conditions stated above. The following twenty-four hours the pDNA was incubated with the treatments (TEABs, TEPBs, Lipofectamine LTX, or Xfect) in quintuplicate. The wells containing Lipofectamine or Xfect

were incubated with 500 ng of pDNA at room temperature for 10 minutes and diluted to a volume of 1000 μL using DMEM supplemented with 12% FBS and antibiotics, streptomycin and penicillin. The wells treated with the CS derivatives contained a N+/P- or P+/P- (polymer: pDNA) ratio of 10:1 and diluted to a volume of 1 mL using DMEM supplemented with 12% FBS and antibiotics streptomycin and penicillin. All samples were incubated at 37^{o}C with 5% CO_2 for 24 hours. On the day of quantitation, each sample was washed twice with 0.5 mL of 1X PBS, trypsinized with 0.5 mL of trypsin-EDTA, and neutralized with 0.5 mL of DMEM media containing 12 % FBS and streptomycin and penicillin. The mixture was centrifuged at 1500 rpm for 10 minutes to obtain a pellet. The supernatant was discarded, and the pellet was resuspended in 1000 μL of 1X PBS. The *Attune NxT* flow cytometer was used to visualize the transfected cells using the BL1 channel, with excitation/emission wavelength set of 488/530.

Statistical Analysis

A one-way ANOVA and Dunnett's multiple comparison test were performed using Prism Graphpad version 8.1 to assess significance and p-values of the non-treated cells compared to cells treated with TEAB1-CS (24% TEAB), TEAB2-CS (40.6% TEAB), TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG), TEPB-CS (43% TEPB), and TEPB-mPEG-CS (43% TEPB; 2.6% mPEG), Xfect, and Lipofectamine. p-values and corresponding asterisks are based on $p>0.05$ to $p> 0.001$.

RESULTS

Chitosan Modified Derivative TEAB1-CS (24% TEAB) did Not Exhibit Any Cytotoxicity

Cytotoxicity of these chitosan derivatives can be a limiting factor of their ability to serve as gene delivery agents in biological systems *in vivo* or *in vitro*. A previous study reported on the cytotoxic effects of unmodified chitosan, upon treatment with concentrations ranging from 0-100 μg/mL. The results showed that the amount of viable BEL-7402 cells (a hepatoma cell line) decreased in a dose dependent manner [9]. Thus, this prompted me to assess cytotoxicity effects of the CS derivatives.

The cytotoxicity or reduction of cell viability induced by the derivatives was evaluated by an *in vitro* XTT assay 7 hours after the treatment of XTT and PMS. To generate a standard curve, known number of HeLa cells, ranging 0-10,000 cells without treating any CS derivatives, were used to obtain A450-A630nm values (Figure 2A). Hour zero refers to the timepoint before the addition of the activated XTT solution and hour seven is seven hours following addition of the activated XTT solution. The standard curve also exhibits a positive control bar of 10,000 cells using RIPA buffer (Fig. 2A, far right).

The TEAB2-CS (40.6% TEAB) derivative displayed cytotoxicity at concentrations greater than 10 μg/mL (Fig. 2B). Interestingly, the cytotoxicity experiment for TEAB1-CS (24% TEAB) showed a dose dependent increase in cell viability when treated with 0-100 μg/mL concentration of TEAB1-CS (24% TEAB), suggesting that TEAB1-CS (24% TEAB) positively affects cell viability in a mild way (Fig. 2B). In contrast, the TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) derivative reduced cell viability at 100 μg/mL (Fig. 2B). At a concentration range of 0.1-1 μg/mL TEAB2-CS (40.6% TEAB) saw a 32-64% increase in viability, but at

concentrations greater than 10 μg/mL the viability was reduced by 40% or greater (Fig. 2, B&E) Moreover, TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) had a 68% viability reduction at 100 μg/mL (Fig. 2, B&E) Whearas TEAB1-CS (24% TEAB) exhibited a 10-50% increase in viability in a dose dependent manner (Fig. 2, B&E). Treatment with TEPB-CS (43% TEPB) and TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) showed mild defects in viability from 10-100 μg/mL (Fig. 2C). The TEPB-CS (43% TEPB) derivative saw a 34-46% decrease in cell viability in the 10-100 μg/mL range (Fig. 2, C&F). Whearas, TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) only exhibited a 37% decrease in cell viability at the concentration of 100 μg/mL (Fig. 2, C&F). Together, all tested CS derivatives except TEAB1-CS (24% TEAB), regardless of the presence or absence of mPEG, induced a mild defect in cell viability with higher concentration of these derivatives.

To assess the effectiveness of CS derivatives on reducing HeLa cell viability, we determined the half maximal inhibitory concentration (IC_{50}). The IC_{50} value for TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) was 396 μg/mL, while the IC_{50} values TEAB2-CS (40.6% TEAB) was 10.17 μg/mL (Fig. 2D), suggesting that of these two derivatives, TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) is a safer intracellular cargo delivery agent than TEAB2-CS (40.6% TEAB). TEAB1-CS (24% TEAB)'s IC_{50} value was determined to be 0 because it seem to have promoted viability, confirming that this derivative had no negative effects on HeLa cell viability (Fig. 2D). IC_{50} values for TEPB-CS (43% TEPB) and TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) were not determined because they had minor to no effects on the cell's viability. In conclusion, both TEAB1-CS (24% TEAB) and TEPB-CS (43% TEPB) are the safest derivatives because they do not suppress viability at all dosages used in this study (0-100 μg/mL) and could potentially be used as gene delivery agents.

