DEVELOPMENT OF MUCOADHESIVE CHITOSAN-BASED DRUG DELIVERY SYSTEM

Mariya Konovalova[1], Balzhima Shagdarova[2], Anastasia Zubareva[2], Alexander Generalov[1], Maria Grechikhina[1], Elena Svirshchevskaya[1]*

[1] Shemyakin&Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, GSP-7, 16/10, Miklukho-Maklaya st., Moscow 117997, Russia

[2] Institute of Bioengineering, Research Centre of Biotechnology of the Russian Academy of Sciences. 33, bld. 2 Leninsky Ave., Moscow 119071, Russia
**e-mail: esvir@ibch.ru*

Abstract

Chitosan is a highly versatile biopolymer characterised by low toxicity, biocompatibility, and slow but complete biodegradation in the human body, possessing multiple reactive groups. One of the most well-known properties of positively charged chitosan derivatives is their ability to bind mucous membranes. The aim of this work was the analysis of mucoadhesion of unmodified 20 kDa chitosan and its hydrophobic (HC) and hydrophobic quaternised (QHC) derivatives in vitro and ex vivo. Unmodified chitosan formed large aggregates in vitro in keratinocyte and colon cell cultures and ex vivo in murine small intestine and muscle explants. At the same time, HC and especially QHC bound cells in vitro and ex vivo in a fine dotted manner, as evidenced by confocal microscopy. Such a pattern of hydrophobic derivatives distribution provides the possibility to develop mucoadhesive drug delivery systems with increased local drug release and improved chitosan biodegradation.

Key words: *chitosan, hexanoylchitosan, quaternised chitosan, mucoadhesion, drug delivery systems*

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1. Introduction

The development of carriers for targeted drug delivery is one of the priority areas of research. The directed delivery of drugs can overcome many of the difficulties caused by random drug distribution in the body and reduce the effective dose and systemic toxicity. Targeted drug delivery systems of a new generation should meet a number of requirements, including the high selectivity of accumulation in specific tissues, low toxicity of the carrier, and the ability to immobilise different types of drugs without losing their functional activity. Chitosan, the nearest deacetylated derivative of the natural chitin polysaccharide, meets these criteria in many aspects. Chitosan, unlike many other biocompatible polymers, has a large number of reactive amino groups, which allows the synthesis of derivatives with specified properties in respect to hydrophilicity/hydrophobicity, size, charge, and the ability to attach drugs covalently or incorporate them via hydrophobic and/or van-der-Waals interactions.

The most well-known property of chitosan and its positively charged derivatives is the ability to adhere to mucosal surfaces and to open intercellular contacts called tight junctions [1–4]. Currently, chitosan and its derivatives are permitted for medical use in the form of wound-healing films [5,6]. The major mechanism of wound-healing by chitosan-based films results from its mucoadhesive properties and high positive charge, which mediates antimicrobial activity [1,2]. Chitosan also increases the activity of polymorphonuclear leukocytes, macrophages and fibroblasts, which accelerate tissue formation, and promotes wound healing through the restoration of mesothelial cells [7,8]. However, the application of chitosan films is mostly limited to the skin application. Delivery systems for topical, oral, or parenteral application require a soluble form of the vehicle. A major way to develop such soluble carriers is the production of different forms of chitosan or chitosan/copolymer based nanocontainers, nanogels, or nanoparticles [2,9–11]. However all nanosized delivery systems have limitations: they poorly bypass epidermal and mucosal barriers as topical applications and are delivered mostly to the liver and kidney after parental injection due to their relatively large size. Direct conjugates of molecular chitosan-drugs can have some advantages. We previously showed that positively charged 200 kDa chitosan modified by anhydride of hexanoic acid can be retained for several days on cell membranes [12]. Shelma *et al.* demonstrated that N-acylation of chitosan by hexanoyl, lauroyl, or oleoyl groups increased mucoadhesion, with the best results for oleoyl-chitosan [13]. Due to low solubility at neutral pH, positively charged chitosan derivatives form large aggregates on cell membranes. Chitosan solubility can be increased by its quaternisation [14,15]. There have been multiple attempts to use quaternised chitosan for the development of drug delivery systems, most of which are nanocarriers [16–19]. Dual chitosan derivatives containing both hydrophobic and ammonium groups are rarely used. Amphiphilic chitosan derivatives were synthesised by de Oliveira *et al*. from trimethylammonium and dodecyl aldehyde-modified chitosan [20]. The aim of this work was the analysis of mucoadhesive properties of hydrophobic quaternised low molecular weight chitosan *in vitro* and *ex vivo*.

