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Synthesis and Characterization of Fatty Acid Grafted Chitosan Polymer and Their Nanomicelles for Nonviral Gene Delivery Applications

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Abstract

The aim of this study was to synthesize and characterize fatty acid-grafted-chitosan (fatty acid-*g*-CS) polymer and their nanomicelles for use as carriers for gene delivery. CS was hydrophobically modified using saturated fatty acids of increasing fatty acyl chain length. Carbodiimide along with *N*-hydroxysuccinimide was used for coupling carboxyl group of fatty acids with amine groups of CS. Proton nuclear magnetic resonance and Fourier transform infrared spectroscopy were used to quantify fatty acyl substitution onto CS backbone. The molecular weight distribution of the synthesized polymers was determined using size exclusion high performance liquid chromatography and was found to be in range of the parent CS polymer (~50 kDa). The critical micelle concentration (cmc) of the polymers was determined using pyrene as a fluorescent probe. The cmc was found to decrease with an increase in fatty acyl chain length. The amphiphilic fatty acid-*g*-CS polymers self-assembled in an aqueous environment to form nanomicelles of ~200 nm particle size and slightly positive net charge due to the cationic nature of free primary amino groups on CS molecule. These polymeric nanomicelles exhibited excellent hemo- and cytocompatibility, as evaluated by in vitro hemolysis and MTT cell viability assay, respectively, and showed superior transfection efficiency compared to unmodified chitosan and naked DNA. The surface of these nanomicelles can be further modified with ligands allowing for selective targeting, enhanced cell binding, and internalization. These nanomicelles can thus be exploited as potential nonviral gene delivery vectors for safe and efficient gene therapy.

Graphical Abstract

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The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconj-chem.7b00505.

¹H NMR spectra of chitosan and fatty acid-grafted chitosan polymers (PDF)

INTRODUCTION

Gene therapy is a promising tool for treating a wide range of diseases such as cancer, Alzheimer's disease, diabetes, AIDS, hemophilia, and several other genetic disorders.¹ Gene therapy involves introduction of exogenous nucleic acids (transgene) in an attempt to deliver missing genes or to repair, replace, or silence a faulty gene in the cells to treat pathological conditions.² Given the large size, hydrophilic nature (owing to negatively charged phosphate groups), poor cellular uptake, low transfection efficiency, and susceptibility to enzymatic degradation of genetic material, their delivery is typically mediated by carriers or vectors. However, compared to the widely promising therapeutic applicability, the clinical success of gene therapy is eclipsed by delivery and safety concerns that are mostly associated with the carriers or vectors employed for transgenesis. A massive portion of gene delivery in clinical trials is carried out using viruses or modified virus-based vectors due to their favorable cellular uptake, but their success is substantially limited owing to serious intrinsic issues including carcinogenesis, immunogenicity, broad tropism, limited nucleic acid packaging capacity, and cost effectiveness.^{2,3} Nonviral vectors have the advantage of being biosafe but are limited in their potential to successfully protect and/or deliver the gene of interest. Consequently, the overall success of gene therapy relies on successfully overcoming the prerequisites for an ideal gene delivery system.

Chitosan (CS) is a natural cationic copolymer of randomly distributed β -(1, 4)-linked D-glucosamine and N-acetyl-D-glucosamine derived from alkaline partial deacetylation of chitin. Chitin is a long chain polymer of N-acetylglucosamine found in the exoskeleton of crustaceans and insects. CS-based vectors have gained a lot of interest due to their biocompatibility, low immunogenicity, negligible cytotoxicity, biodegradability, favorable physicochemical properties, and ease of chemical modification. Under acidic conditions protonated primary amine groups of CS interact with negatively charged plasmid DNA (pDNA) to form nanoscale complexes while still possessing a net positive surface charge.⁴ This allows them to bind to negatively charged molecules.⁵ These complexes are taken up by the endosomal-lysosomal system, internalized into the endosomes. Inside the endosomes the cationic polymer acts as a "proton sponge", trapping protons pumped into the endosome. As a result, membrane potential rises causing additional chloride ions to diffuse into the endosome inducing subsequent osmotic swelling and rupture of the endosome. This releases the polyplex into the cytoplasm.^{4,6} The released pDNA then passes and integrates into the nuclear DNA.⁴ Therefore, CS-pDNA polyplex formation facilitates its cellular uptake by adsorption mediated endocytosis, allows efficient protection of the genetic material from lysosomal nuclease degradation, and triggers endosomal escape due to the intrinsic buffering capacity of CS. However, unmodified CS shows poor gene transfection efficiency primarily

attributed to its poor solubility at physiological pH, low cellular uptake, and strong ionic interactions with pDNA resulting in inefficient release of pDNA.^{7–9} Hydrophobic modification of CS has shown encouraging results in overcoming some of these limitations. Conjugation of hydrophobic fatty acid segments on CS results in amphiphilic cationic polymers with the ability to self-assemble in aqueous environment to form polymeric micelles.¹⁰

Superior gene transfection is achieved owing to enhanced adsorption onto the lipophilic cellular membranes and effective intracellular dissociation releasing free pDNA.^{7,9,11,10,12} The surface of these polymeric micelles can further be modified with ligands allowing for selective targeting, enhanced cell binding, and internalization.^{12,13}