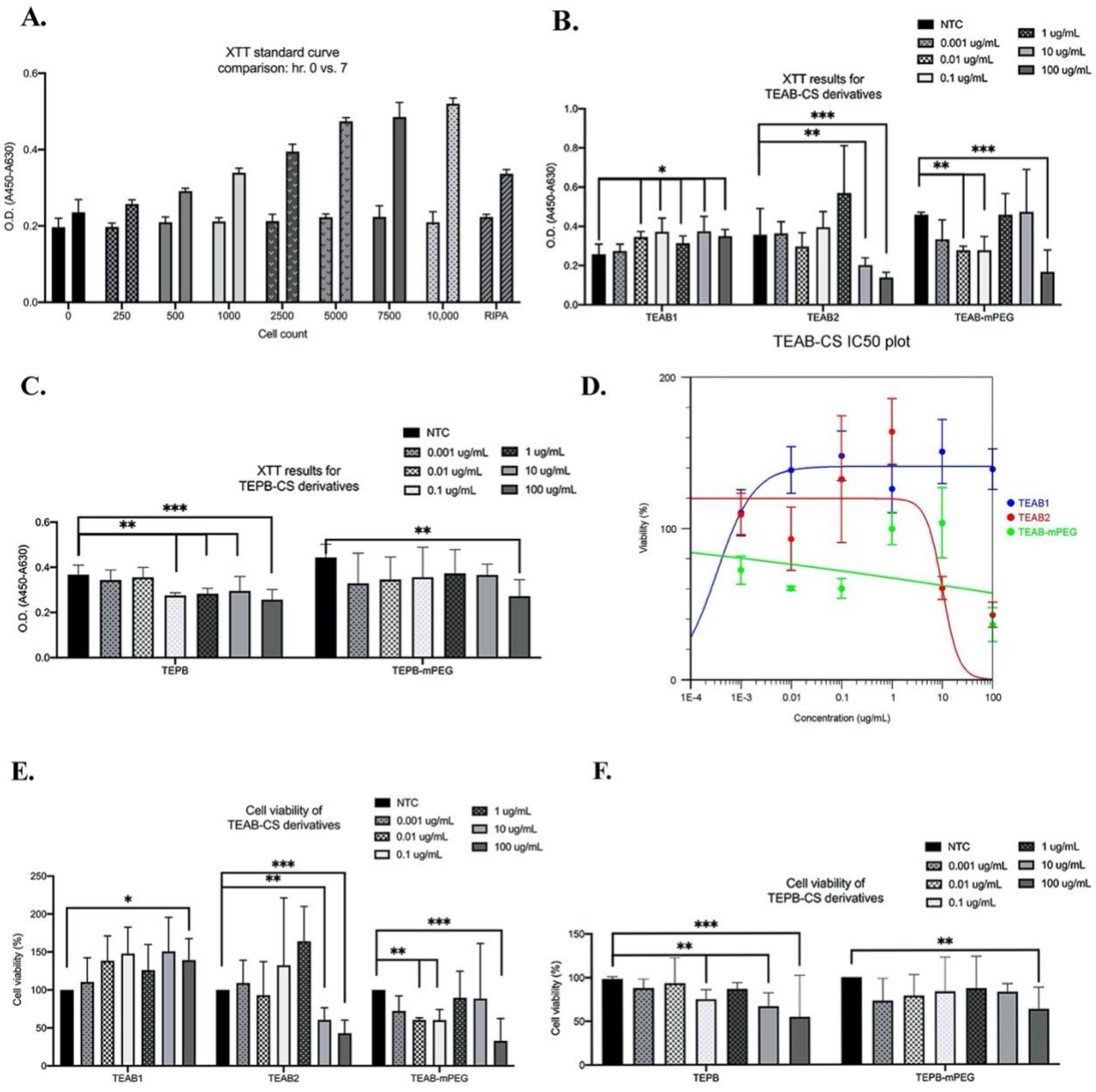

Figure 2: Chitosan derivative cytotoxicity measurements**.** Two separate experiments were performed in triplicate sets **A.** Standard curve with optical density (O.D.) of A450-A630 was measured at hour 0 (before the addition of XTT solution to the 96-well plate) and hour 7 after the addition of the XTT solution. The standard curve served as reference to determine viable cells and compare to the optical densities of the HeLa cells containing the CS derivatives; RIPA buffer was used as a positive control. **B.** TEABs-CS derivatives effect on cytotoxicity. TEAB2-CS (40.6% TEAB) showed defects in cell viability at concentrations higher than 10 µg/mL, while TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) showed defects in viability at the concentration of 100 µg/mL. Whereas TEAB1-CS (24% TEAB) showed an increase in viability. **C.** TEPBs-CS derivatives effect on cytotoxicity). The TEPB treatment shows no defect in cell viability. TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) treatment showed defects in viability at

100 μg/mL, same as TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG). Represented by * are p-values from 0.01 to 0.05, p-values from 0.001 to 0.01 are represented by **, and p-values from 0.0001 to 0.001 are represented by ***. **D.** IC_{50} values for TEAB derivatives: TEAB1-CS (0.0 μg/mL), TEAB2-CS (10.17μg/mL), and TEAB-mPEG-CS (396.25 μg/mL). **E.** Displays the collective cell viability results of all TEAB-containing chitosan derivatives. This graph correlates with Fig. 2B TEAB2-CS (40.6% TEAB) was more toxic to the HeLa cells above 10 μg/mL. TEAB1-CS (24% TEAB) was not defective at any concentration and TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) was only defective at 100 μg/mL. **F.** Displays the collective cell viability results for all TEPB-containing CS derivatives. TEPB-CS (43% TEPB) was defective at concentrations higher than 10 μg/mL, while TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) was defective mainly at 100 μg/mL.

Apoptosis Not Induced By TEAB1-CS

To further understand the cytotoxicity of the CS derivatives, an apoptosis assay was performed. A previous study reported that apoptotic effects of unmodified chitosan on three breast cancer cell lines (MDA-MB-231, MCF-7, and T47D) [66]. In light of the finding of viability defects following treatment with concentrations of TEAB2-CS (40.6% TEAB), TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG), TEPB-CS (43% TEPB), and TEPBmPEG-CS, ranging from 10-100 μg/mL, I quantitatively evaluated the effect of the CS derivatives on cell apoptosis using Annexin V-APC/PI staining. 50, 000 events were counted for each sample, and the gating for all samples were consistent. My results only focused on viable, early apoptotic cells, and late apoptotic cells because gating in these quadrants showed significant changes, whereas gating from the necrosis quadrant did not exhibit significant differences from 1-100 μg/mL.

No significant change can be seen in early and late apoptosis measurements after treatment with 1 μg/mL and 100 μg/mL TEAB1-CS (24% TEAB). Nevertheless, at concentration 10 μg/mL there is a decrease in viable cells and an increase in apoptotic cells (Fig. 3, A-E). In contrast, dose-dependent treatment with TEAB2-CS (40.6% TEAB) led to a significant decrease in non-apoptotic cells as the percent of early and late apoptotic cells increases significantly (Fig. 4, A-E).

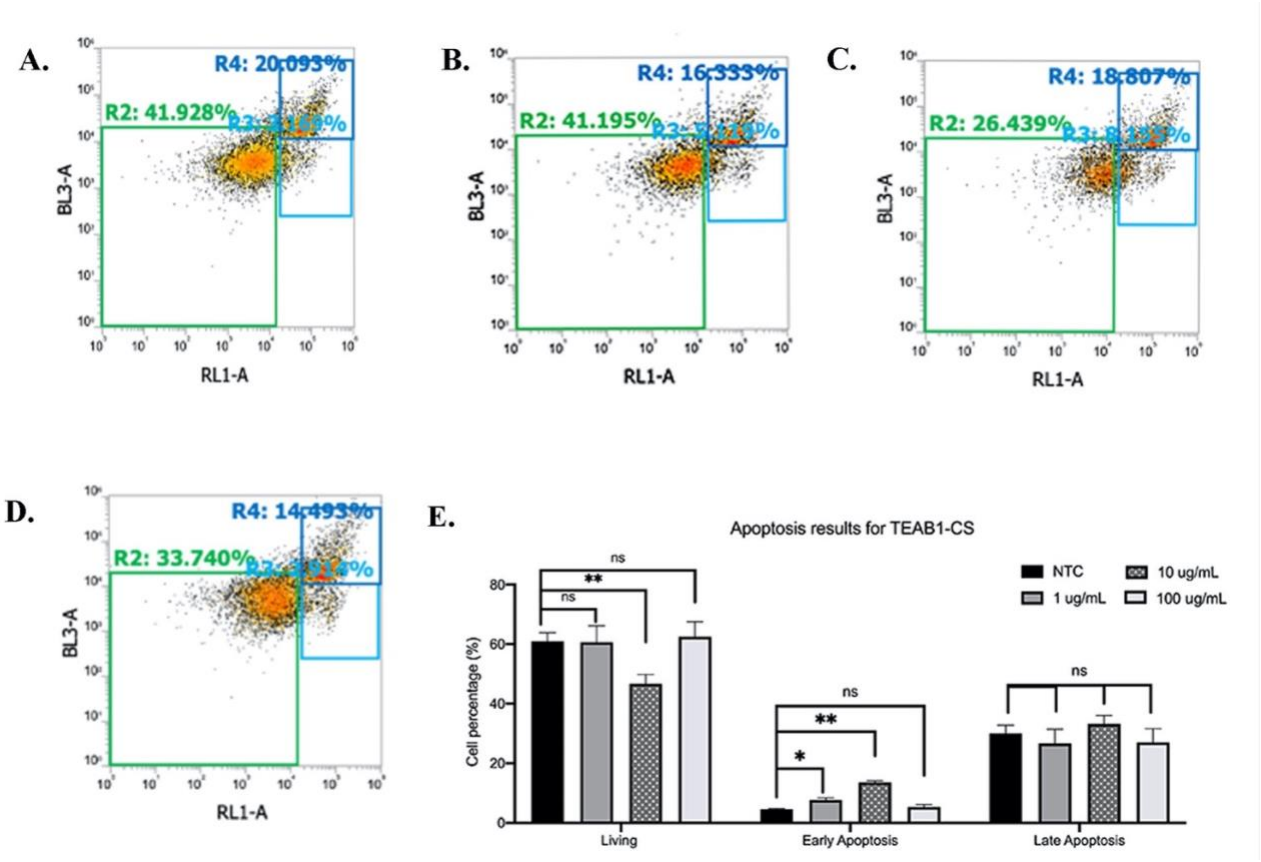

Figure 3: TEAB1-CS (24% TEAB) apoptosis measurements. Two separate experiments were performed in quintuplicate sets **A.** A representative of all five NTC flow cytometry measurements. **B.** A representative of all five 1 µg/mL TEAB1-CS treated flow cytometry measurements **C.** A representative of all five 10 µg/mL TEAB1-CS treated flow cytometry measurements. **D.** A representative of all five 100 µg/mL TEAB1-CS treated flow cytometry measurements. **E.** No significant change can be seen at all concentrations of the TEAB1-CS treatment with viable, early, and late apoptotic cells. Represented by ns are values with p-value >0.05, p-value from 0.01 to 0.05 is represented by *, p-value from 0.001 to 0.01 is represented by **, and p-value from 0.0001 to 0.001 is represented by ***, and p-value<0.0001 is represented by ****.

