

RESEARCH ARTICLE

Preparation and Characterization of Amphoteric Polysaccharides Derived From Chitosan and Gellan Gum

Rysgul N. Tuleyeva^{1,2}  | Gulnur S. Tatykhanova^{1,3}  | Nargiz N. Gizatullina^{1,3}  | Daulet B. Kaldybekov^{1,2}  | Yuliia V. Bardadym⁴  | Vladimir O. Aseyev⁴  | Sarkyt E. Kudaibergenov¹ 

¹Institute of Polymer Materials and Technology, Almaty, Kazakhstan | ²Department of Chemistry and Chemical Technology, Al-Farabi Kazakh National University, Almaty, Kazakhstan | ³Satbayev University, Almaty, Kazakhstan | ⁴Department of Chemistry, University of Helsinki, Helsinki, Finland

Correspondence: Rysgul N. Tuleyeva (risgul_93@mail.ru) | Sarkyt E. Kudaibergenov (skudai@mail.ru)

Received: 30 September 2024 | **Revised:** 30 November 2024 | **Accepted:** 3 December 2024

Funding: This work was supported by Horison 2020, 823883-H2020-MSCA-RISE-2018. Ministry of Science and Higher Education of the Republic of Kazakhstan, AP13067773.

Keywords: carboxymethylated chitosan | degree of quaternization | electrophoretic mobility | isoelectric point | polyampholyte gellan gum | rod-like gold nanoparticles | spherical gold nanoparticles

ABSTRACT

Polysaccharides such as chitosan (Ch) and gellan gum (GG) were chemically modified to produce water-soluble amphoteric polyelectrolytes. These derivatives were synthesized via carboxymethylation and quaternization reactions and characterized using techniques including ¹H NMR, FTIR spectroscopies, elemental analysis, potentiometric titration, and thermogravimetric analysis (TGA). The degree of quaternization of gellan gum (QGG) with trimethylammonium groups was determined to be ~38% as by ¹H NMR spectroscopy; ~35% based on potentiometric titration, and ~39% according to elemental analysis. Similarly, the degree of carboxymethylation of chitosan (CMCh) was calculated as ~37% according to ¹H NMR data, while back potentiometric titration provided a value of ~35%. The modified polysaccharides exhibited distinct isoelectric points (pH_{IEP}) as determined through electrophoretic mobility measurements and conventional viscometric analysis. The data collected from both techniques were in good agreement indicating pH_{IEP} = 2.0–2.5 for the modified gellan gum and pH_{IEP} = 7.0 for the modified chitosan. Amphoteric Ch and GG were used to stabilize spherical (AuNSs) and rod-like (AuNRs) gold nanoparticles, synthesized using “one-pot” and seed-growth methods, respectively. Dynamic light scattering (DLS) and transmission electron microscopy (TEM) confirmed particle binding to the modified polymers. The average diameters of AuNSs stabilized with QGG and CMCh were ~45 and 85 nm, respectively, whereas AuNRs stabilized by QGG and CMCh exhibited dimensions of ~50–55 nm (length) and ~12–14 nm (width). These findings suggest that amphoteric QGG and CMCh-stabilized AuNSs and AuNRs could potentially be used as effective photothermal agents for treating Ehrlich cancer cells, as previously reported by our research group (Macromolecular Chemistry and Physics, 2024, 2400128).

1 | Introduction

Recent advancements have highlighted the potential role of polysaccharide-based materials as stabilizers for gold nanoparticles (AuNPs), improving biocompatibility and therapeutic efficacy while minimizing toxicity [1–5]. The integration of nanotechnology has transformed biomedical engineering, enabling

the development of polymeric nanoparticles for diverse applications, including cancer therapy [6], photothermal treatment [2, 5], and gene-silencing technologies [3, 7]. Chitosan has been extensively used in gene therapy and siRNA delivery, providing protection against enzymatic degradation and facilitating cellular uptake [1, 7]. Similarly, gellan gum has been employed in controlled drug release and as a stabilizer for AuNPs, demonstrating

enhanced cytotoxicity against cancer cells and excellent in vivo biocompatibility [2, 5]. Recently [8] it was shown that the gellan gum is a promising candidate for developing polymeric carriers with extended retention on the ocular surface, aiming to enhance the efficacy of topical ocular drug delivery systems.

Advanced polymeric systems have addressed many limitations of conventional therapies. For instance, surface-functionalized AuNPs, demonstrate remarkable efficiency by selectively targeting cancerous tissues, reducing side effects, and allowing precise heat generation under external stimuli during photothermal therapy. Additionally, gellan-based hydrogels have exhibited significant potential in tissue engineering, gene transfer, and anticancer treatments, showcasing their multifunctional application. Other natural polysaccharides, such as alginate, dextran, and chitosan, further contribute to the stability and delivery efficiency of nanocarriers, extending their applications to regenerative medicine and diagnostic imaging [3, 9–12].

Chitosan (Ch) and gellan gum (GG) are classified as polybases and polyacids, respectively, due to the presence of primary amine in Ch and carboxylic groups in GG within their macromolecular chains. These polysaccharides can be chemically modified into amphoteric polyelectrolytes. Chitosan can be modified into amphoteric forms by introducing carboxylic (or sulfo-) groups, while gellan gum can acquire amphoteric properties through the incorporation of amine (or ammonium) groups [13–24]. For instance, the conjugation of strong anionic sulfonate groups to chitosan (Ch), such as in *N*-(3-sulfo-2-propyl) chitosan salt, is achieved by reacting Ch with 1,3-propane sultone in aqueous medium [25]. Similarly, GG is quaternized by grafting *N*-(3-chloro-2-hydroxypropyl)-trimethylammonium chloride onto the primary hydroxyl groups of gellan through nucleophilic substitution, a process conducted under alkaline conditions [16]. These modifications confer amphoteric properties to the polysaccharides, enhancing their water solubility, biocompatibility, and biodegradability. Amphoteric polysaccharides based on Ch and GG have attracted significant attention for their potential applications as bioactive materials in fields such as drug delivery, antibiofouling coatings, self-healing materials, and bio- and hemocompatible artificial organs [26, 27]. Their dual charge (positive and negative) characteristics enable interactions with diverse molecules, making these biopolymers highly versatile for biomedical and industrial applications.

Various sorption experiments have been conducted using materials including carboxymethyl chitosan based polyampholyte superabsorbents [20, 21], magnetically recyclable macroporous chitosan-g-poly(acrylic acid) hydrogels [28] and cryogels functionalized with aminopolycarboxylic acids. These studies were performed under both batch and fixed-bed column conditions to evaluate their efficacy in removing heavy metal ion from solutions [29]. Furthermore, carboxyethyl chitosan polyampholyte was employed in molecular dynamics (MD) simulations to investigate the structural changes of the polyampholyte in aqueous solutions. The simulations investigated how pH, temperature, and ionic strength influence the polyampholyte's structure, offering valuable molecular-level insights [19].

Chitosan and gellan gum are capable of forming interpolyelectrolyte complexes both with each other [30–32] and with

other polyacids and polybases [22, 33]. Both Ch [34–38] and GG [4, 39–44] are capable of stabilizing the gold nanoparticles (AuNPs) and demonstrate potential in the treatment of Ehrlich cancer cells [5]. Recent reviews [2] have highlighted progress in designing anticancer drug delivery systems involving gellan gum, while advancements [45] include developing pH- and thermo-responsive injectable carboxymethyl chitosan hydrogels containing nanodrugs for combined chemotherapy and photothermal tumor therapy.