2. Materials and methods

2.1. Materials

Chitosan with a molecular mass (MM) of 20 kDa and degree of deacetylation (DD) of 98% was obtained by hydrolysis with hydrochloric acid, as described earlier [21]. Caproic anhydride (Fluka, Germany), WGA-AlexaFluor 584, MitoTreckRed (Invitrogen

MolecularProbes, USA), Hoechst 33342, fluorescienisothyocyanate (FITC), and glycidyl trimethylammonium chloride (Sigma, USA) were used as purchased.

2.2. Synthesis of chitosan derivatives

Chitosan 20 kDa, DD 98% 2 g (12.4 mmol) was dissolved in 120 ml 1% acetic acid and 200 ml methanol. Caproic anhydride (1.5 mmol) was added drop-wise during 20 min of shaking, and then titrated with 12% ammonium until pH 8.5 was reached; the resulting sediment was dialysed against water and lyophilised. The yield of acylated hydrophobic derivative hexanoylchitosan (HC) was 50-70%.

For the synthesis of a quaternised derivative, 1 g (6.2 mmol) of HC was dissolved in 30 ml of distilled water at 85°C, and then 2.12 ml (15.8 mmol) of glycidyl trimethylammonium chloride was added in three equal portions at 1 h intervals. The reaction mix was stirred for 5 h at 85°C. Then, the mixture was slowly added to 33 ml of chilled acetone. The resulting precipitate was separated by decantation, dissolved in 17 ml of methyl alcohol and precipitated in a mixture of acetone/ethanol (4/1 vol/vol). The resulting precipitate was again separated by decantation and freeze-dried. All chitosan derivatives were analysed by ^{1}H-NMR. SD for quaternary ammonium groups was 40-50% and of the hydrophobic groups was 10-15%. The yield of quaternised HC (QHC) was 66-75%.

2.3 Synthesis of chitosan-FITC conjugates

For the *in vitro* analysis of mucoadhesion, fluorescent conjugates of chitosan, HC, and QHC with FITC were synthesised. To this end, 25, 5, or 1 nmol FITC dissolved in DMSO was added to 5 mg of chitosan, HC, or QHC in 1 ml of 50 mM acetate buffer. The reaction mixture was stirred for 1.5 h at room temperature in the dark and dialysed against 50 mM acetate buffer for 24 hours with three changes. The efficiency of FITC immobilisation and purity of chitosan-FITC conjugates from free FITC was analysed by 1% agarose (Fluka, Germany) gel electrophoresis in TAE buffer. Chitosan samples were loaded in DNA loading buffer (Fermentas, USA) and run at 10 V per 1 cm of gel.

2.4. *In vitro* analysis of chitosan mucoadhesion

Mucoadhesion of chitosan, HC, and QHC was studied by confocal microscopy. Human keratinocyte cells HaCaT and murine colon cancer cell line Colon-26 were cultivated in RPMI-1640 containing 7% foetal calf serum, 300 μg/mL L-glutamine, and 100 μg/mL penicillin/streptomycin (all from PanEco, Russian Federation). After cell trypsinisation, 150 μL of suspension containing 10^6 cells/mL was seeded onto sterile cover glasses placed onto 6-well plates and cultivated in a CO_2 incubator at 37°C overnight until cell adhesion. FITC-labelled chitosan derivatives (20 μg/mL) were added to cells and incubated at 37°C for 4 h and 24 h. The nuclei stain Hoechst 33342 and membrane and complex Golgi stain WGA-AlexaFluor 584 were added for the last 30 min of incubation. At the end of incubation, cells were fixed in 1% paraformaldehyde, washed three times with phosphate buffer, and sealed on glass slides by Mowiol 4.88 (Calbiochem, Germany). Slides were analysed using an Eclipse TE2000 confocal microscope (Nikon, Japan).