The present study describes detailed methodology to synthesize and characterize saturated fatty acid conjugates of CS as widely applicable gene delivery vectors in a reproducible manner. A wide range of even chain saturated fatty acids ranging from 6 to 20 carbon atoms were selected. The fatty acid substitution on CS was quantified using proton nuclear magnetic resonance (¹H NMR) and Fourier transform infrared (FT-IR) spectroscopy. Molecular weight distribution of CS and synthesized fatty acid-grafted-CS (fatty acid-*g*-CS) polymers was estimated using size exclusion high performance liquid chromatography (SEC-HPLC). The effect of fatty acid chain length on size, ζ potential, and critical micelle concentration (cmc) of amphiphilic fatty acid-CS conjugates was investigated. Blood compatibility study was performed to demonstrate suitability for intravenous administration. In vitro cytotoxicity study was performed in human embryonic kidney cell line (HEK-293 cell line, ATCC, Rockville, MD, U.S.) using hydrophobically modified CS-polymer nanomicelles to establish their use as a safe and biocompatible nonviral gene delivery vector. Finally, in vitro transfection study was performed using nanomicelles of a model fatty acid-grafted-CS polymer as an example, to demonstrate the effect of fatty acid substitution onto CS backbone on transgenesis.

RESULTS AND DISCUSSION

Synthesis and Characterization of Fatty Acid Grafted Chitosan Polymer

CS of 50 kDa molecular weight and 85% degree of deacetylation (DDA%) was chosen for hydrophobic modification based on previous research done on different molecular weight and DDA% of CS for optimum transfection efficiency, cellular uptake, and protection of CS-pDNA complex against nuclease degradation.^{14,15} The fatty acid-*g*-CS polymers were prepared using carbodiimide mediated amidification reaction where carbodiimide along with *N*-hydroxysuccinimide (NHS) acts as a zero-length cross-linker of carboxyl group of fatty acids with amine group of CS. The reaction is most efficient at acidic pH (pH 4.5–7.2) in conditions devoid of foreign carboxyl and amine groups.¹⁶ In this reaction carbodiimide reacts with the carboxylic group of fatty acid to form *O*-acylisourea active ester intermediate (Scheme 1) which reacts with amine to form a stable amide bond and water-soluble isourea as byproduct. However, failure to react with an amine causes hydrolysis of the unstable intermediate regenerating the carboxylic group and releasing *N*-unsubstituted isourea. Subsequent addition of NHS allows carbodiimide to couple NHS to the carboxyl group forming an amine-reactive ester, which is markedly more stable than the *O*-acylisourea ester,

thus favoring efficient conjugation of carboxylic and amine group with the formation of a stable amide bond in an aqueous environment.¹⁶ The excess carbodiimide and water-soluble byproducts were removed by dialysis against deionized water. Unreacted fatty acid was removed by ethanol washing.

The substitution of fatty acid onto CS backbone was confirmed by ¹H NMR and FT-IR spectroscopy. The ¹H NMR spectra of CS and fatty acid-*g*-CS polymers are shown in Figure 1. For both CS and fatty acid-*g*-CS polymers the resonances of three *N*-acetyl protons of *N*-acetylglucosamine ((-CO)-CH₃) and two protons (H₂) of glucosamine residue were observed at 1.9 and 3.0 ppm, respectively. The ring protons (H-3, -4, -5, -6, -6') of CS backbone are considered to resonate at 3.4–3.8 ppm, as depicted in the spectra. The new proton peaks at 0.8–1.1 ppm can be associated with the resonances of -CH₃ and -CH₂- groups of the fatty acyl residues. The protons of the fatty acyl residue associated with the amide bond ((-CO)-CH₂-) resonated around 2.4–2.7 ppm.^{17,18} FT-IR spectra absorption peaks of CS and fatty acid-CS polymers at 1655 cm⁻¹ can be attributed to the carbonyl stretching of secondary amides, at 1470 cm⁻¹ to the N-H bending vibrations of nonacylated primary amines of glucosamine, and at 1585 cm⁻¹ to the N-H bending vibrations of the amide II band (Figure 2). The absorption peaks at 2850–2950 cm⁻¹ were due to -CH₂- groups of *N*-acyl moiety, and their intensity was proportional to fatty acid chain length. After grafting onto CS, the peak at 1470 cm⁻¹ almost disappeared while more prominent peaks at 1655 and 1585 cm⁻¹ were seen. The graft ratio (GR%) of fatty acid onto CS was calculated by measuring the areas under the peak at 1655 and 2870 cm⁻¹ as the DDA% of CS was >75%.¹⁹ Degree of acetylation (DA%) and GR% determined using ¹H NMR and FT-IR, respectively, are reported in Table 1.

Determination of Molecular Weight Distribution of Synthesized Polymers Using SEC-HPLC

The retention time of the synthesized fatty acid-*g*-CS polymers eluted from SEC-HPLC column was noted and compared with that of CS (average molecular weight of 50 kDa) (Table 2). The molecular weight distribution of the synthesized polymers was in range of the parent CS polymer (~50 kDa) (Figure 3).

Critical Micelle Concentration

The fatty acid-*g*-CS polymers form nanomicelles in aqueous solution owing to their amphiphilic nature. The polymer contains hydrophobic domains, represented by the fatty acid groups, which induce self-aggregation of the polymer forming the core of the micelle structure. Hydrophobic molecules can be efficiently loaded in the core of such micelles.²⁰ The cmc is an important parameter for characterizing a self-aggregating amphiphilic substance. It is a relatively narrow concentration range over which aggregation of the molecules takes place to form micelles, and the physical properties of the dispersion show a sudden change.²⁰ The cmc of these polymers was determined using a fluorimeter with pyrene as the hydrophobic fluorescence probe. Pyrene is considered an efficient fluorescence probe owing to the long lifetime of pyrene monomers and its ability to form pyrene excimers.^{21,22} The fluorescence intensity corresponding to the third vibrational peak of pyrene is particularly sensitive to solvent polarity.²³ Figure 4A shows the changes in the pyrene spectra of amphiphilic fatty acid-*g*-CS polymer over increasing concentration range.