Despite these advancements, a review of the literature reveals that the solution properties of amphoteric derivatives of chitosan and gellan gum remain insufficiently explored. Few studies have addressed their role in stabilizing gold nanoparticles (GG) [46–48]. Nevertheless, amphoteric polysaccharides based on Ch and GG hold significant potential as platforms for advanced material development. Their ability to form stable, biocompatible hydrogels and complexes and the potential for chemical modification, make them as valuable candidates for designing next-generation biomedical devices and therapies. These polysaccharides could find applications ranging from drug delivery to photothermal cancer treatments, with future research focusing on optimization to broaden their application and improve their effectiveness.

In the present study, chitosan and gellan gum were functionalized to produce amphoteric derivatives. These modified polysaccharides were characterized using various techniques, including ¹H NMR, FTIR spectroscopies, thermogravimetric analysis (TGA), elemental analysis, potentiometric and conductimetric titrations, and viscosity measurements. Spherical (AuNSs) and rod-like (AuNRs) gold nanoparticles were synthesized via “one-pot” and seed-growth methods in aqueous media and stabilized with polyampholyte chitosan and gellan gum. The nanoparticles were subsequently characterized for their size and morphology. The application of biocompatible and nontoxic amphoteric polysaccharides for coating and stabilizing AuNSs and AuNRs is expected to enhance the stability of colloidal gold nanoparticles in aqueous solutions. This approach holds promise for developing effective photothermal agents for cancer therapy.

2 | Materials and Methods

2.1 | Materials

Chitosan (Ch; commercial low molecular weight, M_n 65,200 Da determined using a GPC), monochloroacetic acid (99%), 2-Propanol (99.9%) and (3-chloro-2-hydroxypropyl) trimethyl-ammonium chloride (CHPTMAC) were purchased from Sigma-Aldrich (Gillingham, UK). Before use, Ch was dissolved in 0.1 M HCl, stirred overnight, vacuum filtered through a PTFE membrane disc filter with Ø 47 mm and 1 µm pore size to remove insoluble residual chitin particles. Ch solution was then purified by dialysis against distilled water and lyophilized. Gellan gum (GG) was purchased from Zhejiang DSM Zhongken Biotechnology Co. Ltd. (China). GG was purified and fractionated by ultrasonic treatment as described in our previous report [8, 49]. The viscosity-average molecular weight (M_v) of GG was determined using the Mark-Kuhn-Houwink equation in 0.025 M tetramethyl-ammonium

chloride $[\eta] = 7.48 \times 10^{-3} M_w^{0.91}$ [50] and yielded M_v 6.43×10^5 Da. Deuterium oxide (D_2O , 99.9% D, Eurisotop, France) and deuterated trifluoroacetic acid (99.5 atom % D, Sigma-Aldrich, USA) were used in NMR experiments. A standard aqueous solution of tetrachloroauric acid ($HAuCl_4$) with a concentration of 100 mg/mL, cetyltrimethylammonium bromide (CTAB, 99%), sodium borohydride ($NaBH_4$, 98.5%) and ascorbic acid were purchased from Sigma-Aldrich (Germany). Dialysis tubing with a molecular weight cut-off of 12–14 kDa was purchased from Sigma-Aldrich (USA). Distilled water was used in all experiments involving aqueous solutions.

2.2 | Synthesis of Carboxymethylated Chitosan (CMCh)

Chitosan was first pre-treated by immersing 2 g of the sample in 50 mL of 40% NaOH solution overnight to produce alkalinized chitosan [51]. The pre-treated chitosan was then filtered and subsequently dissolved in 25 mL of 50% isopropanol with continuous stirring for 30 min. Separately, 5 g of monochloroacetic acid was dissolved in 5 mL of 50% isopropanol, and this solution was added dropwise to the chitosan solution. The reaction mixture was stirred at room temperature for 10 h, followed by dialysis for 2–3 days against distilled water, and then frozen for 24 h. The resulting product CMCh was lyophilized, collected as a powder and stored in a dry place until further use.

2.3 | Synthesis of Quaternized Gellan Gum (QGG)

In order to synthesize quaternized gellan gum (QGG), 0.5 g of fractionated gellan was dispersed in 100 mL of distilled water with stirring for 2 h to ensure complete dissolution. A solution of CHPTMAC in 50 mL of 1 M NaOH was then added dropwise to the gellan gum solution at 50°C, maintaining a QGG/CHPTMAC molar ratio of 1:10 [16]. The reaction mixture was kept at 50°C overnight. To stop the quaternization reaction, 37% HCl was added to adjust the pH to 5.5. The resulting product was purified by dialysis using a cellulose membrane against distilled water until a neutral pH was achieved, ensuring the removal of unreacted compounds. Finally, the purified QGG was freeze-dried and stored in a dry place until further use.

2.4 | Synthesis of AuNSs and AuNRs

Spherical gold nanoparticles (AuNSs) stabilized with gellan gum (GG) and quaternized gellan gum (QGG) were synthesized using a “one-pot” approach, as described [5]. Briefly, a mixture containing polymer solutions, 5 mL of $HAuCl_4$ (100 mg/mL), and 4 mL of 0.5 M KOH were combined, stirred, and heated up to 100°C for 3–5 min in an Anton Paar Monowave 50 microwave reactor (Graz, Austria) equipped with temperature and time controllers. The reaction produced solutions that changed color from yellow to dark red or purple, indicating the successful formation of AuNSs. The pH of the initial solution, which was 12, reduced to pH 8 following the dialysis against deionized water.

Chitosan-gold nanoparticles were synthesized following the procedure described by Huang, Yuan, and Yang [52]. Initially,

20 mg of chitosan was dissolved in 10 mL of 1% acetic acid to yield a 2 mg/mL solution. The mixture was vortexed until fully dissolved and then stored overnight. Subsequently, 2 mL of this solution was filtered through a 0.22 μ m polyethersulfone (PES) syringe filter and mixed with 1 mL of 10 mM $HAuCl_4$ solution under vigorous stirring for 30 min. A freshly prepared 0.4 mL of cold 100 mM $NaBH_4$ (used as a reducing agent) was then gradually added, resulting in a rapid color change from yellow to wine-red, indicating the formation of gold nanoparticles. The reaction was allowed to continue with stirring for an additional 2 h to complete the process. Chitosan-gold nanoparticles were purified using the dialysis against deionized water (5 L; water changed 8 times).

In order to synthesize gold nanorods (AuNRs), the seed-mediated growth method was employed [4, 5]. Initially, a solution consisting of 5 mL of 0.2 M CTAB was mixed with 5 mL of 0.5 mM $HAuCl_4$ and stirred. Subsequently, 0.6 mL of chilled 0.01 M $NaBH_4$ was added, producing a brownish-yellow solution that contained gold nanoseeds. In parallel, another solution composed of CTAB (0.2 M; 30 mL), $AgNO_3$ (4 mM; 1.5 mL), and $HAuCl_4$ (1 mM; 30 mL) was gently mixed, and 0.42 mL (78.8 mM) ascorbic acid was introduced. Ascorbic acid served as a mild reducing agent, causing the color of the growth solution to change from dark yellow to colorless. Finally, the initial seed solution (72 μ L) was added to the growth solution, and the mixture was allowed to incubate at 30°C overnight. Consequently, a crimson solution containing gold nanorods (AuNRs) was generated. To remove by-products and excess CTAB, the solution underwent centrifugation at 10,650 rpm for 30 min using an Eppendorf 5810R centrifuge (Tuttlingen, Germany). The resulting precipitate was then re-dispersed in 3 mL of deionized water and centrifuged again at 10,650 rpm for 15 min. After repeating the washing cycle three times, the AuNRs were re-dispersed and stabilized in 5 mL of the designated polymer solutions and then dialyzed against deionized water using a cellulose membrane.

2.5 | Characterization

2.5.1 | Nuclear Magnetic Resonance (^1H NMR) Spectroscopy

^1H NMR spectra of the samples were recorded using a 500 MHz Bruker Avance III NMR spectrometer (Bruker UK Ltd., Coventry, UK). The samples were prepared at 10 mg/mL. GG and QGG samples were dissolved in D_2O , while Ch and CMCh samples were dissolved in D_2O by adding a few drops of deuterated trifluoroacetic acid (TFA-d, 99%).