Treatment with 100 µg/mL TEAB2-CS (40.6% TEAB) caused a 2.3-fold increase in late apoptosis (Fig. 4 D&E). With the same concentration of TEAB2-CS (40.6% TEAB), early apoptotic levels increased 6-fold compared to the NTC (Fig. 4 D&E). I observed more viable cells than apoptotic cells at the TEAB2-CS (40.6% TEAB) concentration of 1 µg/mL. At TEAB2-CS (40.6% TEAB) dosages of 10 µg/mL and 100 µg/mL, there were more late and early apoptotic cells than viable cells, with more than 60% of the total cell population being either late

or early apoptosis. Taken together, we can conclude that TEAB2-CS (40.6% TEAB) promotes apoptosis, and therefore, it may not be an effective gene carrier for HeLa cells (Fig. 4). There are no significant defects seen between all doses of TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) and the NTC, suggesting that TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) does not induce apoptosis. Although treatment with 1 µg/mL and 100 µg/mL induce apoptosis, their increase values were not statistically significant when compared to NTC (Fig. 5, A-E).

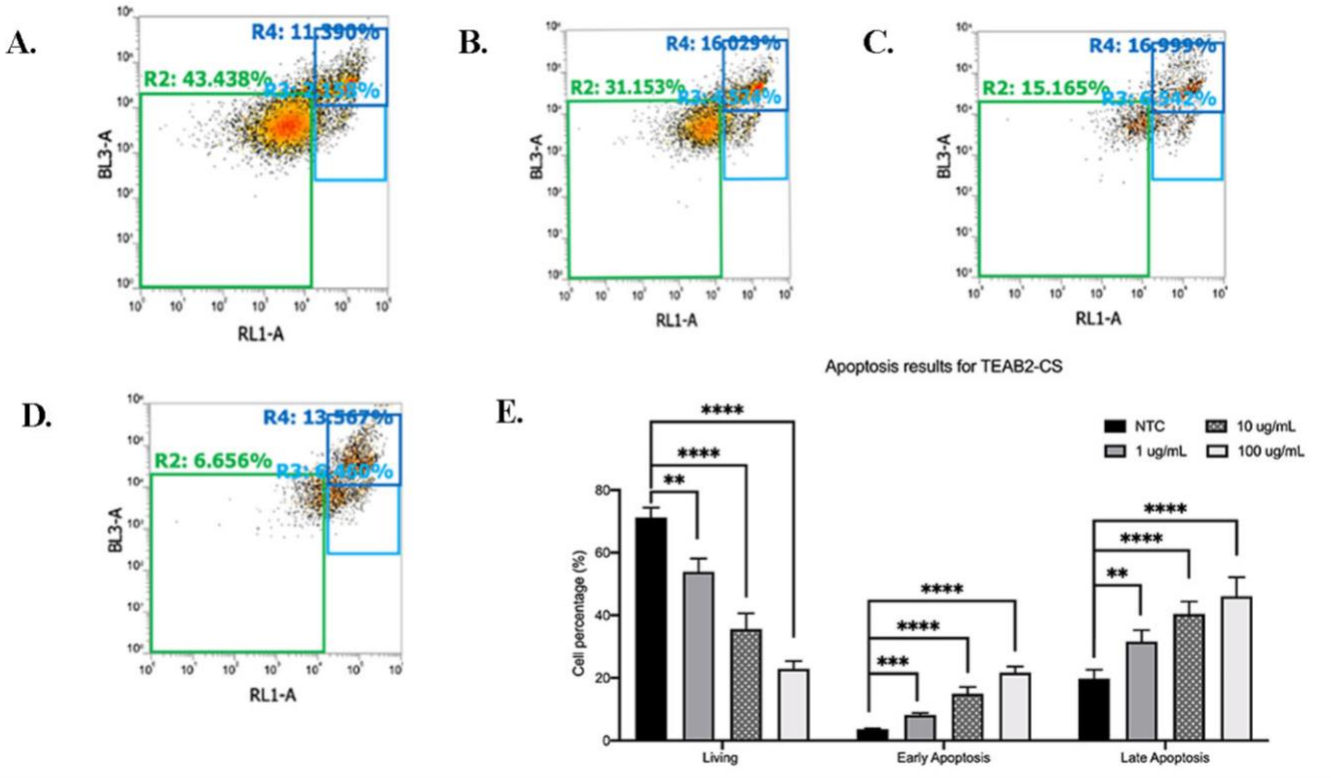

Figure 4: TEAB2-CS (40.6% TEAB) apoptosis measurements. Two separate experiments were performed in quintuplicate sets **A.** A representative of all five NTC flow cytometry measurements **B.** A representative of all five 1 µg/mL TEAB2-CS treated flow cytometry measurements **C.** A representative of all five 10 µg/mL TEAB2-CS treated flow cytometry measurements. **D.** A representative of all five 100 µg/mL TEAB2-CS treated flow cytometry measurements. **E.** A 2.3-fold decrease in percentage of late apoptotic cells and 6-fold decrease in early apoptotic cells, and a 3.11-fold decrease compared to the NTC after treatment with 100 µg/mL of TEAB2-CS. Represented by ns are values with p-value >0.05, p-value from 0.01 to 0.05 is represented by *, p-value from 0.001 to 0.01 is represented by **, and p-value from 0.0001 to 0.001 is represented by ***, and p-value<0.0001 is represented by ****.