The spectra depict that the fluorescence intensity of pyrene decreased with increasing polymer concentration. Also, a red shift is observed for the emission spectrum with increasing polymer concentration indicated by an increase in intensity of the third peak with respect to the first peak. This happens due to internalization of pyrene from water into the less polar micelle domain (hydrophobic core of micelles).²³ The changes in intensity of the first peak to the third peak (I_1/I_3) plotted against the decadic logarithm of polymer concentration reveal a sharp change in the intensity ratio (Figure 4B).

At low polymer concentration the I_1/I_3 remains relatively constant which can be explained by both the amphiphilic polymer and the hydrophobic pyrene molecules remaining in dispersed state. As the concentration reaches cmc, the micelles begin to self-aggregate and incorporate pyrene into their hydrophobic core. As a result, I_3 increases considerably as compared to I_1 , resulting in a sharp decrease in the I_1/I_3 ratio. Furthermore, in general the cmc value was found to significantly decrease with the increase in fatty acid chain length (Figure 4C).

Characteristics of Fatty Acid Grafted Chitosan Polymer Nanomicelles

The size and charge are two essential factors in determining the transfection efficiency of nanomicelles. It has been reported that eukaryotic cells can internalize particles up to 1 μm by different mechanisms.²⁴ Cationic surface charge of nanomicelles allows greater interaction with anionic phospholipids of the cell membrane triggering endocytosis.²⁵ The average hydrodynamic diameter and ζ potential of the fatty acid-*g*-CS polymeric nanomicelles were determined using dynamic light scattering (DLS) technique (Table 3). The particle size of the nanomicelles was found to be ~200 nm, distributed within a fairly narrow range indicated by low polydispersity index (PDI). The ζ potential of the nanomicelles was slightly positive due to the cationic nature of free primary amino groups on CS molecule at acidic pH but was lower compared to CS alone, which can be correlated to substitution of primary amino groups by fatty acids. Additionally, both electrostatic and hydrophobic interactions between pDNA and cationic nanomicelles promote condensation of anionic pDNA with improved stability and compact size. The morphology of the nanomicelles was determined using atomic force microscopy (AFM). The shape of the nanomicelles was observed to be spherical, and the size obtained for caproic acid-*g*-CS and palmitic acid-*g*-CS was found to be 210 and 183 nm, respectively, and was in agreement with that obtained using DLS technique (Figure 5).

Blood Compatibility Study

The nonspecific interactions of cationic polymers with the negatively charged membrane of red blood cells (RBCs) may cause lysis of these cells and adversely affect the safety and biocompatibility of the delivery system.²⁶ Additionally, such interactions may also affect the targeting ability, therapeutic efficacy, and half-life of the system.¹² The release of hemoglobin was used to quantify the membrane-damaging properties of the polymers spectrophotometrically (Figure 6A). The percent hemolysis for all concentrations of the polymers was below 2%. In general, in vitro hemolysis value of less than 10% is considered to be nonhemolytic.²⁷ The visual observation of the hemolytic activity of the cationic polymers was closely comparable to 10 mM phosphate buffer saline (PBS, pH 7.4) treated

cells and in agreement with the spectrophotometric data (Figure 6C). Microscopic examination of the RBCs treated with PBS, CS (500 $\mu\text{g/mL}$), and fatty acid-*g*-CS polymers (500 $\mu\text{g/mL}$) revealed no changes in the morphological structure (Figure 6B). However, cells treated with Triton X-100 (1% v/v) were severely damaged and had lost membrane integrity. All these observations together with microscopic confirmation validate good blood compatibility of the amphiphilic fatty acid-*g*-CS polymeric nanomicelles and support their applicability as an in vivo drug delivery system.

Cytotoxicity Assay

Cytotoxicity of gene delivery vectors is a major obstacle to gene therapy. Cationic polymers are known to possess inherent cytotoxicity due to their interaction with cell membrane and negatively charged cellular components.^{26,28} Cytotoxic action of CS based polymers is generally correlated with the number of free primary amino groups.⁴ In vitro cytotoxicity of the fatty acid-*g*-CS polymers was evaluated by testing different concentrations of the polymer in HEK 293 cell line (ATCC, Rockville, MD, U.S.) by MTT cell viability assay.