2.5.2 | Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra of the samples were recorded using an IRTracer-100 Shimadzu FTIR spectrometer (Kyoto, Japan). The spectra were collected from an average of 16 scans between 4000 and 500 cm^{-1} , employing the absorbance mode with a spectral resolution of 4 cm^{-1} . The spectra were plotted and analyzed using Origin 2022 (version 9.9.0.225) software (OriginLab Corporation, MA, USA).

2.5.3 | Potentiometric Titration

Potentiometric titration of chlorine ions from QGG – $[N^+(CH_3)_3Cl^-]$ groups was performed with $AgNO_3$ solution using an automatic titrator Metrohm 905 Titrando (Metrohm AG, Herisau, Switzerland). A total of 10 mg of QGG was dissolved in 20 mL distilled water and 0.1 M $AgNO_3$ aqueous solution was added dropwise into QGG solution with 0.01 mL increment. The degree of quaternization (DQ) was calculated using the following equation:

$$DQ = \frac{n_{\text{quaternary ammonium groups}}}{n_{\text{polymer repeating units}}} \quad (1)$$

$$n_{\text{quaternary ammonium groups}} = C_{AgNO_3} \times V_{AgNO_3}$$

$$n_{\text{polymer repeating units}} = \frac{m_{\text{polymer}}}{M_{\text{repeating unit}}}$$

where, C is a concentration of $AgNO_3$ (mol/L), V is a volume of $AgNO_3$ (L), m is a mass of quaternized gellan gum used (g), and M is a molecular weight of the repeating unit of gellan gum (g/mol).

2.5.4 | Elemental Analysis

Elemental analysis was conducted to determine the composition of the modified gellan gum in comparison to GG. The measurements were performed using a Vario MICRO cube instrument (Elementar Analysensysteme GmbH, Hanau, Germany), with sulfanilamide as the calibration standard. Three samples, each approximately 2 mg of dry polymer, were analyzed and the mean average values were reported.

2.5.5 | Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was performed using a NETZSCH STA 449 F3 Jupiter instrument (Selb, Germany). Samples (8–10 mg) were heated from 25°C to 600°C at a heating rate of 10°C/min in a nitrogen atmosphere. Thermograms were plotted and analyzed using Origin 2022 software.

2.5.6 | Determination of the Isoelectric Point (IEP) by Electrophoretic and Viscometric Measurements

The isoelectric points of CMCh and QGG were determined using a conventional viscometric technique and electrophoretic mobility analysis using a Zetasizer Nano-ZS90 instrument (Malvern Instruments, Malvern, UK), by varying pH levels of the solutions. All measurements were conducted in triplicate at 25°C. Briefly, 0.05 g of QGG was prepared in 10 mL of deionized water at room temperature and stirred until complete dissolution. Simultaneously, 0.05 g of CMCh was dissolved in 10 mL of 0.03 M HCl. The specific viscosity measurements were carried out using an Ostwald-type capillary viscometer (capillary diameter of 0.86 mm) and calculated as the ratio of flow time (t) to that of water (t_0):

$$\eta_{sp} = \frac{t - t_0}{t_0} \quad (2)$$

The isoelectric point ($IEP_{\text{viscometry}}$) for each sample was determined by measuring the pH at which the polymer solution reached its lowest viscosity, indicating that the net charge of the macromolecules is close to zero at that specific pH.

In electrophoretic mobility experiments, a refractive index of 1.33 and an absorbance of 0.01 were used for all measurements in zeta-potential mode at 25°C. Viscosity (0.8872 cP) and refractive index (1.33) of water served as dispersant parameters. Each sample was analyzed five times, and the results were processed using the Smoluchowski model ($Fka = 1.50$) to calculate the mean electrophoretic mobility $\pm$ standard deviation values. The electrophoretic mobility versus pH curve was generated for each test sample and the point at which the curve intersects zero mobility was considered as an IEP_{EM} [53].

2.5.7 | Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) measurements were performed using a Zetasizer NanoZS90 instrument (Malvern Instruments, Malvern, UK). All solutions were filtered through 0.45 μ m PES syringe filters. The reported values are intensity-weighted diameters, calculated as averages of repetitive measurements at 25°C. Similar refractive index and absorbance values were used as in the electrophoretic mobility measurements mentioned above.

2.5.8 | Transmission Electron Microscope (TEM)

The morphology of the particles was examined using a Hitachi S-4800 FESEM (Tokyo, Japan). For TEM imaging, dilute dispersions (5 μ L) were placed on carbon-coated copper grids with a mesh size of 200 square micrometers and air dried prior to imaging.

3 | Results and Discussion

3.1 | Synthesis and Characterization

Following purification by dialysis and lyophilization, modified polymers were then characterized using ^1H NMR spectroscopy.

The ^1H NMR spectra of both Ch and CMCh recorded in CCl_3COOH/D_2O showed a distinct methyl characteristic peak at 2.01 ppm from $-NHCOCH_3$ methyl protons, as well as a singlet at 3.12 ppm (H2) and multiplets from 3.50 to 3.90 ppm (H3–H6) corresponding to the methine protons in polysaccharide ring (Figure 1). Additionally, new signals appeared at 3.30; 4.10 and 4.24–4.30 ppm in CMCh, which were attributed to the protons of the $-O-CH_2-COO^-$ group substituted at the C2 amino group; the C6 and C3 hydroxyl groups of Ch, respectively. These findings are in good agreement with previous observations [54]. The signal at 4.10 ppm was significantly stronger, while that at 3.30 ppm was lower, suggesting that carboxymethylation occurred mainly at the