2.5. Time-dependent deposition of chitosan on cell membranes

HaCaT and Colon-26 cells were seeded in 24-well plates until adhesion. FITC-labelled chitosan samples were added at 100 μg/mL. Cells were trypsinised in the dynamics of chitosan binding at 5, 120 and 240 min, washed and then analysed by flow cytometry (FACScan, BDm USA). Data are shown as MFI_t/MFI_0 ratios, where MFI_t

corresponds to the mean fluorescent intensity at the time of cell collection.

2.6. *Ex vivo* analysis of chitosan mucoadhesion

For the *ex vivo* analysis of chitosan, HC, and QHC mucoadhesion, explants of the small intestine and muscles were obtained from BALB/c mice (Pushchino Farm, Moscow district). Mice were sacrificed by cervical dislocation, the small intestine was removed, washed of faeces, and cut in 1 cm lengths per sample. The abdominal wall was dissected, washed out, and cut into 0.5 cm^2 per sample. Explants were incubated in 24 well plates in 200 μL saline. FITC-labelled chitosan derivatives were either injected into the intestine or applied onto the muscle sheet at 50 μg/sample. Explants were incubated for 1.5 hours at 37°C, washed from the chitosan samples, fixed in 1% paraformaldehyde for 30 min and frozen in Tissue-Tek freezing media (Sakura, Japan) overnight. Samples were cut into 12 μm sections using a cryotome (ThermoScientific, USA). Cryosections were thawed at room temperature, stained with Hoechst 33342 and mitochondrial dye MitoTreckRed for 30 min and sealed by a cover glass using Mowiol 4.88. Slides were analysed using the Eclipse TE2000 confocal microscope (Nikon, Japan).

3. Results and Discussion

3.1. Characterisation of chitosan derivatives

The ^{1}H-NMR spectra of the original chitosan, HC, and QHC are shown in Figure 1 A-C. The arrows show the signals of H3-H6 protons of the glucopyranose group (dark arrows) and H2 proton (light arrows). Parentheses show the signals of hexanoyl and ammonium protons (Fig. 1, B-C). The SD for quaternary ammonium groups was 40–50% and of the hydrophobic groups was 10–15%.

The efficiency of FITC binding to chitosan was estimated by electrophoresis on a 1% agarose gel. The results were visualised by FITC fluorescence. FITC is a negatively charged molecule that migrates to the anode, which has a positive charge (Fig. 1, D). It contains two fractions with different mobility in the electrophoretic field. All chitosan samples also contained two fractions: a positively charged fraction of chitosan-FITC conjugates, which migrated to the cathode (-) with different velocities, and a negatively charged fraction, migrating to the anode (+) (Fig. 1, D). The latter is likely to represent a fraction of chitosan-FITC conjugates containing the chitosan molecules with the lowest MM conjugated to too many FITC molecules, which forms a net negative charge for this fraction.

Gel electrophoresis showed that the migration of unmodified chitosan was low; the inclusion of hydrophobic groups increased the mobility of HC, while the introduction of ammonium groups in HC resulted in the highest mobility. These results show that hydrophobic and especially quaternised chitosan derivatives are more soluble at neutral pH than the unmodified sample, which corresponds to the data about chitosan solubility presented in the literature [14,15].