It is a colorimetric assay that quantifies the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye by mitochondrial succinate dehydrogenase enzyme present in metabolically active cells to insoluble purple formazan crystals. The formazan crystals formed are then solubilized using dimethyl sulfoxide (DMSO), and absorbance is measured spectrophotometrically at 570 nm wavelength. Only metabolically active cells have the ability to reduce MTT; therefore this activity can be taken as a marker of viable cells. As shown in Figure 7, the polymeric nanomicelles did not adversely affect cell viability compared to the control, tested up to a concentration of 1 mg/mL (~50 times higher than the optimal polymer concentration for transgenesis). On the contrary it was observed that HEK-293 cells grow better in the presence of CS and CS derived polymers at intermediate concentrations due to the positive effect of CS on cell proliferation and attachment.²⁹ It has been previously reported that DDA% affects cell toxicity which can be directly related to the overall surface charge density.⁵ It can be seen from the relative cell viability data in Figure 7 that CS has relatively lower cell viability compared to fatty acid-*g*-CS polymers. Fatty acid substitution can be directly related to decrease in number of primary amino groups, thereby reducing the net positive charge. This can also be seen from the representative ζ potential values in Table 3. Overall cytotoxicity due to CS based polymers is dose-dependent, increasing with increasing dose (1 mg/mL), though several studies have demonstrated high cell viability and negligible cytotoxicity even at higher doses.^{30–36} Some irregularities in the overall cell viability can also be seen with changing concentration which can be attributed to the distinct conformation that the modified polymer chains adopt (three-dimensional arrangement of the functional groups) and net surface charge of the resulting molecule.^{5,36} Negligible cytotoxicity demonstrates good safety profile and biocompatibility of these nanomicelles as nonviral gene delivery vectors without any significant detrimental effect on the cell membrane during uptake. This also exemplifies clinical application of this delivery system for repetitive administrations of therapeutic gene of interest.

In Vitro Gene Transfection

In vitro gene transfection ability of palmitic acid-*g*-CS nanomicelles (midrange cmc value of $\sim 58 \mu\text{g/mL}$) was evaluated in HEK 293 cell line (ATCC, Rockville, MD, U.S.) using β -galactosidase and green fluorescent protein (pGFP) encoding pDNA. The CS/pDNA polyplexes form owing to electrostatic interactions between positively charged CS and negatively charged pDNA. Fatty acid grafting confers amphiphilicity to CS molecule, further allowing both electrostatic and hydrophobic interactions to promote pDNA condensation and formation of self-assembling compact cationic nanomicelles. Cationic polyplex formation with CS/polymer improves the stability of pDNA, facilitates cellular uptake by their interaction with anionic cellular membrane followed by adsorption mediated endocytosis, triggers endosomal escape due to the buffering action of the free amino groups present on CS/polymer, and protects the pDNA against nuclease degradation. Efficient polyplex formation was observed at N to P ratio of 20 with pDNA association efficiency of CS/pDNA and palmitic acid-*g*-CS polymer/pDNA of $98.1 \pm 1.2\%$ and $95.3 \pm 2.7\%$, respectively.

The transfection efficiency at protein level was quantified using β -galactosidase enzyme activity. Cell lysate was assayed using galactosidase enzyme assay system containing *O*-nitro-phenyl- β -D-galactopyranoside (ONPG), a reaction substrate for detection of β -galactosidase enzyme activity, producing a yellow product estimated spectrophotometrically. Total protein content in each cell lysate was also quantified to normalize β -galactosidase activity per milligram of protein (Figure 8A). It was observed that palmitic acid-*g*-CS nanomicelles showed significantly higher transfection in comparison to naked pDNA and CS/pDNA polyplex. Similar trend was observed when cells were transfected with pGFP, showing significantly higher fluorescence with palmitic acid-*g*-CS nanomicelles than CS/pDNA polyplex and naked pDNA (Figure 8B). As mentioned earlier, electrostatic interactions between positively charged CS and negatively charged pDNA allow formation of polyplexes with net positive surface charge which helps in protecting the pDNA and enhances its cellular uptake. Fatty acid substitution on CS further enhances cellular uptake and transfection of genetic material by forming nanoscale polyplexes with pDNA which show optimum balance between polyplex stability and intracellular unpacking of pDNA.

CONCLUSIONS

A series of hydrophobically modified chitosan polymers were synthesized using even numbered carbon chain saturated fatty acids. High toxicity is one of the major limitations of gene delivery vectors. These fatty acid-grafted-chitosan polymers formed cationic nanomicelles with exemplary hemo- and cytocompatibility. Additionally, fatty acid-grafted-chitosan nanomicelles have been shown to effect high transfection in different cell lines.^{9,10,12} In our study, transfection efficiency of chitosan was found to be significantly increased after modification with palmitic acid. These fatty acid-grafted-chitosan polymers can further be functionalized using surface ligands for targeting ability and higher cell penetrability.^{37–39} In this report we have demonstrated a method for easy and cost-effective preparation and characterization of nanomicellar nonviral gene delivery vectors with negligible toxicity. These polymeric nanomicelles are potentially a superior alternative to

viral and other nonviral gene delivery vectors which possess limited biosafety and compliance.

EXPERIMENTAL SECTION

Materials

Chitosan (Mw = 50 kDa, 85% deacetylated) (CS), palmitic acid, stearic acid, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-Hydroxysuccinimide (NHS) was procured from Alfa Aesar (Ward Hill, MA, U.S.). Caproic, caprylic, capric, and myristic acid were procured from MP Biomedicals (Solon, OH, U.S.). Lauric acid was purchased from EMD Millipore Corporation (Billerica, MA, U.S.). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) was obtained from Creosalus Inc. (Louisville, KY, U.S.). Human embryonic kidney (HEK 293) cell line, Dulbecco's modified Eagle's medium (DMEM), and phosphate buffered saline (PBS) were purchased from American Type Culture Collection (ATCC, Rockville, MD, U.S.). Plasmid DNA encoding green fluorescent protein (gWiz-GFP) and β -galactosidase (gWiz- β Gal) were acquired from Aldevron LLC (Fargo, ND, U.S.). β -Galactosidase enzyme assay kit with reporter lysis buffer was procured from Promega (Madison, WI, U.S.). All other reagents were of analytical grade and were used without further modification.