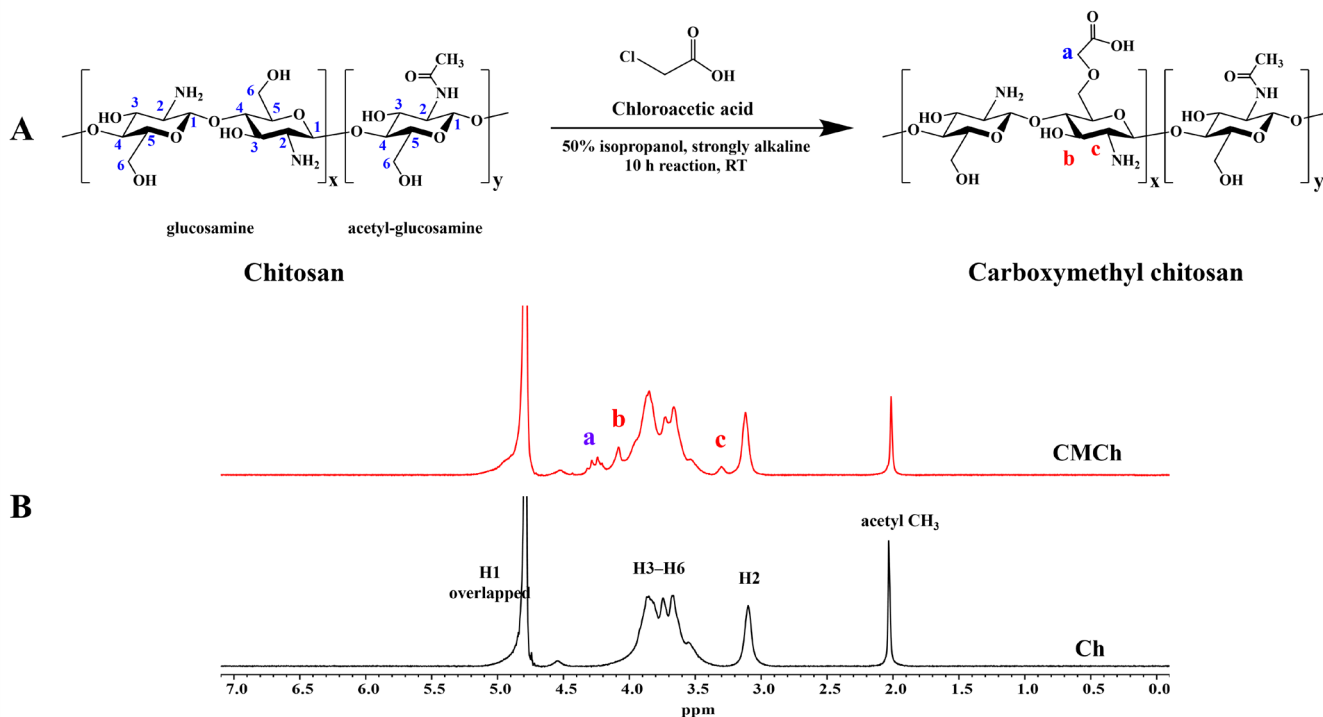


FIGURE 1 | Synthesis and characterization of carboxymethyl chitosan (CMCh). (A) Schematic illustration of the carboxymethylation reaction. Please note that the schematic structure shows only one possibility of a chloroacetic acid reaction with the hydroxyl groups (which are the reactive sides) at C6 in the polysaccharide backbone. In reality, it could also react with any primary amino (C2) and/or hydroxyl (C3) groups present in chitosan and (B) ^1H NMR spectra of chitosan (Ch) and carboxymethyl chitosan (CMCh) recorded in $\text{CCl}_3\text{COOH}/\text{D}_2\text{O}$.

C3 and C6 hydroxyl (or O-carboxymethylation), but much less at the amino C2 groups, that is, N-carboxymethylation. Perhaps this depends on different reaction conditions that favor obtaining either O-carboxymethyl or N-carboxymethyl chitosan [51, 55]. Nevertheless, the chitosan backbone allows carboxymethylation at three potential locations: C6-O-, C3-O-, and C2-N-. Based on ^1H NMR analysis, it was observed that the carboxymethylation preferentially occurs at hydroxyl groups, specifically favoring the C6-O- site when the reaction temperature exceeds 20°C . As such, the synthesized CMCh includes both amino (C2-N site) and carboxymethyl (C6-O site) functional groups [14]. The degree of substitution (DS%) was calculated by integrating the corresponding peaks and using the following equation:

$$\text{DS\%} = \left\{ \frac{\frac{I_{\text{C3 and C6-substituted}}(-\text{O}-\text{CH}_2-\text{COOH})}{4} + \frac{I_{\text{C2-substituted}}(-\text{N}-\text{CH}_2-\text{COOH})}{2}}{I_{\text{C2,H}}} \right\} \times 100, \quad (3)$$

where, $I_{\text{C3 and C6-substituted}}(-\text{O}-\text{CH}_2-\text{COOH})$ are the integral of the signals of methylene protons from C3 and C6 substituted $-\text{O}-\text{CH}_2-\text{COOH}$ moieties, while $I_{\text{C2,H}}$ is the integral of the signal corresponding to the methine protons in the polysaccharide's glucosamine unit. Figures S1 and S2 display the ^1H NMR spectra of modified chitosan with corresponding peak integrals as well as the monomer, respectively.

In ^1H NMR spectrum of gellan gum, distinct signals can be observed: the peak at 1.30 ppm corresponds to the methyl protons of the rhamnose unit, while the peak at 4.53 ppm is assigned

to the hydrogen bonded to C1 of glucose II. Additionally, the peak at 5.15 ppm is associated with the hydrogen attached to C1 of the rhamnose unit. The solvent peak (D_2O) is assigned at 4.80 ppm and chemical shifts of all protons of the tetrasaccharide repeating unit formed by glucose I, glucuronic acid, glucose II, and rhamnose can be observed in the range between 3.34 and 4.25 ppm (Figure 2). In the ^1H NMR spectrum of QGG, a new peak at 3.22 ppm is identified, which corresponds to the vibrations of three pairs of protons from $[\text{N}^+(\text{CH}_3)_3]$ group. The degree of quaternization (DQ%) for QGG was quantified by integrating the corresponding peaks and using the following equation:

$$\text{DQ\%} = \left\{ \frac{I_{\text{N}(\text{CH}_3)_3}}{I_{\text{CH}_3(\text{rhamnose})}} \times \frac{3}{9} \right\} \times 100, \quad (4)$$

where, $I_{\text{N}(\text{CH}_3)_3}$ represents the integral of the signal corresponding to the methyl protons of newly substituted cationic N,N,N-trimethyl moiety observed at 3.22 ppm, while $I_{\text{CH}_3(\text{rhamnose})}$ refers to the integral of the signal associated with the methyl protons of the rhamnose unit that appeared at 1.30 ppm. Figures S3 and S4 depict the ^1H NMR spectra of modified gellan gum with corresponding peak integrals and the monomer, respectively.

The FTIR spectra of chitosan and CMCh are illustrated in Figure 3A. In the FTIR spectrum of chitosan, strong characteristic peaks corresponding to the amino groups were observed at approximately 3328 (O—H stretch and N—H stretch), 1651 cm^{-1} (C=O stretching, amide I), and 1375 cm^{-1} .