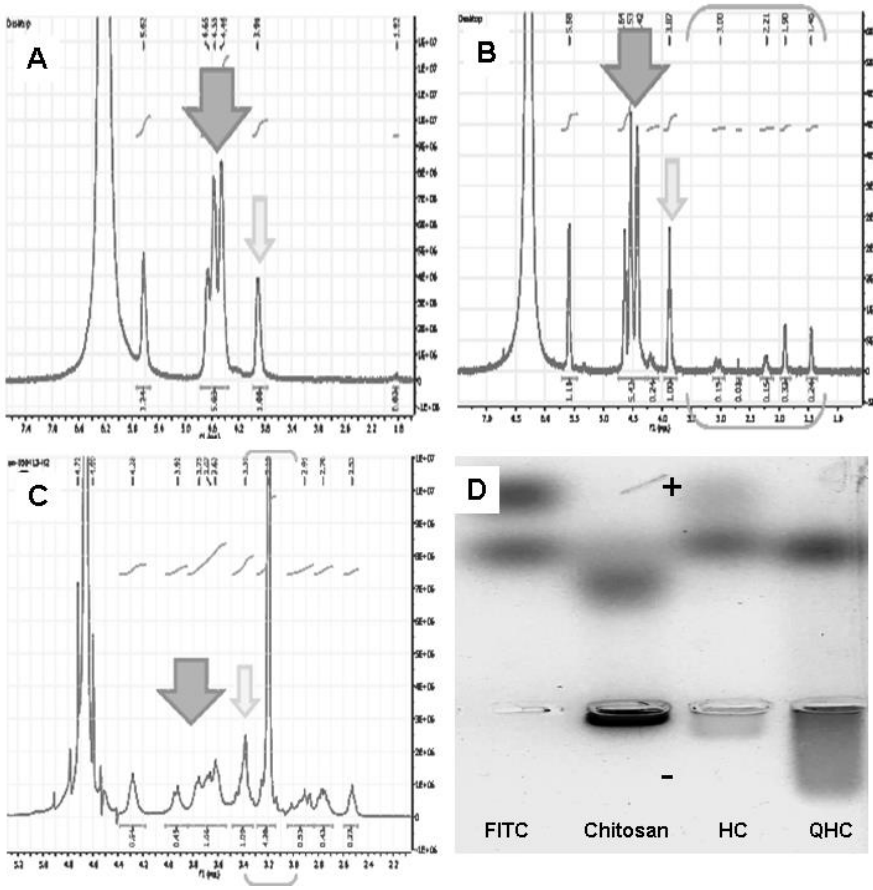

Figure 1. Characterisation of chitosan derivatives. A-C. ^{1}H-NMR spectra of chitosan (A), HC (B), and QHC (C). The arrows show the signals of H3-H6 protons of the glucopyranose group (dark arrows) and H2 proton (light arrows). Parentheses show the signals of hexanoyl and ammonium protons. D. Gel-electrophoresis in 1% agarose gel of FITC-labelled chitosan conjugates. The cathode and anode are marked "-" and "+", respectively. The FITC images were obtained using a UV transilluminator.

3.2. Analysis of chitosan mucoadhesion *in vitro*

Mucoadhesion is the property shown by biopolymers to bind mucous membranes, such as in the eyes, oral cavity, or gastrointestinal tract. Mucoadhesion of positively-charged biopolymers is mostly as a result of polyelectrolyte complex formation with negatively-charged cell membranes. The binding of polymers to cell membranes *in vitro* mimics its ability for mucoadhesion. To this end, the human keratinocyte cell line HaCaT and murine colon carcinoma cell line Colon-26 were incubated with FITC-labelled chitosan or its derivatives for 2 and 24 hours and analysed by confocal microscopy. The results demonstrated that unmodified chitosan forms aggregates which were located around the cells but not on their membranes (Fig. 2 A, D).