Synthesis of Fatty Acid Grafted Chitosan Polymer

Synthesis of fatty acid-*g*-CS polymer was performed using carbodiimide mediated coupling of the carboxyl group of fatty acids with amine group of CS^{37,40} (Scheme 1). Briefly, CS (500 mg) was dissolved in 100 mL of deionized water acidified to pH 4.5 using hydrochloric acid. Fatty acid (0.3 mol/mol of monomer unit of CS) was dissolved in 50 mL of ethanol with sonication treatment (required for higher chain fatty acids) followed by the addition of EDC·HCl (5 mol/mol of fatty acid) and stirred on a magnetic stirrer. After 1 h, NHS (5 mol/mol of fatty acid) was added to the mixture of fatty acid and EDC·HCl in ethanol, and this resultant mixture was then added dropwise to CS solution under constant stirring. The reaction was allowed to take place at 90 °C for 12 h. The resultant product was dialyzed using a 3.5 kDa molecular weight cut-off dialysis membrane (Thermo Scientific, IL, U.S.) against deionized water for 48 h to remove the water-soluble byproducts completely, followed by freeze-drying of the dialyzed product to remove water. The lyophilized product was washed three times by dispersing it in ethanol followed by filtration through 0.2 μ m nylon filter paper (Cole-Parmer, IL, U.S.). The precipitate was vacuum-dried to obtain the fatty acid-*g*-CS polymer.

Structural Characterization of Fatty Acid Grafted Chitosan Polymer

Fatty acid grafting on CS backbone was confirmed by ¹H NMR and FT-IR techniques. For ¹H NMR experiments, CS and fatty acid-*g*-CS polymers were dissolved in deuterium oxide containing 1% deuterium chloride as a solvent at a concentration of 10 mg/0.6 mL. ¹H NMR spectra were recorded using a Bruker 400 MHz spectrometer at 25 °C (96 scans and 1.5 s delay) and analyzed using Bruker TopSpin 3.2.b.69 software. DA% of CS and fatty acid-*g*-CS polymers was calculated using eq 1.

$$DA\% = \frac{\frac{1}{3}A\delta_{1.9}}{A\delta_{3.0}} \times 100 \quad (1)$$

where $A\delta_{1.9}$ is the integral of the peak at 1.9 ppm and $A\delta_{3.0}$ is the integral of the peak at 3.0 ppm.

FT-IR analyses were made using a Thermo Scientific Nicolet iS-10 FT-IR spectrometer equipped with a Smart iTX accessory comprising of a diamond crystal. Samples were carefully added to cover the diamond crystal, and the knob was pressed in closely over the sample. Background was taken before each polymer sample, and the scan parameters were set to % transmittance mode, 64 scans, 4.0 resolution, and 0.482 cm^{-1} data spacing. All spectra were analyzed using the OMNIC 9.3.30 software by Thermo Scientific. The degree of fatty acid substitution on CS (GR%) was calculated by the method given by Moore and Roberts (1978) using eq 2.^{19,41}

$$GR\% = \frac{A_{1655}}{A_{2870}} \times \frac{100}{1.33} \quad (2)$$

where A_{1655} is the area under the % absorbance peak at 1655 cm^{-1} and A_{2870} is the area under the % absorbance peak at 2870 cm^{-1} .

Molecular Weight Distribution of Synthesized Polymers Using SEC-HPLC

Average molecular weight distribution of chitosan (50 kDa) and fatty acid grafted chitosan polymers was determined using size exclusion high performance liquid chromatography (SEC-HPLC).¹¹ Samples were prepared at a concentration of 10 mg/mL in 1% acetic acid (v/v) in deionized water. An Ultrahydrogel 250 (Waters, MA, U.S.) column was used in combination with the Agilent 1120 Compact LC system coupled with an Agilent 1200 series refractive index (RI) detector (Agilent, DE, U.S.) set at $35\text{ }^{\circ}\text{C}$, with 1% (v/v) acetic acid in deionized water as mobile phase, 0.5 mL/min flow rate, and 60 min run time. Data were analyzed using EZChrom Elite 3.3.2 software (Agilent, CA, U.S.).

Critical Micelle Concentration Determination

The fatty acid-*g*-CS polymers dispersed in water self-assemble to form micelle-like structures owing to their amphiphilic nature. The critical concentration at which such micellar assembly takes place is termed as the cmc. The cmc of the fatty acid-*g*-CS polymers was determined using pyrene as the hydrophobic probe.¹² Pyrene was dissolved in acetone at a concentration of $24\text{ }\mu\text{g/mL}$, and $10\text{ }\mu\text{L}$ of this solution was added to 10 mL glass test tubes followed by drying at $50\text{ }^{\circ}\text{C}$ to remove acetone. Increasing concentrations of polymers ranging from 1.0×10^{-3} to 1.0 mg/mL were prepared in 20 mM glacial acetic acid solution in deionized water, and 2 mL of each concentration was added to the glass test tube containing pyrene, keeping the final concentration of pyrene in each sample at $0.6\text{ }\mu\text{M}$. The test tubes were vortexed at 1800 rpm for 5 min followed by sonication treatment in a bath sonicator for 30 min. The solutions were then allowed to equilibrate overnight in the dark, and the fluorescence emission spectrum of pyrene was noted for each sample using

Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon, NJ, U.S.). The emission wavelength range was selected as 360–450 nm, emission slit width 5 nm, excitation wavelength 336 nm, and excitation slit width 2 nm. The emission intensity ratio of the first peak (I_1 , 373 nm) to the third peak (I_3 , 393 nm) of pyrene was calculated and plotted against the decadic logarithm ($\log_{10}$) of concentration to determine the cmc value. All measurements were performed in replicates of four.