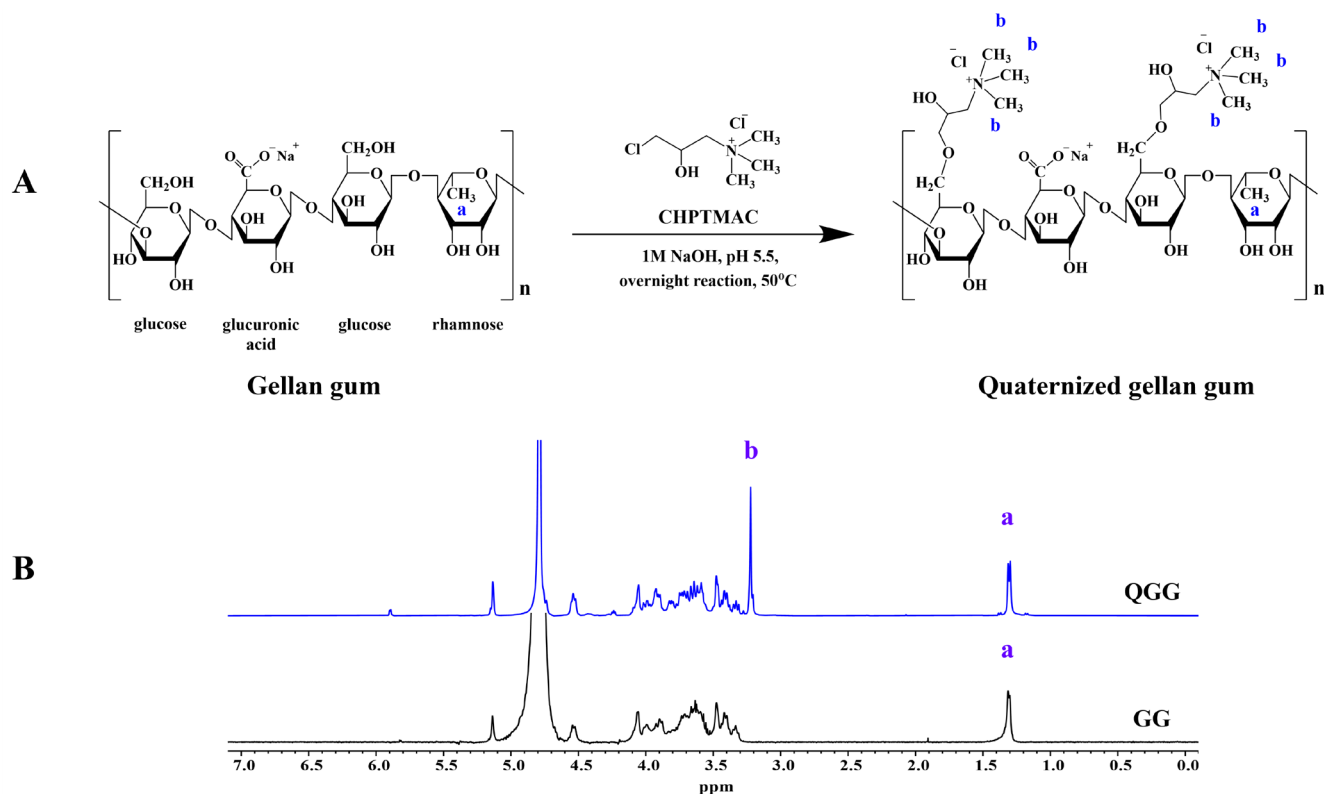


FIGURE 2 | Synthesis and characterization of quaternized gellan gum (QGG). (A) Schematic illustration of the quaternization reaction. Please note that the schematic structure illustrates only one possibility of (3-chloro-2-hydroxypropyl)trimethyl-ammonium chloride (CHPTMAC) reaction with $-\text{CH}_2-\text{OH}$ groups of gellan gum. In reality, it could react with any hydroxyl groups present in gellan gum and (B) ^1H NMR spectra of gellan gum (GG) and quaternized gellan gum (QGG) recorded in D_2O at 60°C .

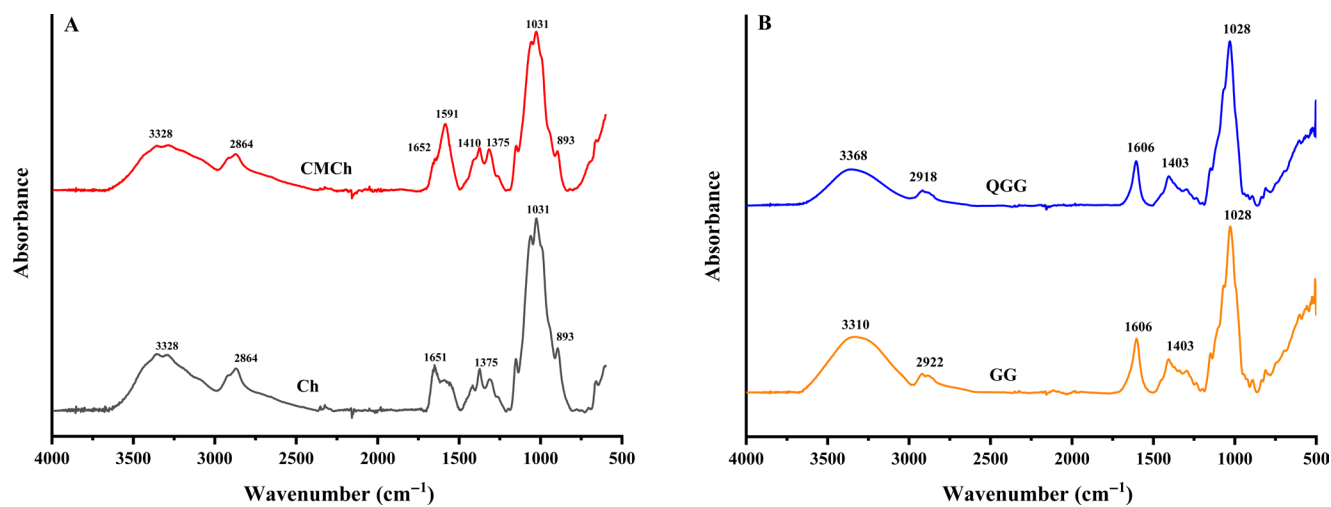


FIGURE 3 | The FTIR spectra of (A) chitosan (Ch) and carboxymethyl chitosan (CMCh); and (B) gellan gum (GG) and quaternized gellan gum (QGG).

Additionally, peaks associated with the polysaccharide structure were identified at 1153cm^{-1} (C—H stretching) and 1031cm^{-1} (C—O stretching) [56]. In the CMCh spectrum, the broad band observed at 3328cm^{-1} is attributed to the vibrational stretching of the O—H bonds and the symmetric stretching vibration of C=O bonds within $-\text{COOH}$ groups, which both overlapped in that region. This peak, along with the peak at 1591cm^{-1} , which originates from the symmetric stretching vibration of the $-\text{COO}^-$ group, substantiates the

incorporation of carboxymethyl groups onto the chitosan backbone. Additionally, two bands at 1652 and 1410cm^{-1} , assigned to NH_3^+ , suggest that carboxymethylation has occurred at the OH positions. The peaks observed at 1375 and 1309cm^{-1} correspond to the symmetric angular deformation of C—H bonds and the C—N stretching vibration (amide III), respectively. The peak at 2864cm^{-1} is attributed to the C—H stretching vibration, while the stretching vibration of the C—O bonds in the $-\text{OCH}_2\text{COOH}$ group accounts for the peak at

1260 cm⁻¹. Peaks in the range between 1031 and 893 cm⁻¹ are attributed to the vibrations of C—O and C—O—C bonds, along with other bonds constituting the polysaccharide chain [57]. Thus, analysis of FTIR spectra confirms the successful introduction of carboxymethyl groups onto the chitosan backbone.

The modification of gellan gum was further confirmed using FTIR spectroscopy (Figure 3B). FTIR analysis of spectra clearly revealed key absorbance peaks: the spectrum of pristine gellan gum displays a broad band (3000–3600 cm⁻¹) with a maximum peak at 3310 cm⁻¹ corresponding to O—H stretching vibrations, and a band at 2922 cm⁻¹, which is attributed to asymmetric and symmetric C—H stretching vibrations in CH— and CH₂— groups. Additionally, peaks at 1606 and 1403 cm⁻¹ are assigned to the asymmetric and symmetric vibrations of the —COO⁻ group, respectively. The peak at 1028 cm⁻¹ indicates the characteristic vibration of the C—O—C bond within the rhamnose ring or the β-(1–4) glycosidic linkage [58, 59], with the additional C—O

TABLE 1 | Elemental analysis of gellan gum (GG) and quaternized gellan gum (QGG).

Biopolymer	Element content (wt.%)			
	Nitrogen	Carbon	Hydrogen	Sulfur
GG	0.05	36.31	5.60	0.00
QGG	1.14	38.07	6.67	0.00

Abbreviations: GG: gellan gum; QGG: quaternized gellan gum.

TABLE 2 | Degree of quaternization of GG and carboxymethylation of chitosan (CMCh).

Method	¹ H NMR	Elemental analysis	Potentiometric titration
QGG (%)	38	38.87	34.55
CMCh (%)	37	—	35.20

Abbreviations: CMCh: carboxymethylated chitosan; QGG: quaternized gellan gum.

bond from the incorporated cationic hydroxypropyl group overlapping in the 1000–1150 cm⁻¹ region and an increase in peak intensity. A characteristic absorption band (symmetric bending) typical for C—H of the methyl groups in the quaternary ammonium should appear at ~1450–1480 cm⁻¹ in modified gellan gum spectrum; however, in our case, it was overlapped by the broader signals of the —COO⁻ group [60]. Nevertheless, the incorporation of the cationic moiety caused shifts in the hydroxyl bands of gellan gum to higher frequencies (3368 cm⁻¹). Overall, similar infrared spectra were acquired for QGG and GG, suggesting the limitations of FTIR spectroscopy in confirming the effectiveness of gellan gum derivatization. However, elemental analysis confirmed the presence of amine groups in QGG.