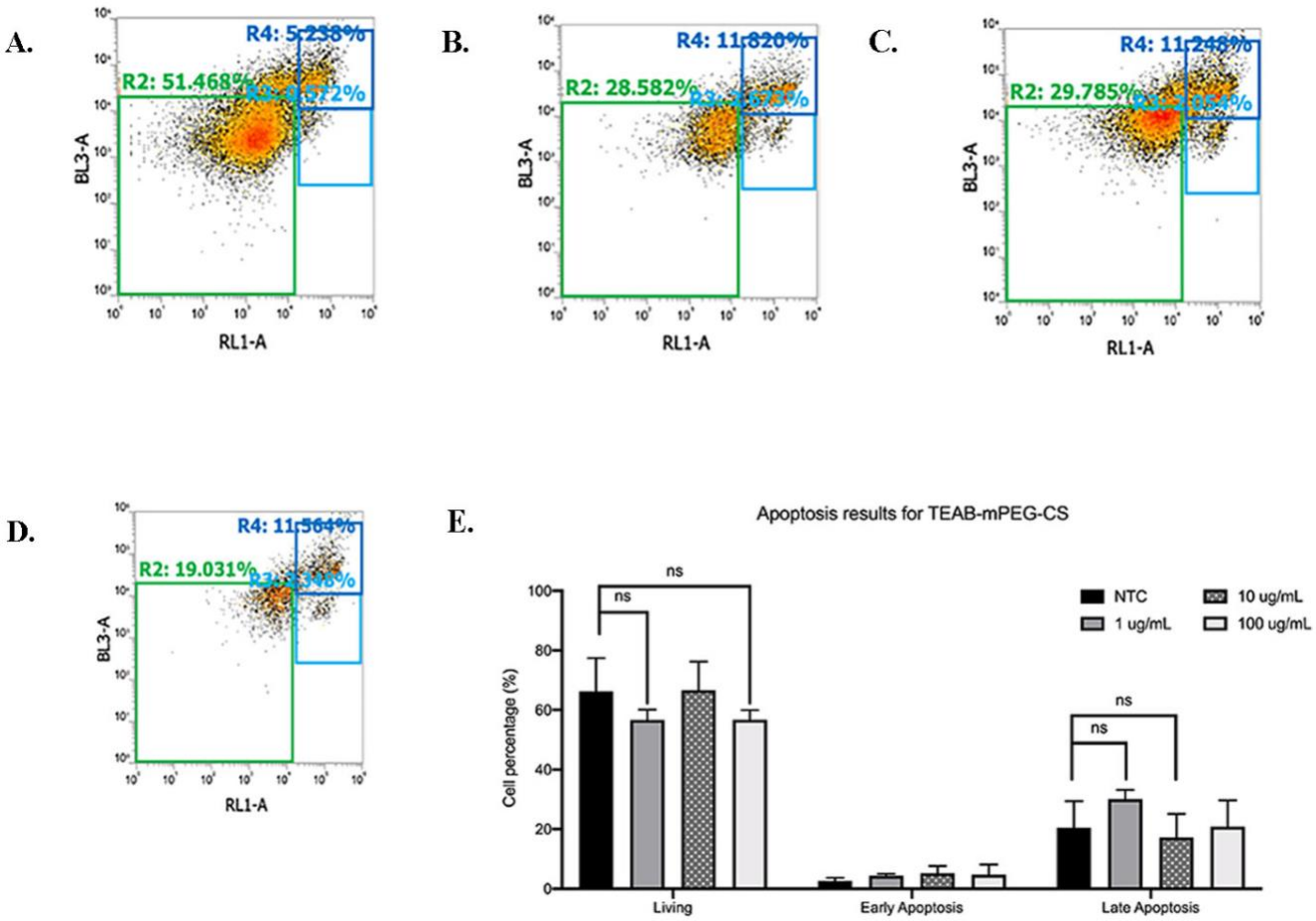

Figure 5: TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) apoptosis measurements. Two separate experiments were performed in quintuplicate sets **A.** A representative of all five NTC flow cytometry measurements **B.** A representative of all five 1 µg/mL TEAB-mPEG-CS treated flow cytometry measurements **C.** A representative of all five 10 µg/mL TEAB-mPEG-CS treated flow cytometry measurements. **D.** A representative of all five 100 µg/mL TEAB-mPEG-CS treated flow cytometry measurements. **E.** The change between the treatments using TEAB-mPEG-CS and the non-treated control is non-significant, because fold change from NTC to 100 µg/mL is less than 1. Represented by ns are values with p-value >0.05, p-value from 0.01 to 0.05 is represented by *, p-value from 0.001 to 0.01 is represented by **, and p-value from 0.0001 to 0.001 is represented by ***, and p-value<0.0001 is represented by ****.

As viable cells decrease at 1-100 µg/mL of TEPB-CS (43% TEPB), early apoptotic cells increase after treatment with 1-100 µg/mL of TEPB-CS (Fig. 6). At a concentration of 1 µg/mL and 10 µg/mL, less than 50% of the cell population were experiencing early apoptosis (Fig. 6, B&C&E). In contrast, dosage at 100 µg/mL induced early apoptosis in more than 50% of the cell

population (Fig.6 D&E). The fold change between the non-treated control and 100 µg/mL of TEPB was a 2-fold viability decrease in the percent of viable cells and 7.25-fold increase in early apoptotic cells (Fig. 6, A&D&E). These results are somewhat similar to TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) which significantly induced late apoptosis at concentrations of 1 to 100 µg/mL (Fig. 7, A-E). At the concentrations of 1 µg/mL and 10 µg/mL, less than 50% of the cell population were late apoptotic (Fig.7, B&C&E), whilst, at the concentration at 100 µg/mL late apoptosis was induced in more than half of the cell population (Fig. 7, D&E). Concentration of 100 µg/mL TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) displayed 2.8-fold increase in late apoptotic cells compared to the NTC (Fig. 7E).

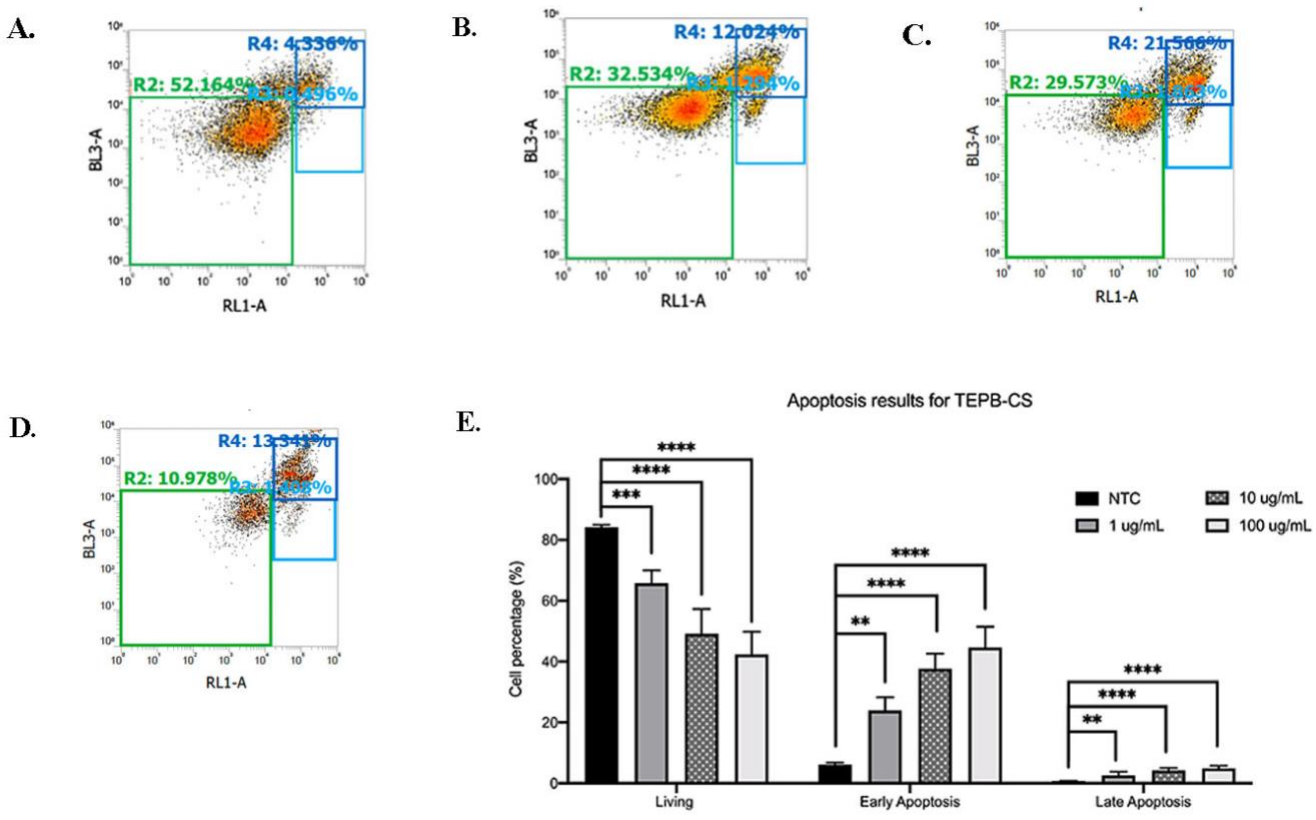

Figure 6: TEPB-CS (43% TEPB) apoptosis measurements. Two separate experiments were performed in quintuplicate sets **A.** A representative of all five NTC flow cytometry measurements **B.** A representative of all five 1 µg/mL TEPB-CS treated flow cytometry measurements **C.** A representative of all five 10 µg/mL TEPB-CS treated flow cytometry

measurements. **D.** A representative of all five 100 µg/mL TEPB-CS treated flow cytometry measurements. **E.** An increase in early apoptosis can be seen following treatment with 100 µg/mL TEPB when compared to the NTC. A 2-fold decrease in percentage of viable cells and 7.25-fold decrease in early apoptotic cells compared to the NTC after treatment with 100 µg/mL of TEPB-CS. Represented by ns are values with p-value >0.05, p-value from 0.01 to 0.05 is represented by *, p-value from 0.001 to 0.01 is represented by **, and p-value from 0.0001 to 0.001 is represented by ***, and p-value<0.0001 is represented by ****.