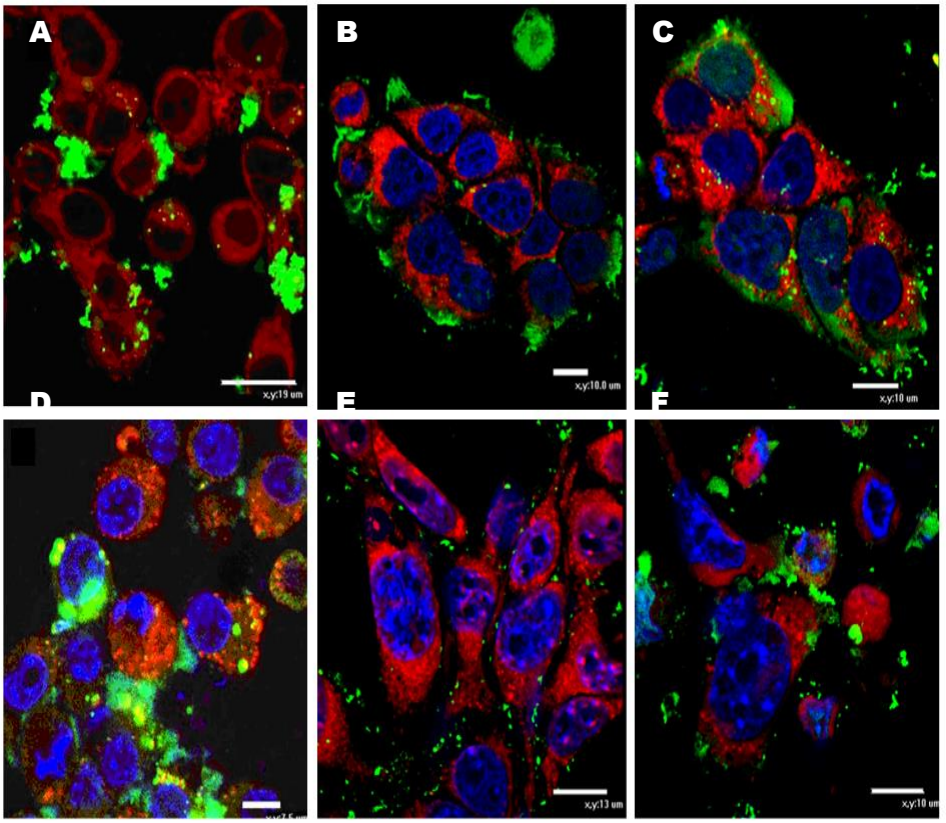

Figure 2. Analysis of chitosan mucoadhesion *in vitro*. Confocal images of HaCaT (A-C) and Colon-26 (D-F) after 2 h incubation with FITC-labelled chitosan (A, D), HC (B, E), and QHC (C, F) (green). Counterstaining was conducted by mitochondrial tracker Red CMXRos (red) and nuclear stain Hoechst (blue). Scale bar 10 μm.

The hydrophobic derivatives HC and QHC bound cell membranes after 2 h (Fig. 2, B, C, E, F) and much better after 24 h of incubation (Fig. 3). The pattern of HC and QHC binding was comparable. HC membrane localisation was additionally shown by co-localisation with a complex Golgi tracker (Fig. 3, A). The absence of the intracellular localisation of QHC was supported by mitochondrial staining (Fig. 3, B). The dotted pattern of chitosan derivative distribution in cell membranes show that it forms 200–300 nm particles immersed in the cell glycocalyx.

The dynamics of chitosan binding was analysed by flow cytometry. All chitosan samples were associated with cells for at least 240 h. Flow data showed that not only hydrophobic derivatives but also unmodified chitosan was associated with cells in spite of its aggregation. Chitosan binding and prolonged deposition on cell membranes is shown by many groups [22–24].