The results of the elemental analysis for both GG and QGG are displayed in Table 1. The analysis reveals a trace amount of nitrogen (0.05 wt.%) in pristine GG is likely attributed to the residual cell proteins or nutrients from the fermentation process [61]. In contrast, the nitrogen content in modified gellan gum is notably significant. This increase substantiates the successful grafting of quaternary ammonium groups onto the gellan gum backbone. The degree of quaternization (DQ) determined according to Equation (1) is 34.55%.

Table 2 summarizes the degrees of modification for QGG and carboxymethylated chitosan (CMCh), as determined by ¹H NMR spectroscopy, elemental analysis, and potentiometric titration. The data indicate that all methods yield results that are in satisfactory agreement. Degrees of substitution calculated based on analysis of ¹H NMR spectra of modified polymers are in good agreement with the values reported in the literature [16, 62].

Figure 4A presents the thermogravimetric analysis (TGA) thermograms of chitosan and CMCh. The TGA curve for chitosan displays two distinct stages of weight loss within the temperature range of 28°C–490°C. The initial weight loss, occurring between 28°C and 124°C, is attributed to the evaporation of absorbed water, accounting for ~5% (0.5 mg) of the total weight. The primary degradation of chitosan commences at 250°C and concludes around 490°C, resulting in a total weight loss of about

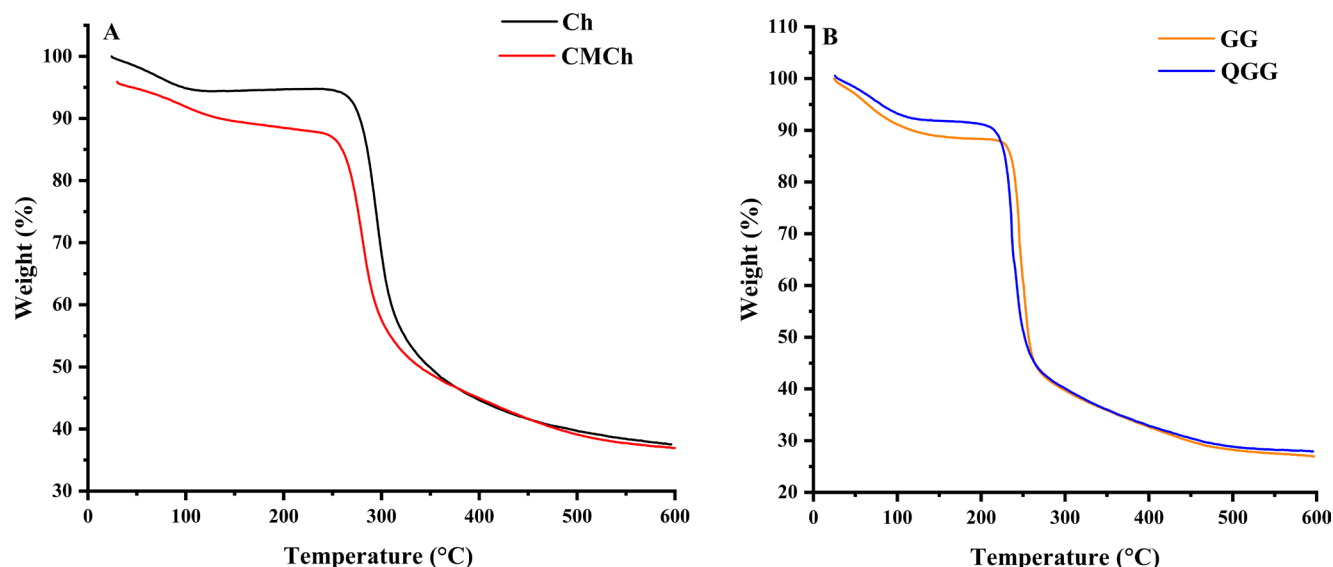


FIGURE 4 | Thermogravimetric analysis of Ch, CMCh (A), GG and QGG (B).

55% (5.5 mg). In the case of CMCh, the TGA curve similarly exhibits two distinct stages of weight loss (Figure 4A). The initial stage, occurring between 40°C and 159°C, is likely due to the loss of adsorbed water with 9% weight loss (1.0 mg). The subsequent stage of decomposition, spanning from 232°C to 447°C, corresponds to thermal degradation and results in a weight loss of approximately 54% (5.9 mg). These results suggest a slight reduction in the thermal stability of CMCh compared to pure chitosan. Similar results were reported for other chitosan derivatives [63, 64].

Figure 4B shows the TGA results for both gellan gum and quaternized gellan gum. All samples exhibit a weight loss within the temperature range from room temperature up to ~100°C resulting in evaporation of absorbed or bound water with around 11% (1.08 mg). The initial degradation temperature and the temperature at which the main degradation phase occurs are slightly lower for the modified gellan gum compared to unmodified gellan gum, shifting from 247°C to 237°C with a total weight loss of 58.53% (5.07 mg). This observation is in good agreement with the findings reported for similar quaternized polysaccharides [65, 66].

3.2 | The Isoelectric Points (IEP) of Polymers

CMCh and QGG possess both carboxylic and amino groups, which enable them to exhibit anionic, cationic, or quasi-electroneutral properties depending on the pH of the solution. In acidic and basic environments, the macromolecules of CMCh and QGG are either positively or negatively charged. At the isoelectric point (IEP), these polymers achieve a quasi-electroneutral state due to the balanced compensation of opposing charges. At this point, the polyampholyte chains adopt their most compact conformation, resulting in a minimum in solution viscosity [67].

In this study, the IEPs or isoelectric pH values (pH_{IEP}) of both CMCh and QGG macromolecules were determined using electrophoretic mobility measurements and by analyzing changes in solution viscosity as a function of pH. Aqueous solutions (0.5% w/v) of CMCh and QGG were titrated by adding drops of 0.03 M HCl or 0.02 M NaOH, and the resulting pH changes were recorded using a pH meter. The IEPs of the samples were estimated by determining the pH at which the electrophoretic mobility curve intersected zero (Figure 5) or at which the polyampholyte solution reached a minimum specific viscosity (Figure 6). It was observed that the IEP_{EM} of CMCh and QGG are pH 7.0 and pH 2.0, respectively (Figure 5). The IEP determined by viscometric technique for CMCh is pH 7.0 (Figure 6A), which is in good agreement with the value obtained through electrophoretic mobility measurement. The $\text{IEP}_{\text{viscometry}}$ of QGG was found to be pH ~2.50 (Figure 6B). This discrepancy is likely attributed to the measurement accuracy of each technique. It should be noted that the pH-dependent electrophoretic mobility of GG remains negative across the entire pH range due to the presence of only carboxylic groups. In contrast, chitosan (Ch), which contains amine groups, exhibits a zero crossing of electrophoretic mobility at pH ~8.60, remaining negative at higher pH levels (Figure 5). This behavior of Ch has been previously explained in literature [68] by the

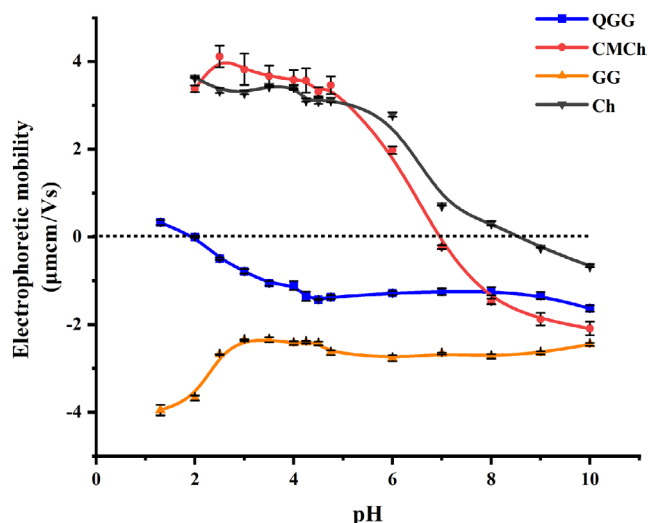


FIGURE 5 | Electrophoretic mobility of GG, QGG, Ch, and CMCh as a function of solution pH.

fact that the protonated $-\text{NH}_2$ groups are significantly compensated by the accumulation of counterions in the diffusive part of the electric double layer in line with Manning theory [69] and experimental studies on poly(*N,N*-dimethyl-*N,N*-diallylammonium chloride) [70] and poly-L-lysine [71]. Our findings are consistent with the explanations provided by the studies observed in [68].