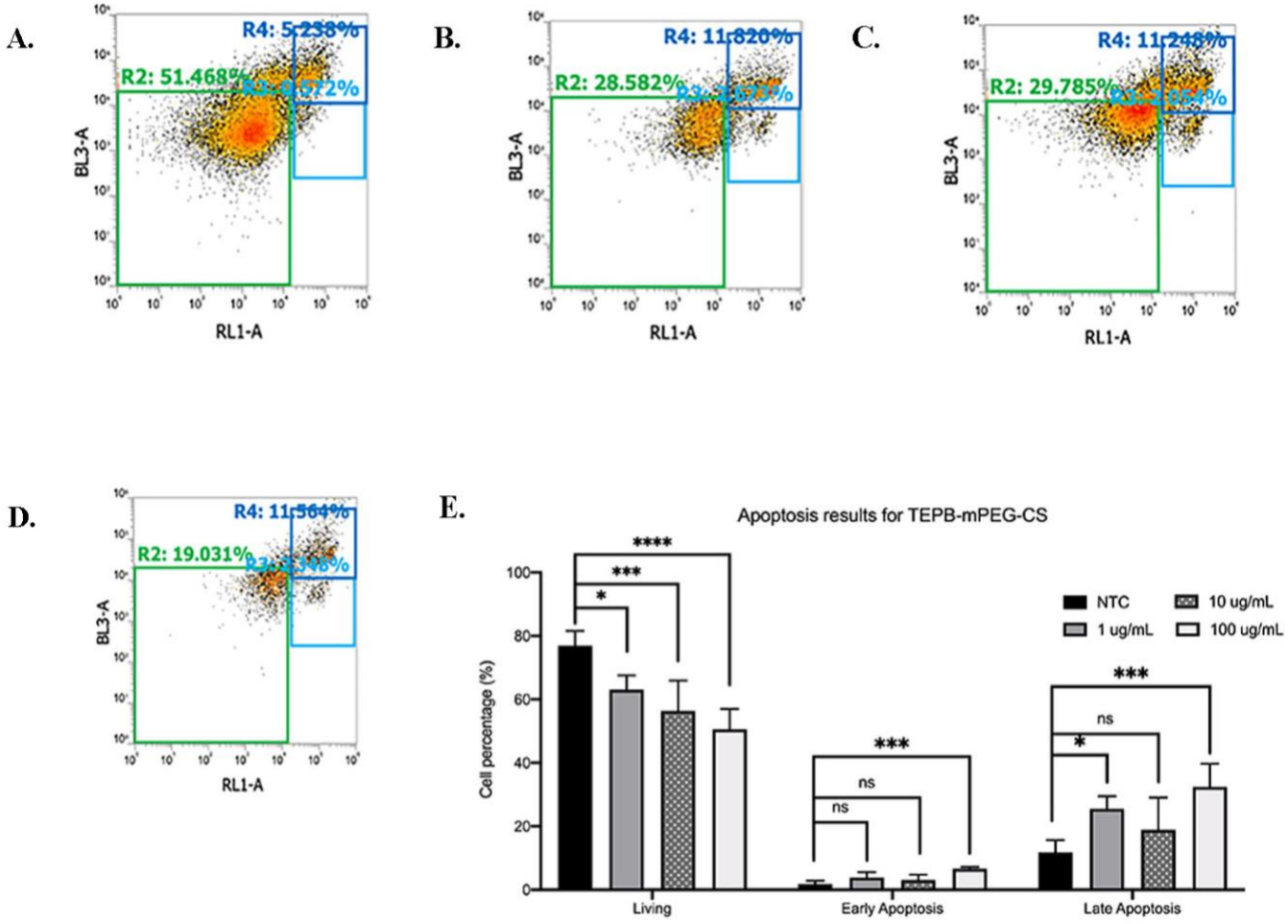

Figure 7: TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) apoptosis measurements. Two separate experiments were performed in quintuplicate sets. **A.** A representative of all five NTC flow cytometry measurements **B.** A representative of all five 1 µg/mL TEPB-mPEG-CS treated flow cytometry measurements **C.** A representative of all five 10 µg/mL TEPB-mPEG-CS treated flow cytometry measurements. **D.** A representative of all five 100 µg/mL TEPB-mPEG-CS treated flow cytometry measurements. **E.** An increase in early apoptosis can be seen following treatment with 100 µg/mL TEPB-mPEG-CS when compared to the NTC. A 1.5-fold decrease in percentage of viable cells and 2.8-fold decrease in late apoptotic cells compared to the NTC after treatment with 100 µg/mL of TEPB-mPEG-CS. Represented by ns are values with p-value

>0.05, p-value from 0.01 to 0.05 is represented by *, p-value from 0.001 to 0.01 is represented by **, and p-value from 0.0001 to 0.001 is represented by ***, and p-value<0.0001 is represented by ****.

Taken together, these apoptotic measurements suggest that TEAB2-CS (40.6% TEAB) induces apoptosis, whereas TEAB1-CS (24% TEAB) and TEAB-mPEG-CS seem to have little to no effect on cell death (Figs. 3, 4, and 5). TEPB and TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) were shown to have defects on the HeLa cells (Figs. 6 and 7), suggesting that high concentrations of TEPB causes an increase in early apoptosis, whereas high concentrations of TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) cause an increase in late apoptosis. Therefore, TEAB2-CS (40.6% TEAB), TEPB-CS (43% TEPB) and TEPB-mPEG-CS induce apoptosis and might not be effective gene delivery agents in HeLa cells.

TEAB1-CS Proved to be the Most Effective Gene Delivery Vehicle of the Five Derivatives

N+/P- ratio is the ratio between the ammonium group on chitosan and DNA phosphate [2]. Unmodified chitosan nanoparticles with N+/P- ratio between 1CS:16 pDNA showed efficient pDNA delivery into CHO cells [2]. The binding affinity is the confirmation of polyplex formation. The single band as a result of TEPB-CS (43% TEPB) and pDNA polyplex formation is at a P+/P- ratio of 4:1 (Fig. 8, A&B). However, TEAB-CS had a binding affinities at N+/P- ratios of 4:1 and 8:1 on the gel (Fig. 8, A&B). We also see a band retardation in terms of mobility as a result of a reduction in the negative charge of the CS-pDNA complex (Fig. 8, A&B) . However, the N+/P- ratio used in my study, for all five derivatives was 10 CS derivatives: 1 pDNA because we were not able to make more pDNA for the 4:1 ratio.