Preparation and Characterization of Fatty Acid Grafted Chitosan Polymer Nanomicelles

The fatty acid-*g*-CS polymers were dissolved in 20 mM glacial acetic acid solution in deionized water (pH 6.5) at a concentration of 1 mg/mL.

The solution was extruded through 0.2 μm Whatman Nucleopore polycarbonate membrane (GE Healthcare, PA, U.S.) using Avanti Mini Extruder (Avanti Polar Lipids, Inc., AL, U.S.) to obtain uniform sized micelles. Average hydrodynamic diameter and ζ potential were measured using Zetasizer Nano-ZR (Malvern Instruments, Malvern, U.K.) equipped with a 5 mW He–Ne laser operating at 633 nm wavelength at a 90° constant scattering angle. The average hydrodynamic diameter was calculated by the Stokes–Einstein equation, while the Smoluchowski approximation was utilized to obtain ζ potential from the electrophoretic mobility of the micelles. The size and surface morphology of the micelles were also observed using a DI-300 AFM (Veeco, MN, U.S.A.) operating in tapping mode. Samples were prepared as before, and 3 μL of solution was placed onto freshly cleaved mica plates (grade V-4; $15 \times 15 \times 0.15 \text{ mm}^3$).

The plates were air-dried for a few minutes followed by drying under a flow of nitrogen gas. A pyramidal cantilever with a spring constant range 1.2–6.4 N/m, resonant frequency range 47–90 kHz, and a tip curvature radius of 10 nm was employed, and images were captured at a scan rate of 1 Hz. NanoScope Analysis software version 1.50 (Bruker corporation, CA, U.S.) provided with the instrument was used for analyzing the images. All measurements were performed in replicates of four.

Blood Compatibility Study

To substantiate biocompatibility for in vivo applications, the interaction between amphiphilic fatty acid-*g*-CS polymer and anionic RBCs was evaluated. Blood was collected from adult Sprague-Dawley rats into polypropylene centrifuge tubes precoated with 0.5 M ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2) solution as an anticoagulant. RBCs were harvested by centrifuging the blood at 1500 rpm for 10 min, and the pellet was washed three times using 10 mM PBS (pH 7.4). The RBCs were then suspended in PBS at a final concentration of 5×10^9 cells/mL. The hemolytic activity of CS alone as well as the fatty acid-*g*-CS polymer solutions at increasing concentrations (25, 50, 100, 200, and 500 $\mu\text{g/mL}$) was tested by incubating 900 μL of sample solution with 100 μL of resulting RBCs suspension in a reciprocal water bath at 37 °C for 1 h. PBS treatment was taken as negative control (0% hemolysis), and 1% (v/v) Triton X-100 solution treatment was considered as positive control (100% hemolysis). The samples were then centrifuged at 1500 rpm for 10 min, and the absorbance of the supernatant was measured using a spectrophotometer at 540 nm wavelength ($A_{540\text{nm}}$). The percent hemolysis was calculated using eq 3.

$$\text{hemolysis (\%)} = \frac{A_{\text{polymer}} - A_{\text{PBS}}}{A_{1\%(\text{v/v})\text{TritonX-100}} - A_{\text{PBS}}} \times 100 \quad (3)$$

where A_{polymer} is $A_{540\text{nm}}$ for samples incubated with test polymer samples, A_{PBS} is $A_{540\text{nm}}$ for samples incubated with PBS, and $A_{1\%(\text{v/v})\text{TritonX-100}}$ is $A_{540\text{nm}}$ for samples incubated with 1% (v/v) Triton X-100.

The microscopic examination of incubated RBCs was also performed to observe for morphological changes. The RBCs were observed under 40× magnification of Am Scope inverted light microscope (Melville, NY, U.S.). The images were captured and processed using Am Scope MT version 3.0.0.1 software.⁹

In Vitro Cytotoxicity

The cytotoxicity of the polymers was tested using MTT colorimetric assay in HEK 293 cells (ATCC, Rockville, MD, U.S.).^{42,43} Cells were seeded in 96-well plates at 5×10^3 cells/well in 150 μL of DMEM containing 10% fetal bovine serum (FBS) and cultured for 24 h at 5% CO_2 and 37 °C in an incubator.¹² The cells were then treated with 200 μL of increasing concentrations (100, 200, 500, 1000 $\mu\text{g/mL}$) of CS and fatty acid-*g*-CS polymers in serum-free DMEM. After 48 h of incubation the medium containing the polymer samples was carefully removed and 100 μL of MTT solution (1 mg/mL in serum-free DMEM) was added to each well and allowed to react for 3 h. Consequently, the unreacted MTT was carefully aspirated and the cells were rinsed with cold PBS. Finally, 150 μL of dimethyl sulfoxide was added to dissolve the formazan crystals formed by viable cells and the absorbance was recorded at 570 nm using a microplate reader. The relative cell viability was calculated for the treated cells by taking the cells without any treatment as negative control using eq 4.

$$\text{cell viability (\%)} = \frac{A_{\text{polymer}}}{A_{\text{DMEMonly}}} \times 100 \quad (4)$$

where A_{polymer} is the average absorbance of wells incubated with polymer samples and A_{DMEMonly} is the average absorbance of the control wells incubated with serum-free DMEM.