The observed difference in the isoelectric points (IEP) between quaternized gellan gum (pH ~2.0–2.5) and carboxymethyl chitosan (pH ~7.0) can be explained by the varying ratios of acidic and basic groups in each modified polysaccharide. QGG, with approximately 62% carboxylic groups, exhibits an IEP at a lower pH. In contrast, CMCh has a greater proportion of amine groups (~63%), resulting in a higher IEP. This behavior is consistent with established findings that the IEP of polyampholytes shifts to lower pH as the content of acidic groups increases, and to higher pH with more basic groups present in the macromolecular chain [72, 73].

As anticipated, the difference between the IEP values determined by electrophoretic mobility and viscometry likely arises from the fundamental principles underlying each technique. Electrophoretic mobility, or electrophoresis, is generally more precise for IEP determination as it directly measures the net charge of the macromolecule. For instance, in a quasi-neutral state, the polyampholyte macromolecule does not migrate toward either electrode under an applied electric field, allowing for an accurate determination of zero charge. In contrast, the viscometric method observes changes in solution viscosity, that is, changes in the hydrodynamic volume, which vary with the macromolecular conformation. Nonetheless, both techniques place the IEP of QGG between pH 2.0 and 2.5, demonstrating good overall agreement.

The relationship between the hydrodynamic size of polyampholytes and pH is depicted in Figure 6. For CMCh, the minimum hydrodynamic size of macromolecules is observed at pH 7.0, which is in good agreement with the results obtained

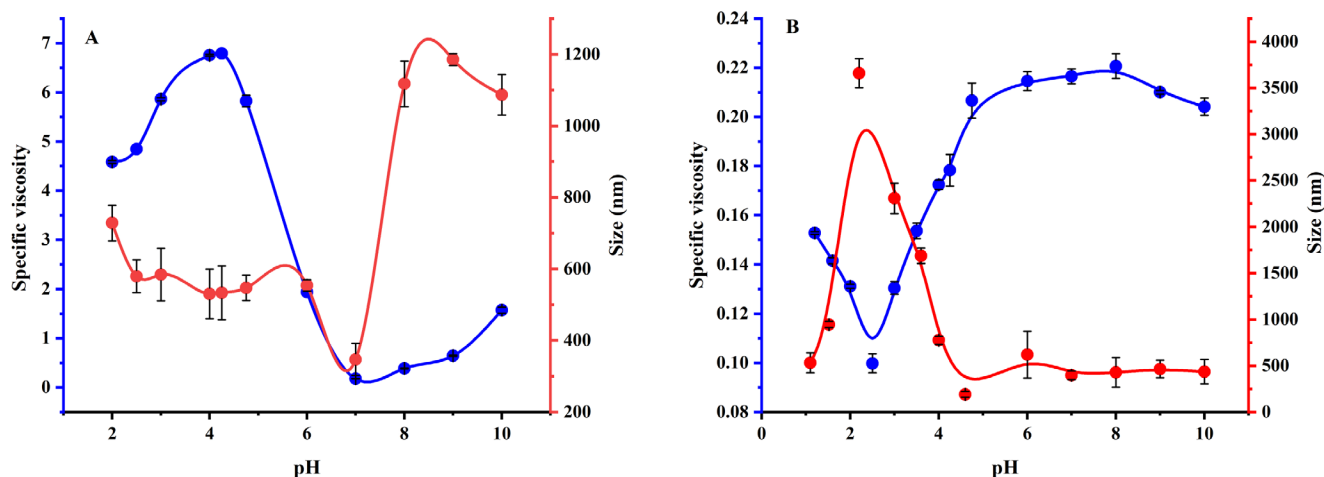


FIGURE 6 | Determination of the IEP and hydrodynamic size of CMCh (A) and QGG (B) by viscometric and DLS techniques.

TABLE 3 | Size and zeta-potential values of polymer-protected AuNSs determined by DLS at pH 6.50–7.0.

Type of polymer	Concentration of polymer (% w/v)	Mean diameter (nm)	PDI	Zeta-potential (mV)
Ch	0.2	99 ± 2	0.442	42 ± 2
CMCh	0.2	85 ± 1	0.414	40 ± 1
GG	0.5	170 ± 2	0.402	−15 ± 1
QGG	0.5	45 ± 2	0.318	−25 ± 2

Note: Data are expressed as mean ± standard deviation values of five measurements ($n = 5$).

Abbreviations: Ch: chitosan; CMCh: [O-carboxymethyl chitosan](#); GG: gellan gum; PDI: polydispersity index; QGG: quaternized gellan gum.

from electrophoretic and viscosity measurements. At an IEP, the polyampholyte chains reach their compact conformation due to electrostatic attractions between oppositely charged functional groups [73]. Deviations from the IEP lead to changes in the overall charge of the polyampholyte, causing it to become either positively or negatively charged. This results in increased electrostatic repulsion between uniformly charged groups and, consequently, an expansion of macromolecular chains. In contrast, for QGG the average hydrodynamic size of the macromolecules sharply increases near the IEP. This phenomenon is likely attributed to the aggregation of high molecular weight QGG (~ 10^5 Da) around the IEP due to intra-macromolecular complexation of the polyampholytes [74] and no precipitation of polymer from the solution was detected.

3.3 | Stabilization of Gold Nanoparticles (AuNPs) by Amphoteric Gellan Gum and Chitosan

A water-soluble amphoteric chitosan derivative, 6-O-carboxymethyl chitosan, which possesses a nearly equivalent number of cationic and anionic functional groups, was used as a pH-sensitive template for the reversible assembly of gold nanoparticles (AuNPs) [46, 47]. Aggregation of AuNPs was observed at extreme pH values of 2.0 and 12.0, resulting in aggregates with markedly different morphologies. TEM microphotographs at pH 9.60 reveal that the average diameter of the spherical nanoparticles is ~5 nm. At pH 12.0, the AuNPs aggregate into needle-like structures, with particle–particle distances within these aggregates measuring less than 3 nm. Conversely,

at pH 2.0, the gold nanoparticles form larger, more densely packed aggregates. The morphology of these aggregates is more regular, with a length of 360 nm and a width of 150 nm, displaying symmetrical angles at the cluster ends. Depending on the pH level, the color of the colloidal dispersions changes from purple at pH 2.0 to pink at pH 9.60, and pale purple at pH 12.0. The reversibility of these changes upon pH alteration was confirmed by a shift in the plasmon resonance UV-absorption band, which moved from 526 nm (at pH 2.0) to 510 nm (at pH 9.60), and then to 515 nm (at pH 12.0).