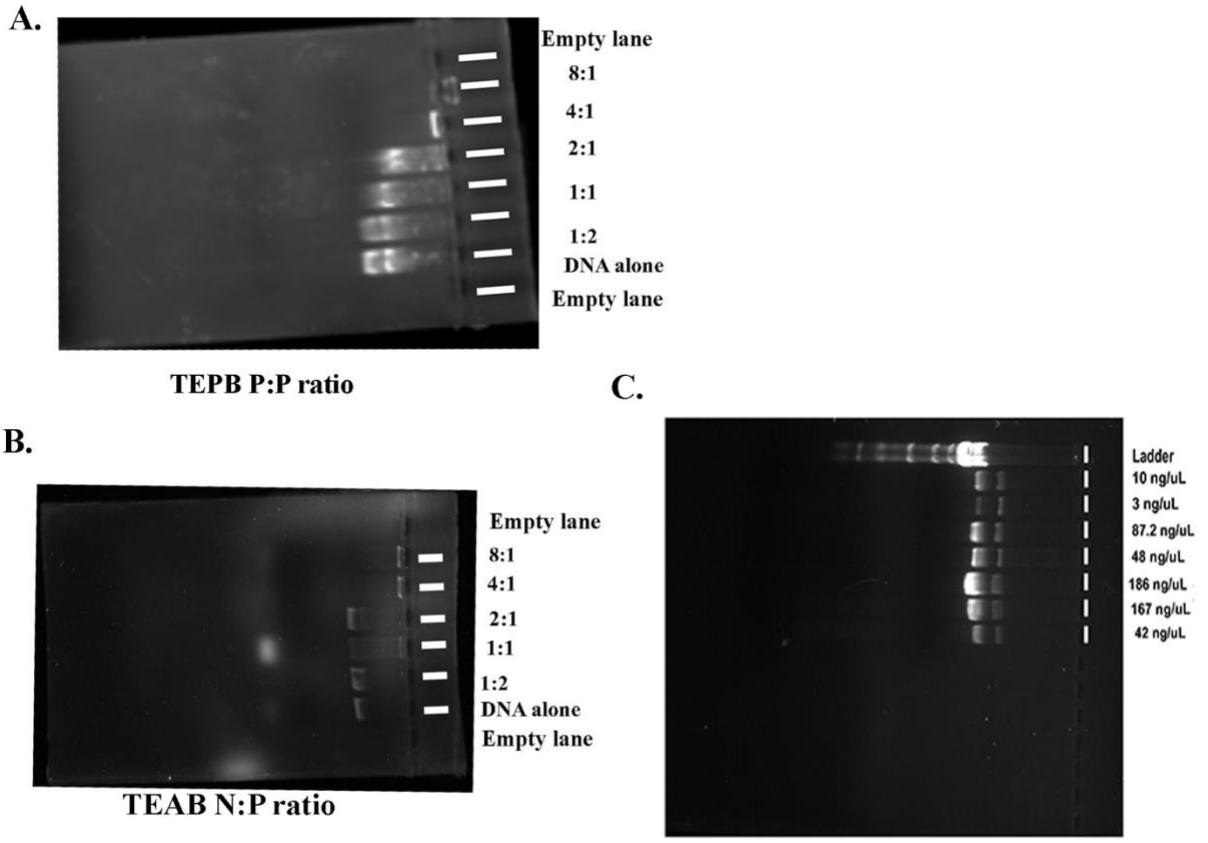

Figure 8: Agarose gel electrophoresis for transfection assays. Each gel was performed in 1% agarose gel containing 0.5 μg/mL of ethidium bromide (EtBr) **A.** TEPB derivatives P+/P- ratio. Lanes show 8:1, 4:1, 2:1, 1:1, 1:2 ratios, and DNA alone. Ratio 4:1 was determined to be ratio at which TEPB-DNA complexation was stable. **B.** TEAB derivatives N+/P- ratio. Lanes show 8:1, 4:1, 2:1, 1:1, 1:2 ratios, and DNA alone. Just like the CS derivatives containing TEPB, the ratio of most stable TEAB-DNA conjugation was 4:1. **C.** Gel electrophoresis of plasmid DNA following mini-prep. Following mini prep, pDNA concentration of186 ng/μL was used for all transfection experiments. Both TEAB and TEPB gels showed that the ammonium and phosphonium polyelectrolytes bound at N+/P- and P+/P- ratios of 4:1.

To investigate the effectiveness of each derivative to be used as a gene delivery vehicle, a transfection assay was performed. TEAB1-CS exhibited the highest transfection efficiencies of all the TEABs, while TEPB-mPEG-CS exhibited the highest transfection efficiencies of all the TEPBs (Fig 9, A&B). The positive controls, Xfect and Lipofectamine were found to facilitate the highest level of TPST1-EGFP gene expression at 80% (± 4.4%) and 80% (± 7.59%),

respectively, followed by TEAB1-CS (24% TEAB) had a transfection efficiency of about 65% (± 2.72%). While TEAB2-CS (40.6% TEAB) and TEPB-CS (43% TEPB) had transfection efficiencies of 47% (± 1.58%) and 49% (± 3.04 %), respectively.

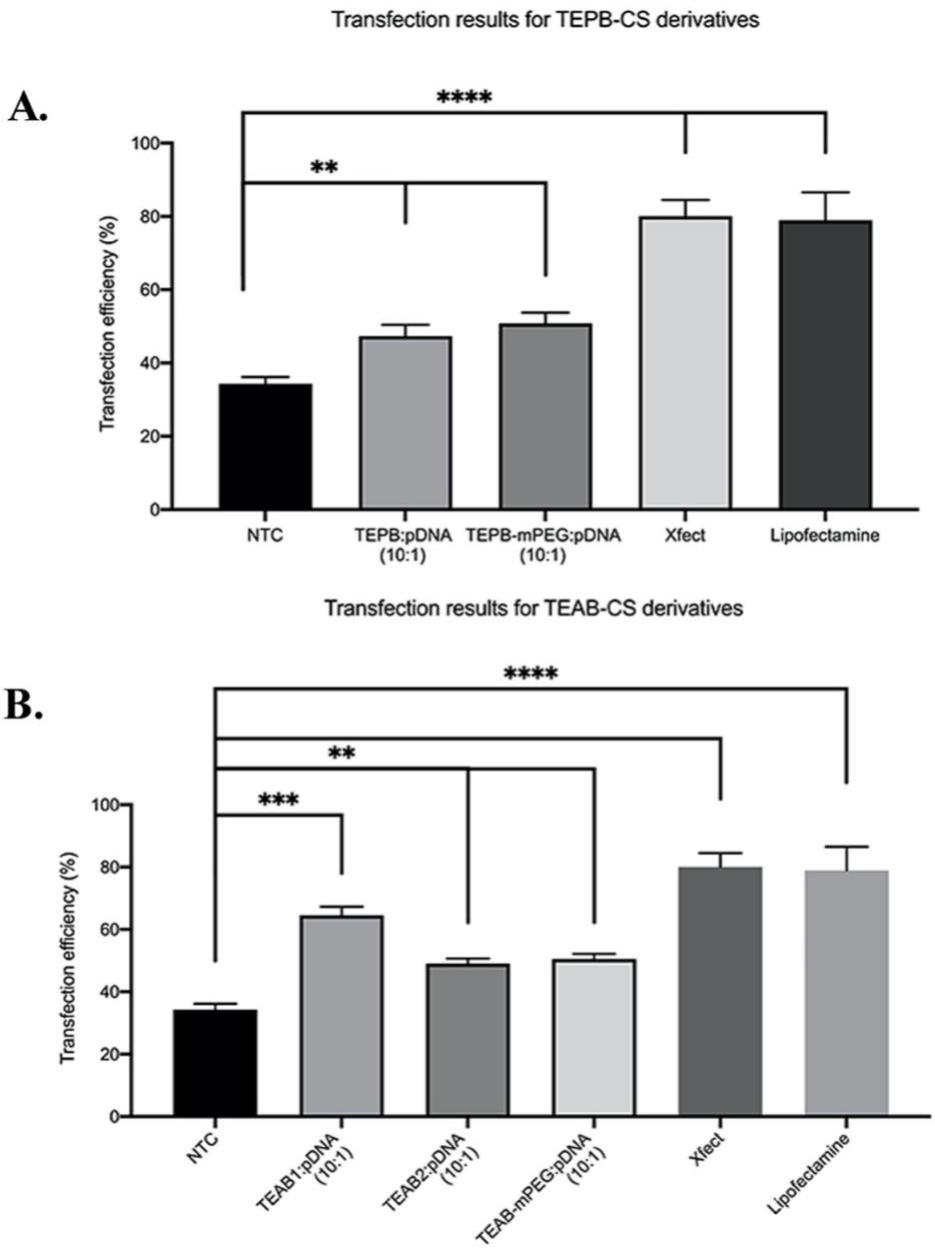

Figure 9: TEABs and TEPBs transfection efficiency comparison. Two separate experiments were performed in quintuplicate sets with a 24-well plate. Non-treated controls containing the naked pDNA and the HeLa cells had 34% transfection efficiency **A.** Gene delivery efficiency of TEPBs. -CS (43% TEPB) and TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) both exhibited transfection efficiencies of 47% and 50%, respectively. **B.** Gene delivery efficiency of TEABs.

TEAB1-CS (24% TEAB) displayed gene delivery efficiency of 65%. TEAB2-CS (40.6% TEAB) and TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) displayed transfection efficiencies of 49% and 50%, respectively. The positive controls, Xfect and Lipofectamine were found to facilitate 80% of gene expression. Represented by ns are values with p-value >0.05, p-value from 0.01 to 0.05 is represented by *, p-value from 0.001 to 0.01 is represented by **, and p-value from 0.0001 to 0.001 is represented by ***, and p-value<0.0001 is represented by ****.

TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) and TEPB-mPEG-CS exhibited transfection efficiencies of 50% (± 1.7 %) and 50% (± 2.94 %), respectively. These results suggest that out of the five CS derivatives, TEAB1-CS (24% TEAB) exhibited the greatest transfection efficiency, followed by the TEPB-mPEG-CS and TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG). TEAB1-CS only showed a 19% reduction in transfection efficiency in comparison to the positive controls, proving that chitosan derivatives with lower triethylammonium butanamide or triethylphosphonium butanamide modifications can serve as better gene delivery vectors than those with higher modifications.