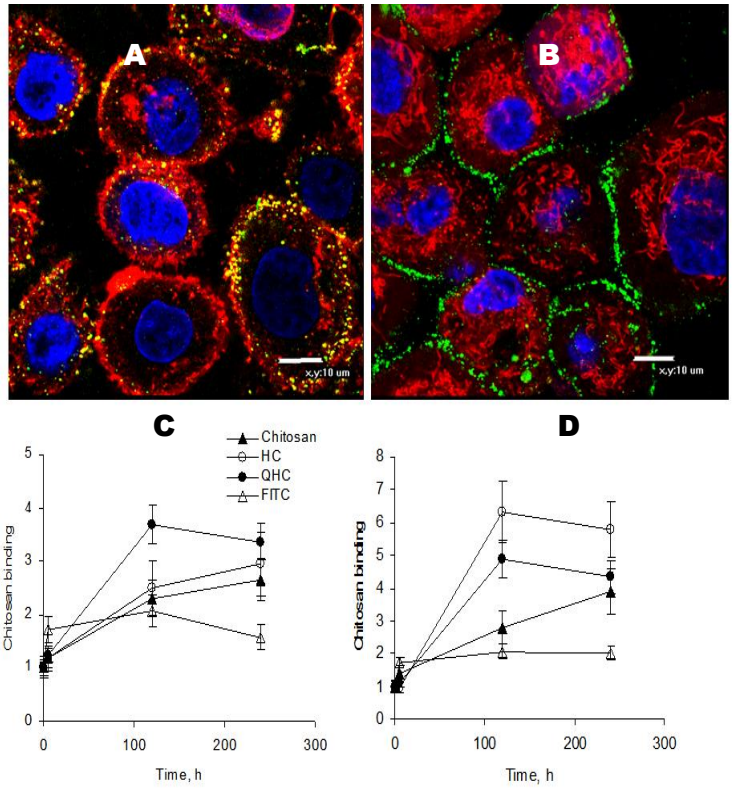

Figure 3. Analysis of chitosan mucoadhesion *in vitro*. A-B. Confocal images of Colon-26 (B) cells after 24 h incubation with HC (A) or QHC (B) (green) counterstained by a membrane tracker WGA-AlexaFluor 584 (A, red) or mitochondrial tracker Red CMXRos (B, red) and nuclear stain Hoechst (blue). Scale bar 10 μm. C-D. Flow cytometry analysis of the time-dependent deposition of chitosan derivatives on HaCaT (C) and Colon-26 (D) cells. Data are shown as the MFI_t/MFI_0 ratio, where MFI_t corresponds to the mean fluorescent intensity at the time of cell collection.

3.3. Analysis of chitosan mucoadhesion *ex vivo*

Finally, we have studied the mucoadhesion of chitosan and its hydrophobic derivatives to tissue explants. The incubation of chitosan samples with fragments of the small intestine for 1.5 h resulted in the diffusion of chitosans to the basal membrane (Fig. 4, white lines). QHC was the only sample which bound specifically to the villi of epithelium, because QHC localised within individual cells (Fig. 4, D, H, white arrows).

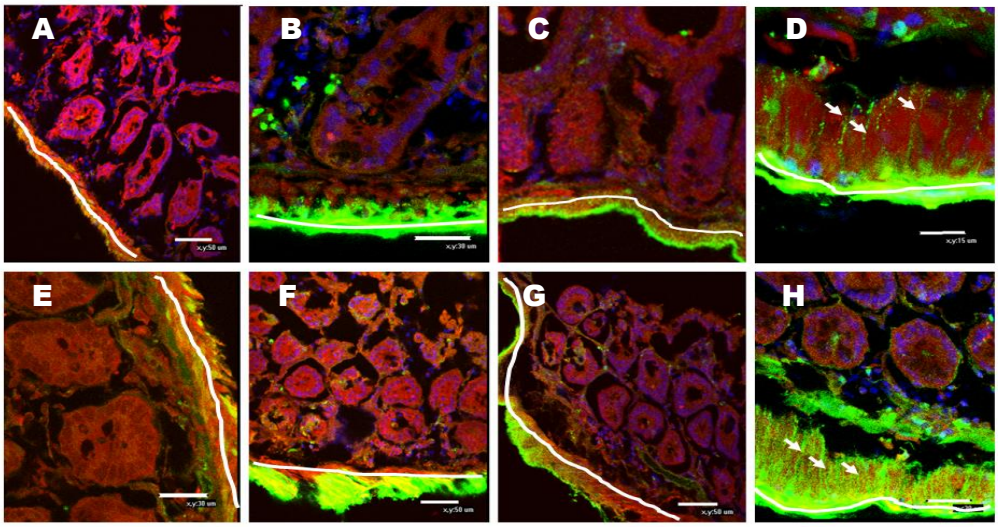

Figure 4. Analysis of chitosan mucoadhesion *ex vivo*. Confocal images of a mouse small intestine after incubation with FITC (A, E), chitosan (B, F), HC (C, G), or QHC (D, H) (green). Samples were incubated with chitosan samples for 1.5 h, fixed by paraphormaldehyde, and cryosectioned. Fixed samples were counterstained by mitochondrial tracker Red CMXRos (red) and nuclear stain Hoechst (blue). The basal membrane is shown as a white line. Scale bar 30 μm.