In Vitro Gene Transfection

In vitro gene transfection assay was performed in HEK 293 cells (ATCC, Rockville, MD, U.S.) to evaluate transfection ability of fatty acid-*g*-CS polymeric nanomicelles. Palmitic acid-*g*-CS polymer having a midrange cmc value of $\sim 58 \mu\text{g/mL}$ was selected as an example to demonstrate the effect of fatty acid grafting onto CS backbone on transfection efficiency (Figure 4C). The pDNA stock solution (100 $\mu\text{g/mL}$) was prepared in sodium acetate buffer (20 mM, pH 6.5). The CS/polymer solution was then added dropwise to the pDNA solution at N to P ratio of 20. The final concentration of polymer was maintained above its cmc. The CS/pDNA and polymer/pDNA solutions were then vortexed for 10 s followed by sonication treatment for 10 min using model 75-T Aquasonic bath sonicator (VWR Scientific Products, GA, U.S.) at room temperature. The polyplexes were allowed to equilibrate at room temperature for 30 min prior to use. The association efficiency of pDNA was determined by

taking 5 mL of the polyplex solutions and centrifuging them at 30 000g for 30 min at 4 °C. pDNA encoding for enzyme β -galactosidase was used for this experiment. The supernatant solution was mixed with Hoechst dye 33342 (1 μ g/mL) at 1:1 ratio (v/v) and analyzed for free pDNA content using a spectrofluorophotometer at excitation and emission wavelengths of 350 and 450 nm, respectively. Total pDNA content was measured using an aliquot of the polyplex solution before centrifugation. The association efficiency was calculated using eq 5.

$$\text{association efficiency (\%)} = \frac{\text{pDNA}_{\text{total}} - \text{pDNA}_{\text{free}}}{\text{pDNA}_{\text{total}}} \times 100 \quad (5)$$

Two different pDNAs encoding for enzyme β -galactosidase and green fluorescent protein were employed to evaluate gene transfection efficiency at protein and cellular levels, respectively. Cells were seeded in 48-well plates at a density of 4×10^4 cells/well in DMEM containing 10% FBS and cultured for 24 h at 5% CO₂ and 37 °C in an incubator to achieve ~60–70% confluency. The culture medium was replaced with serum-free DMEM prior to transfection, and the cells were treated with CS/pDNA and palmitic acid-*g*-CS nanomicelles/pDNA polyplexes containing 1 μ g of pDNA/well at N to P ratio of 20.

Nontreated cells and cells treated with naked pDNA were used as negative and passive controls, respectively. Cells were then incubated for 6 h at 5% CO₂ and 37 °C, following which the transfection medium was replaced with DMEM containing 10% FBS and further cultured for 48 h. The β -galactosidase expression was quantified using Promega β -galactosidase enzyme assay system with reporter lysis buffer (Madison, WI, U.S.) according to the manufacturer's protocol. Total protein content of the cell lysate was quantified using Pierce BCA protein assay kit (Waltham, MA, U.S.) as per the manufacturer's protocol. The β -galactosidase activity was represented as milliunits of β -galactosidase per milligrams of the total protein. For GFP expression images were taken at 4 $\times$ magnification using a Leica DFC 3000G fluorescence microscope (IL, U.S.). All transfection experiments were performed in replicates of six.

Statistical Analysis

Data are expressed as the mean $\pm$ SD. Statistical analyses were performed using two-tailed Student's *t* test and ANOVA. A *p* value of less than 0.05 was considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CS chitosan (50 kDa)

pDNA	plasmid DNA
GFP	green fluorescent protein
¹H NMR	proton nuclear magnetic resonance
FT-IR	Fourier transform infrared spectroscopy
SEC-HPLC	size exclusion high performance liquid chromatography
cmc	critical micelle concentration
HEK 293	human embryonic kidney cell line
EDC·HCl	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
NHS	<i>N</i> -hydroxysuccinimide
DDA%	degree of deacetylation
DA%	degree of acetylation
GR%	graft ratio
DLS	dynamic light scattering
PDI	polydispersity index
AFM	atomic force microscopy
RBC	red blood cell
PBS	phosphate buffered saline
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium

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Figure 1.
 ^1H NMR spectra of chitosan and fatty acid-grafted-chitosan polymers.

Figure 2.
FT-IR spectra of chitosan and fatty acid-grafted-chitosan polymers.

Figure 3. Overlay of SEC-HPLC chromatogram for chitosan (50 kDa) and fatty acid-grafted-chitosan polymers ($n = 3$).

Figure 4.

(A) Changes in the fluorescence spectra of hydrophobic probe pyrene with increasing concentration of fatty acid-grafted-chitosan polymer as it approaches critical micelle concentration. (B) Plot of pyrene fluorescence intensity ratio (I_1/I_3) versus decadic logarithm of polymer concentration in deionized water at room temperature for fatty acid-grafted-chitosan polymer. Arrow indicates the critical micelle concentration of the polymer. (C) Critical micelle concentration ($\mu\text{g/mL}$) of fatty acid-grafted-chitosan polymers with increasing chain length of fatty acid. Data represent the mean $\pm$ SD ($n = 4$); statistically

significant difference ($p < 0.05$) between the cmc values of increasing fatty acyl chain length polymers, analyzed using single factor ANOVA.

Figure 5. Atomic force microscopy images of caproic (top row) and palmitic acid (bottom row) grafted chitosan nanomicelles. A total of $3\mu\text{L}$ of polymer solution in DI water was placed on a freshly cleaved untreated mica plate, and images were captured at a scan rate of 1 Hz using tapping mode. (A) Normal representation. (B) Three-dimensional representation in normal view. (C) Three-dimensional representation in phase contrast view.