DISCUSSION

Cationic polymers have been widely investigated, specifically chitosan due to its many characteristics. In this study I examined the effects of novel chitosan derivatives on viability and as well as their ability to serve as gene delivery vectors. Here I compare my derivatives to other commonly used non-viral synthetic polymers. I also discuss potential effects induced on the HeLa cells by my derivatives such as anticancer effects. I also discuss how degree of quaternization (DQ), mPEG addition, N+/P- and P+/P- ratio, quaternization using phosphonium versus ammonium groups, and IC_{50} influenced the gene delivery capability of the derivatives. My findings might contribute to future studies using non-viral vector gene therapy for genetic diseases.

DQ Modification Affects Properties of CS

It is well known that high degrees of quaternization (DQ) of CS enhances its solubility and can cause it to strongly attach to the cell's plasma membrane, yet high DQ leads to cytotoxicity and deteriorating delivery efficiency [22, 25, 68]. However, there is controversy about the topic of DQ, especially in cancer cells. One article reported having better pDNA transfection with high DQ of the N-(4-pyridinylmethyl) CS derivative (69% DQ) in Huh 7 cells [69]. Another article discovered that TMC derivitives with lower DQ (30% DQ) were much safer in HeLa cells and were better delivery agents than TMC with higher DQs (DQ of 60% and higher) [70]. The DQ in this project ranged from 24%-43%. Furthermore, in the present study, I report that the TEAB1-CS derivative (DQ of 24%) minutely induced apoptosis at a dosage of 10 µg/mL and had undetectable viability defects (via XTT), which I speculate might be due to the

its low DQ (Fig. 2E and 3, C&E). Regardless of the contrasting reports by these studies, I can assume that the effect of DQ on transfection is dependent on cell type and the specific type of derivative used for quaternization.

Moreover, N-substitution of chitosan with alkyl group such as TEAB, TEPB, and methyl (in the case of TMC) can lead to weakened hydrogen bonds [5]. These hydrogen bonds interact with the negative charge on nucleic acids, which allows for more efficient delivery. An increase in DQ leads to low DNA uptake by CS derivatives due to fewer hydrogen bonds as the amount of quaternized amino groups on chitosan increases. Based on this, TEAB1-CS (24% TEAB) among my five CS derivatives was the most consistent with this model. Nevertheless, as these hydrogen bonds decrease through higher DQ, the transfection efficiency of the CS derivative also decreases and when used to treat cells can cause defects in the cell [5]. Research has also shown that high degrees of quaternization often decrease viability and can cause damage in the cell such as apoptosis [22, 24, 71]. Among my four derivatives with higher DQ, TEAB-mPEG-CS (DQ of 40.6%; 3.1% mPEG) is the only one that contradicts this theory because its viability and apoptotic results do not correlate with one another (Fig. 2E & 5E. More than likely, this opposing evidence is due to the high mPEG units in the TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) derivative . From this I gather that low DQ correlates with high cell viability and high DQ correlates with high levels of apoptosis. Thus my CS derivatives prove that derivatives with low DQ are much safer than those with high DQ.

Addition of mPEG Units Impact Cytotoxicity: TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) Vs. TEPB-mPEG-CS (43% TEPB; 2.6% mPEG)

In addition to DQ, mPEG addition might have influenced toxicity. Xu et. al reported that grafting methoxy polyethylene glycol (mPEG) units unto TMC led to an increase in solubility, polyplex colloidal stability, and transfection efficiency and a reduction in cytotoxicity [22]. Moreover, after introduction of PEGylated 50 kDa TMC (PTMC50) and 50 kDa N-trimethyl chitosan (TMC50) in two murine and two human cell lines, PTMC50 demonstrated better transfection efficiency and a decrease in cytotoxicity compared to TMC50 and unmodified chitosan [25]. The MW of the mPEG added was 5,000 kDa and thus the TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) should not have exhibited significant apoptotic effects (Fig. 7E). However, the opposite was seen, which might have been due to a combination of two things. The first being the low percentage (below 3%) of PEG units in TEPB-mPEG-CS (43% TEPB; 2.6% mPEG), second being the higher modification with TEPB, which had 43% of the entire polymer modified with the phosphonium groups. This higher modification percentage could have contributed to TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) displaying lower apoptotic effect than TEPB-CS (Fig. 6E).

In contrast, TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG), although it's IC_{50} was 396.25 μg/mL, did not induce apoptotic cells. I speculate that its ammonium groups interacted with the membrane components cell and led to the toxicity seen only at 100 μg/mL. However, because it did not induce apoptosis I can only assume that it had inhibitory effects on the metabolism of the cancer cells. The difference in the degree of mPEG substitution between the TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) and TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) is very small (0.5%) and to my knowledge this percentage of mPEG substitution is actually quite

big considering the MW of the mPEG. This difference as well as the degree to which chitosan was modified with TEAB/TEPB groups, might have induced apoptosis (Fig. 5E and Fig. 7E). Subsequently, it can be hypothesized that the derivatives with the high modification might be less efficient pDNA delivery agents compared to TEAB1-CS (Fig. 9, A&B).

N+/P- Ratio Influences Transfection Efficiency

N+/P- or P+/P- ratio is a critical factor in the formation of the polyplex because it affects the surface charge of the polyplex, the condensing ability of the DNA and the delivery of the genetic material [15]. N+/P- ratio is the ratio between the positively charged ammonium groups in the polymer and the negatively charged phosphate on the pDNA, whereas the P+/P- ratio is the ratio between the phosphonium groups and the phosphate group of the pDNA [72]. High N+/P- ratio leads to strongly formed polyplexes through electrostatic interaction, and if the bond between the DNA and the polymer is too strong, this can prevent the nucleic acid from being released once it arrives at its site of action [2, 28]. At lower P+/P- ratios, phosphonium-based biopolymers bind better to nucleic acids [73] A study stated that N+/P- ratio between 1:1 and 16:1 allowed unmodified chitosan nanoparticles to efficiently deliver pDNA into the CHO cell line [2]. The observable optimum N+/P- ratio for my ammonium CS derivatives were 8:1 and 4:1, and P+/P- ratio for my phosphonium CS derivatives was 4:1 (Fig. 8A&B). However, due to pDNA shortage I was only able to perform the transfection assays at N+/P- and P+/P- ratios of 10:1. Although the transfection efficiency at a N+/P- or P+/P- ratio of 10:1 presented somewhat appreciable results despite the questionable transfection efficiency results from the non-treated controls. In conclusion a ratio of 4:1 is acceptable for both TEAB and TEPB chitosan derivatives.

Quaternization With Ammonium Groups Vs. Phosphonium Groups Comparing TEAB1-CS (24% TEAB), TEPB-CS (43% TEPB), and TEAB2-CS (40.6% TEAB)

Quaternization of chitosan has been proven to cause an increase in cytotoxicity and gradual inhibition of cell proliferation, but it also has led to better transfection efficiencies than unmodified chitosan [74]. An example is seen with TMC (N-trimethyl chitosan), which has shown promising gene delivery ability [13, 24, 25]. Furthermore, it was proven that biopolymers substituted with ammonium groups improve chargeability of the biopolymer, which is a useful property for gene transfection studies [24]. Modification of chitosan with quaternary ammonium salts has also been reported to decrease toxicity, as well as promote biodegradability and antibacterial properties [5, 24]. To my knowledge, TEAB2-CS (40.6% TEAB), exhibiting the highest toxicity and one of the lowest transfection efficiencies might have be due the higher TEAB modification degree on CS than TEAB1-CS (24% TEAB).

In contrast, some report that biopolymers with ammonium groups are highly toxic compared to the those modified with phosphonium analogues. Substitution of ammonium salts with quaternary phosphonium salts on biopolymers also allowed for increased cellular delivery of DNA [75]. Phosphonium-substituted biopolymers exhibited increased DNA binding and gene delivery efficiency of pDNA than ammonium-substituted biopolymers [75]. For instance, when a triethyl-phosphonium polystyrene complex, with siRNA had better cell viability than its ammonium equivalent [72]. However, TEPB-CS (43% TEPB) exhibited the same apoptotic defects at all concentrations of treatment and it is unclear why this happened, because due to charge distribution this derivative should have had the opposite effects. From this information, it can be assumed that each derivative behaves differently in terms of its effects on the HeLa cell

viability. It can be hypothesized from the results obtained above that low modification with TEAB, would increase delivery efficiency and reduce cytotoxicity.