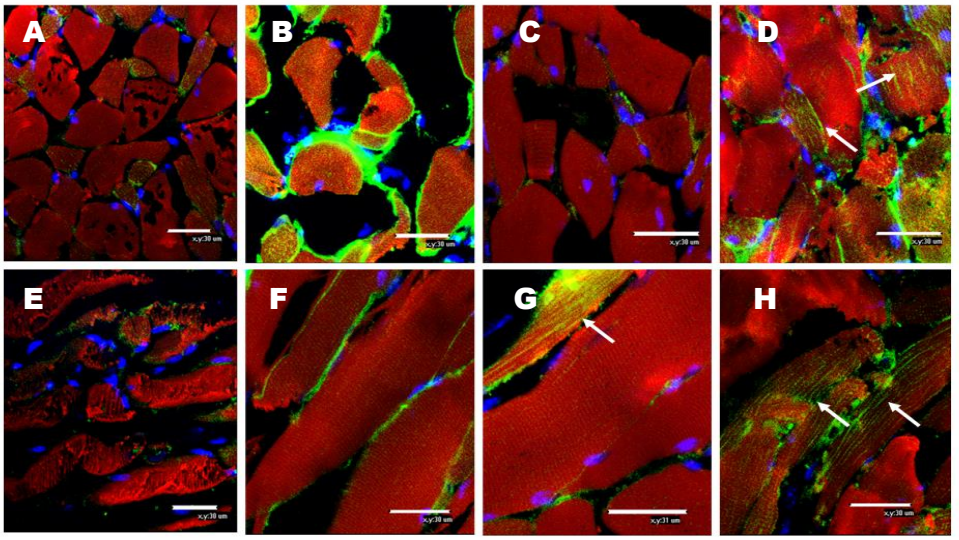

Figure 5. Analysis of chitosan mucoadhesion *ex vivo*. Confocal images of mouse abdominal wall muscles after incubation with FITC (A, E), chitosan (B, F), HC (C, G), or QHC (D, H) (green). Samples of muscles were incubated with chitosan samples for 1.5 h, fixed by paraphormaldehyde, and cryosectioned. Fixed samples are counterstained by mitochondrial tracker Red CMXRos (red) and nuclear stain Hoechst (blue). Scale bar 30 μm.

Similar results were obtained for muscle explants. All chitosan samples, but not free FITC, were visualised between the muscle fibres (Fig. 5). At the same time, unmodified chitosan formed thick deposits along the fibres (Fig. 5, B, F), while HC and especially QHC localised along individual muscle cells (Fig. 5, C, D, G, H, white arrows).

4. Conclusions

Chitosan is a highly versatile biopolymer characterised by low toxicity, biocompatibility, and slow but complete biodegradation, possessing multiple reactive groups. Mucoadhesive chitosan derivatives can be widely used for the development of immuno-conjugates and nanosized drug delivery systems. Here, we have shown that all positively-charged chitosans demonstrate mucoadhesive properties. However, unmodified chitosan forms large aggregates dispersed between the cells. At the same time, hydrophobic derivatives and especially quaternised hydrophobic derivatives spontaneously form small 200–300 nm particles associated with cell membranes where they are retained for several days. Such a pattern of hydrophobic derivative distribution increases local drug release and improves chitosan biodegradation. The inclusion of hydrophobic moieties provides the ability to include drugs without chemical conjugation, leading to increased drug release, decreased production costs, and the exclusion of toxic components used for chitosan-drug conjugation.

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