Figure 6.

(A) Graphical representation of percent hemolysis in rat red blood cells upon incubation with chitosan and fatty acid-grafted-chitosan polymers at different concentrations. Data represent the mean $\pm$ SD ($n = 4$). (“*” indicates significantly ($p < 0.05$) higher hemolysis than all fatty acid-grafted-chitosan polymers.) (B) Microscopic images of red blood cells exposed to chitosan and fatty acid-grafted-chitosan polymers at 500 $\mu\text{g/mL}$ concentration, phosphate buffered saline (PBS, pH 7.4), and 1% (v/v) Triton X-100, respectively, taken at 40 $\times$ magnification using Am Scope inverted light microscope. (C) Picture images of tubes after centrifugation following a 1 h incubation with different concentrations of chitosan and fatty acid-grafted-chitosan polymers.

Figure 7. Graphical representation of percent relative cell viability at different concentrations of chitosan and fatty acid-grafted chitosan polymers. Cytotoxicity was evaluated in HEK-293 cell line using MTT assay. Data represent the mean $\pm$ SD ($n = 4$).

Figure 8.

In vitro gene transfection efficiency of chitosan and palmitic acid-grafted-chitosan nanomicelles in HEK 293 cells at N/P ratio of 20. (A) Transfection efficiency at protein level using β -galactosidase encoding pDNA. Data represent the mean $\pm$ SD ($n = 6$). (“*” indicates significantly ($p < 0.05$) higher transfection than naked pDNA, and “#” indicates significantly ($p < 0.05$) higher transfection than chitosan/pDNA polyplex.) (B) Fluorescence microscopy images of green fluorescent protein pDNA transfected HEK 293 cells at 4 $\times$ magnification 48 h after transfection.

Scheme 1.
 Hydrophobic Modification of Chitosan Using Fatty Acids via Carbodiimide Mediated Coupling Reaction

Table 1.

Degree of Acetylation (%) and Graft Ratio (%) of Chitosan and Fatty Acid-Grafted-Chitosan Polymers Determined Using ^1H NMR and FT-IR, Respectively

polymer sample	degree of acetylation (%) (using ^1H NMR)	grafting ratio (%) (using FT-IR)
chitosan (CS)	14.64	
caproic acid- <i>g</i> -CS	21.32	32.39
caprylic acid- <i>g</i> -CS	24.40	34.28
capric acid- <i>g</i> -CS	19.19	32.25
lauric acid- <i>g</i> -CS	13.40	40.08
myristic acid- <i>g</i> -CS	24.56	33.54
palmitic acid- <i>g</i> -CS	21.86	24.59
stearic acid- <i>g</i> -CS	26.48	29.57
arachidic acid- <i>g</i> -CS	29.52	28.57

Table 2.Retention Time of Chitosan and Fatty Acid-Grafted-Chitosan Polymers Determined Using SEC-HPLC^a

sample	RT ₁ (min)	RT ₂ (min)	RT ₃ (min)
chitosan (50 kDa, CS)	22.85		29.00
caproic acid- <i>g</i> -CS	24.19	26.56	29.32
caprylic acid- <i>g</i> -CS	24.21	26.54	29.28
capric acid- <i>g</i> -CS	24.20	26.56	29.28
lauric acid- <i>g</i> -CS	24.20	26.76	29.64
myristic acid- <i>g</i> -CS	24.50	26.24	28.88
palmitic acid- <i>g</i> -CS	24.65	25.93	28.53
stearic acid- <i>g</i> -CS	24.12	25.89	28.53
arachidic acid- <i>g</i> -CS	24.31	26.60	29.51

^aRT₁ = retention time 1; RT₂ = retention time 2; RT₃ = retention time 3.

Average Hydrodynamic Size, Polydispersity Index, and ζ Potential of Chitosan and Fatty Acid-Grafted-Chitosan Polymers in Deionized Water at Room Temperature^a

Table 3.

polymer sample	average hydrodynamic diameter $\pm$ SD (nm)	polydispersity index (PDI) $\pm$ SD	ζ potential $\pm$ SD (mV)
chitosan (CS)	216.90 $\pm$ 4.60	0.30 $\pm$ 0.17	22.08 $\pm$ 2.07
caproic acid- <i>g</i> -CS	294.63 $\pm$ 3.62	0.24 $\pm$ 0.04	2.65 $\pm$ 0.49
caprylic acid- <i>g</i> -CS	215.18 $\pm$ 7.21	0.19 $\pm$ 0.05	2.80 $\pm$ 1.20
capric acid- <i>g</i> -CS	213.08 $\pm$ 4.83	0.18 $\pm$ 0.03	5.04 $\pm$ 0.46
lauric acid- <i>g</i> -CS	229.80 $\pm$ 2.77	0.20 $\pm$ 0.07	8.21 $\pm$ 2.69
myristic acid- <i>g</i> -CS	223.09 $\pm$ 12.66	0.31 $\pm$ 0.01	2.31 $\pm$ 0.64
palmitic acid- <i>g</i> -CS	187.20 $\pm$ 3.01	0.08 $\pm$ 0.04	3.37 $\pm$ 0.37
stearic acid- <i>g</i> -CS	190.03 $\pm$ 6.16	0.11 $\pm$ 0.06	2.87 $\pm$ 0.47
arachidic acid- <i>g</i> -CS	213.68 $\pm$ 8.08	0.21 $\pm$ 0.06	2.50 $\pm$ 1.49

^aData represent the mean $\pm$ SD ($n=4$).