Comparing TEAB- and TEPB-Containing Chitosan Derivatives to PAMAM, PEI, and PLL

Synthetic polymers such as PEI, PAMAM (Polyamidoamine), and Poly-L-Lysine (PLL) are oftentimes compared to chitosan, because they are much efficient at gene transfection than chitosan. Among those three dendrimers, PAMAM is recognized as the best gene delivery vector, however, in a clinical setting all three polymers are noticebly toxic [76]. PAMAM and PEI are toxic to cells because they have many ammonium groups and these groups because of their charge distribution inflict damage on the cell membrane by interacting with its components [22, 38]. In HeLa cells, PEI was reported to have low cell viability (cell viability of 20-40%) at concentrations ranging 5-25 μg/mL. Whereas, PAMAM had high cell viability at concentrations lower than 100 μg/mL, which is why it is said to be one of the best polymer delivery agents [22]. On the other hand, PLL is highly toxic in HeLa cells, with cell viability as low as 50% [77].

The low cell viability results for TEAB2-CS (40.6% TEAB) and TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) at concentrations of 10 μg/mL and/or 100 μg/mL could have been as a result of damage to the cell membrane as well. PEI and PAMAM induce damage to the cell membrane because of their ammonium groups and thus results in low cell viability. Given this information, I speculate that at least one of my derivatives (TEAB1-CS) would be safer than PEI, PAMAM, and PLL due to its high cell viability in a dose dependent manner (Fig. 2E). However the same cannot be said for the other two TEAB-containing derivatives that contain higher modification with the ammonium groups. These ammonium groups might have inflicted cellular damage which is most likely similar with to literature reports of PEI, PAMAM, and PLL [22].

Highly modified derivatives synthetic polymers result in low transfection efficiencies, whearas the reverse is observed with derivatives with low degrees of modification [22]. My transfection experiments confirm this theory. Furthermore, research has shown that replacement of ammonium groups with phosphonium moieties is safer and enhances transfection efficiency in HeLa cells [64] [72]. However, my results conflicted with such findings, except in the case of TEAB1-CS (24% TEAB). Transfection rates for TEPB-CS (43% TEPB) and its ammonium analogue TEAB2-CS (40.6% TEAB) remained relatively similar, as did TEPB-mPEG-CS (43% TEPB; 2.6% mPEG) and its counterpart TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG). The phosphonium analog for TEAB1-CS was insoluble in PBS and thus was excluded from this project. Although, based on these reports I speculate that if we were able to include the phosphonium counterpart of TEAB1-CS it might have been a better gene carrier than TEAB1-CS. Moreover, it has been reported that PEG addition to polymers such as PLL can increases transfection, yet this was not the case in my study [78]. My PEG containing derivatives (TEAB-mPEG-CS and TEPB-mPEG-CS) displayed similar or reduced transfection efficiency compared to their non-mPEG containing counterparts (TEAB1-, TEAB2-, and TEPB-CS). However it is unclear why such results were seen.

IC_{50} Values of TEAB2-CS and TEAB-mPEG-CS Inhibited HeLa Cell Viability

IC_{50} is the concentration at which the HeLa cell viability is 50% inhibited.[1] Normally, the median half maximal inhibitory (IC_{50}) concentration value of CS derivatives ranges from 0.2-2.5 mg/mL in most cell models [2]. This means that the threshold at which chitosan derivatives such as TMC inhibit cell proliferation is within 0.2-2.5 mg/mL. Whereas, my data displays IC_{50} values for TEAB2-CS (40.6% TEAB) and TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG)

ranging from 0.01-0.396 mg/mL. Moreover, both both TEAB2-CS (40.6% TEAB) and TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG) derivatives remained belowed this threshold, thus proving that both are more toxic than TMC (Fig. 2D).

Potential Anticancer Effect of TEAB2-CS, TEPB-mPEG-CS, and TEPB

Chitosan displays anticancer activity induced by activation of procaspase, which then accelerates the cascades that amplify signals for cell death [79]. Once endocytosed into tumor cells derivatives such as LMWC induces extrinsic apoptosis in which caspase 8 and cell cycle arrest are activated, thus inhibiting tumor growth. However, tetrazolium-based tests, such as XTT cannot differentiate between cytotoxicity or cell death which would explain why TEAB-mPEG had the low viability results but did not induce apoptosis [78]. Given this information, I cannot denounce the possibility of my derivatives exhibiting anticancer activity due to the results of my apoptosis assay. The reduction in viability at 10-100 μg/mL for TEAB2-CS (40.6% TEAB) and at 100 μg/mL for TEAB-mPEG-CS could also be as a result of anticancer activity could be induced by my derivatives (Fig. 2E, 4E, and 5E). While TEPB-CS (43% TEPB) exhibited minor toxicity, it did extremely induce apoptosis at a concentration-dependent manner and a possible hypothesis for this could be that TEPB-CS (43% TEPB) is able to enter the cell without significantly harming the cell membrane, but once it is in the cytoplasm, induction of apoptosis begins (Fig. 2F, 7E, and 6E) [71, 80]. TEAB1-CS (24% TEAB) and TEAB-mPEG-CS (40.6% TEAB; 3.1% mPEG), however by exhibiting little to no apoptotic defects on the cells, demonstrate that they both lack anti-cancer activity. I speculate that this is due to charge on TEAB compared to TEPB groups. TEPB groups possess a more positive charge than their ammonium analogues and this difference in charge distribution would allow for more interaction

with negatively charged components of the cell membrane and lead to growth inhibition of the cancer cells [72].

Several chitosan-based transfection findings in cancer models commonly transfect using siRNA and have shown increased anticancer activity such as apoptosis signaling [80]. However, few report anticancer activity after pDNA transfection into cancer cells. My study has shown that anticancer activity could be induced upon addition of some of the derivatives, though this is difficult to prove given that my derivatives were not tested on a non-cancerous cell line. It would be interesting to determine the specific mechanism by which the derivatives interact with the cell when complexed to pDNA. Such results might support or denounce the anticancer claim.

CONCLUSIONS

Here I report the safety and gene transfection efficiency of five derivatives of chitosan modified with ammonium/phosphonium salts by using a viability assay, an apoptosis assay, and transfection assay. Quaternization of 24% of the ammonium salt TEAB (TEAB1-CS) served as the optimum derivative for transfection. This derivative also promoted viability at treatment concentrations and had limited apoptotic effects in the HeLa cell line.

Limitations of the Study

My non-treated control for both transfection efficiencies showed high background fluorescence, thus the transfection experiment would need to be replicated and the flow cytometer would need to be adjusted in order for a more accurate representation of the transfection efficiencies using my derivatives. Another limitation with the research was usage of the flow cytometer before fluorescence imaging using the confocal microscopy. By first using confocal microscopy, I would have been able to verify that the derivatives were actually able to deliver the pDNA into the HeLa cells and then follow this up using the flow cytometer to measure the transfection efficiency. In the future, I hope to verify the results reported here by performing confocal microscopy in hopes of proving that my derivatives are efficient delivery agents.

Future Directions

I originally hypothesized that the phosphonium CS derivatives would have the least cytotoxic effects as well as promote delivery of the pDNA, *in vitro*, due to reports from previous

studies comparing phosphonium-based and ammonium-based polymers.[64, 72] These studies reported that the polymers with the phosphonium groups had high transfection efficiency and high cell viability than the ammonium-based groups. However, in my study TEAB1-CS (24% TEAB) was less cytotoxic and had a better transfection efficiency compared to my other ammonium-based derivatives. In contrast, my phosphonium based CS derivatives were toxic by inducing apoptosis and had moderate transfection efficiencies. Due to the novelty of this research, there are currently no in-depth studies about the intercellular processes following treatment with these chitosan derivatives. Furthermore, to understand the differences between the phosphonium-based and ammonium-based polymers used in this study, more research needs to be performed on how these derivatives interact with cancer cells as well as normal cells.

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