The chemical modification of chitosan with phthalimide moieties results in the synthesis of two water-soluble bisphthalimides (BPIs), specifically, *N,N'*-(1,3-phenylene)bis(phthalimide-5-carboxylic acid) (BPI-1) and *N,N'*-(1,4-phenylene)bis(phthalimide-5-carboxylic acid) (BPI-2). These bisphthalimides, with relatively low molecular weights, are effective in the green synthesis of gold nanoparticles (AuNPs) [48]. ¹H NMR, FTIR, UV/Vis spectroscopy analyses and TEM imaging demonstrate that the BPIs can reduce gold ions and stabilize AuNPs in colloidal solutions. Consequently, the AuNPs exhibited a quasi-spherical shape, with average diameters of 3 nm for BPI-1 and 12 nm for BPI-2. The reaction to produce AuNPs was most rapid with the polyampholyte derivative of chitosan, completing in 5 min, which is attributed to the high number of oxidizable groups present in the polymer structure and its high molecular weight. The average size of AuNPs stabilized with this polyampholyte was 10 nm. Thus, polyampholyte derivatives of chitosan can effectively stabilize gold nanoparticles across a broad pH range, producing quasi-spherical particles with average diameters of 3, 5, 10,

TABLE 4 | Aspect ratio (measured using TEM) and zeta-potential values of AuNRs synthesized and stabilized using Ch, CMCh, GG, and QGG at pH 6.50–7.0.

Type of polymer	Concentration of polymer (% w/v)	Average length (nm)	Average width (nm)	Aspect ratio	Zeta-potential (mV)
Ch	0.5	55 ± 4	14 ± 2	4 ± 1	70 ± 1
CMCh	0.5	54 ± 3	14 ± 1	4 ± 1	17 ± 2
GG	0.5	50 ± 6	13 ± 3	4 ± 1	−31 ± 2
QGG	0.5	51 ± 3	12 ± 2	4 ± 1	−25 ± 2

Note: Data are presented as mean ± standard deviation of five measurements (*n* = 5).
Abbreviations: Ch: chitosan; CMCh: O-carboxymethyl chitosan; GG: gellan gum; QGG: quaternized gellan gum.

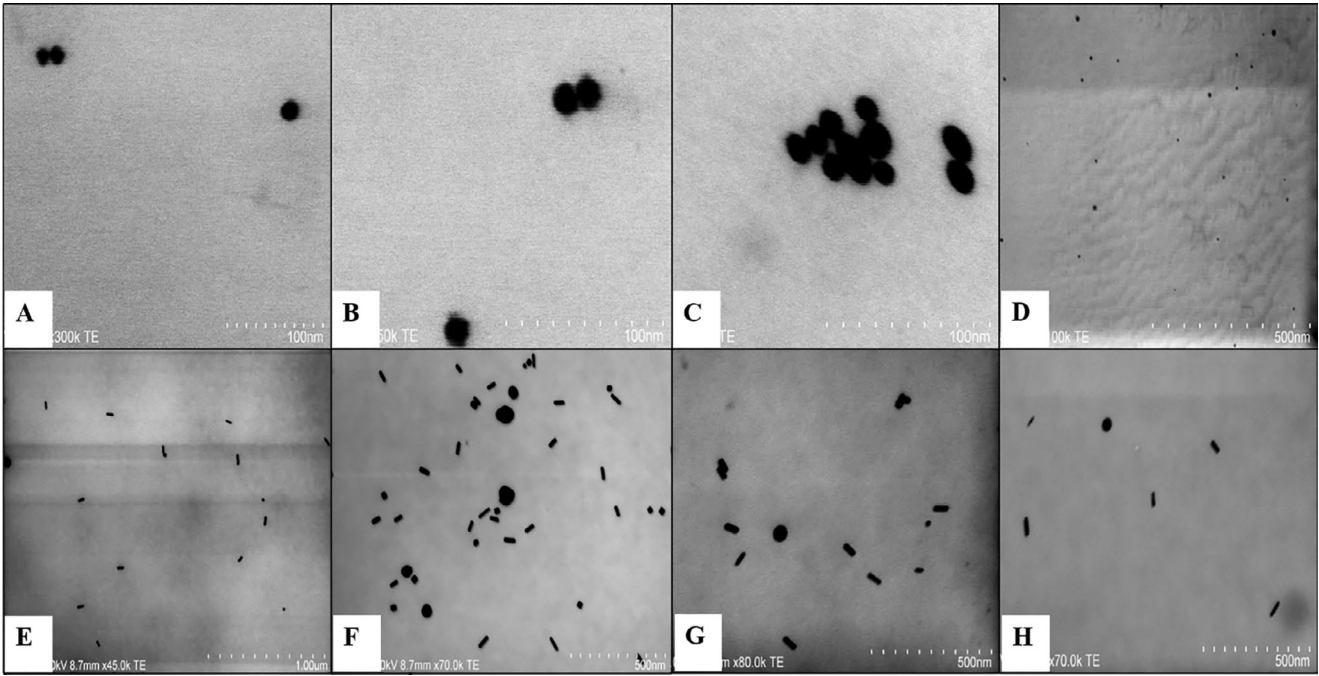


FIGURE 7 | TEM microphotographs of AuNSs and AuNRs stabilized using Ch (A and E), CMCh (B and F), GG (C and G), and QGG (D and H).

and 12 nm, as well as rod-like aggregates measuring 360 nm in length and 150 nm in width (aspect ratio of 2.4).

In this study, spherical and rod-shaped gold nanoparticles were synthesized and subsequently stabilized by QGG and CMCh. Their average hydrodynamic sizes and zeta-potential values of are summarized in Tables 3 and 4. The mean size and zeta-potential values of the spherical gold nanoparticles (AuNSs) were measured using dynamic light scattering (DLS). Generally, all spherical gold nanoparticles exhibited polydispersity. To evaluate the surface charge of the nanoparticles, the zeta-potential values of the various AuNPs were determined, as detailed in Table 3. Figure 7 displays the representative TEM microphotographs for AuNSs and AuNRs stabilized by Ch, GG, CMCh, and QGG. For the gold nanorods (AuNRs) the aspect ratio, that is, length/width, was $\approx 4 \pm 1$.

In summary, the AuNPs stabilized by amphoteric polysaccharides described in this study may serve as a potential platform for the effective photothermal therapy of Ehrlich cancer cells. This prospective application extends our previous research [5].

4 | Conclusion

Amphoteric polysaccharides derived from chitosan and gellan gum were synthesized using a procedure involving monochloroacetic acid and (3-chloro-2-hydroxypropyl) trimethylammonium chloride as modification agents. The resulting polysaccharides, namely carboxymethylated chitosan (CMCh) and quaternized gellan gum (QGG), were characterized using various physico-chemical techniques. The degree of quaternization for QGG and the degree of carboxymethylation for CMCh were determined to be $\sim 38\%$ and $\sim 37\%$, respectively. Both QGG and CMCh demonstrated polyampholytic behavior in aqueous solutions, with isoelectric points (IEPs) observed at pH 7.0 for CMCh and pH 2.0–2.5 for QGG. Spherical (AuNSs) and rod-like (AuNRs) gold nanoparticles were synthesized in the presence of these polysaccharaides through “one-pot” and seed-growth methods, respectively. Dynamic light scattering (DLS) and transmission electron microscopy (TEM) analyses revealed that the average diameter of AuNSs stabilized with modified polymers (QGG and CMCh) were determined to be ~ 45 and ~ 85 nm, respectively, whereas the average dimensions of AuNRs stabilized by QGG

and CMCh were ~50–55 (length) as well as ~12–14 (width). Future research will aim to evaluate the light-to-heat conversion efficiency of these gold nanoparticles stabilized by amphoteric polysaccharides. Additionally, toxicological assessments and mucoadhesive studies will be conducted to optimize the anti-cancer efficacy of the developed nanoparticle systems.

Acknowledgments

This research was funded by the Science Committee of the Ministry of Science and High Education of the Republic of Kazakhstan (Grant No. AP13067773) and was supported by the Horizon 2020 research and innovation program of the European Union Maria Skłodowska-Curie (grant agreement 823883-NanoPol-MSCA-RISE-2018). The authors are grateful to ALD center Finland research infrastructure (University of Helsinki) for their help with transmission electron microscope. Yuliia Bardadym is thankful to the Academy of Finland (No. 353886 and 359456 for Ukrainian researchers) for her financial support.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.