Modification of selectively acid-etched halloysite by mucoadhesive chitosan derivatives: new bionanocomposites with improved functional properties

MODIFICATION OF SELECTIVELY ACID-ETCHED HALLOYSITE BY MUCOADHESIVE CHITOSAN DERIVATIVES: NEW BIONANOCOMPOSITES WITH IMPROVED FUNCTIONAL PROPERTIES

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ABSTRACT

In this study, the modification of selectively acid-etched halloysite (eHal) by low molecular weight chitosan (LChi) and its methacrylated derivative (MeLChi) is investigated to form new bionanocomposites with improved functional properties. The formation of nanocomposites was confirmed by various instrumental techniques (ζ-potential measurements, SEM, FT-IR, DSC, XRD and ^{1}H-NMR). Both nanocomposites exhibited improved functional properties while preserving the tubular structure of halloysite. The cytotoxic activity of eHal and eHal-polycation nanocomposites against normal human lung fibroblasts (MRC5) was determined using MTT assay. The results showed good biocompatibility of the prepared nanocomposites, with a cell survival rate of more than 90%. The mucoadhesive properties of eHal and the corresponding chitosan nanocomposites were investigated *in vitro*. Primarily, ζ-potential measurements revealed electrostatic interactions between the eHal-polycation nanocomposites and mucin. The absorption study showed that the eHal-MeLChi nanocomposites could adsorb a greater amount of mucin (≈82%) than the eHal-LChi nanocomposites (≈72%) and eHal (≈58%) after 8 hours of incubation. Furthermore, the compacts of the tested samples were prepared by direct compression, and the detachment force against the mucin compact was measured using a texture analyzer. A significantly higher detachment force of the eHal-MeLChi nanocomposite compact (1.59 ± 0.07 N) from the mucin compact was determined compared to the eHal-LChi (1.35 ± 0.08 N) and eHal (0.98 ± 0.08 N) samples, demonstrating their improved mucoadhesive potential. In summary, this study demonstrates that functionalization of eHal with MeLChi may be a useful approach for the preparation of nanocomposites as potential carriers for mucoadhesive dosage forms.

Keywords: acid-etched; halloysite; chitosan; methacrylated chitosan; mucoadhesive properties.

1. Introduction

In recent decades, mucoadhesive drug delivery systems have received much attention because they can prolong the residence time of the dosage form at the site of absorption, allowing controlled drug release and improving therapeutic effect [1-3]. Various polymers have been used to develop mucoadhesive formulations [4-7]. Among them, chitosan is widely used due to its remarkable properties, such as nontoxicity, low cost, biocompatibility, biodegradability, and excellent mucoadhesive properties [8-10]. Chitosan is a naturally occurring cationic polysaccharide produced by the deacetylation of chitin. This polymer exhibits significant mucoadhesive properties, mainly due to electrostatic interactions between positively charged amino groups in chitosan and negatively charged groups in the mucin layer, at low pH<6.5 [11]. The mucoadhesive properties of chitosan can be further improved by chemical modification through interaction with methacrylic anhydride. Methacrylated chitosan is able to form covalent bonds with the thiol groups of mucin, resulting in improved adhesion [12], [13].

The preparation of new drug delivery systems based on the combination of chitosan and clay minerals has evoked intense research in recent years. Clay minerals can be considered naturally available materials that can be used as drug carriers because of their high retention capacity and colloidal properties [14], [15]. Among them, halloysite (Hal) is of particular interest due to its biocompatibility, hollow tubular structure, large specific surface area, tunable surface chemistry, positive lumen, and negative external surface charge [16-18].

The Hal nanotubes are two-layered aluminosilicate clay minerals, consisting of silica tetrahedral layer situated on the outside and an octahedral layer of alumina on the inside of the tubes [19]. Despite numerous advances, the use of Hal as a drug carrier is limited by its low loading capacity (5–10 wt%) [18] and mucoadhesiveness [20], [21]. This can be overcome by various approaches

such as selective etching of alumina from the lumen [22-25] and functionalization of Hal with mucoadhesive polymers [21].

Owing to their excellent biological and mechanical properties, Hal-chitosan nanocomposites have been used in various biomedical applications including wound healing [26], drug delivery [27-30], implant coatings [31] and as scaffolds for tissue engineering [32-34]. Our previous study showed that a combined strategy of Hal etching under mild acidic conditions and functionalization with medium molecular weight chitosan had a significant effect on increasing drug loading (≈81%) and sustained release of aceclofenac (≈87% of the drug after 12 h) [23]. Low molecular weight chitosan has more flexible chains than medium and high molecular weight chitosans, which facilitates its efficient binding to the external surface of halloysite. The simultaneous effect of acid etching of Hal and functionalization with LChi on the mucoadhesive properties of nanocomposites has not yet been investigated. To fill this knowledge gap, the present study investigated the effect of these two approaches, which represent a significant contribution to the understanding of the interactions within these systems and their importance for the development of novel drug delivery systems.Hence, this study contributes to the understanding of the effects of methacrylation of LChi on the mucoadhesive properties of eHal-LChi nanocomposites. The combination of acid etching of halloysite and modification with low molecular weight chitosan and its methacrylate derivative represents a novel approach to improve the mucoadhesive properties of halloysite which may prolong the therapeutic effect of loaded drugs. Primarily, lumen enlargement was performed under mild acidic conditions [23]. Moreover, the obtained eHal was modified with LChi and MeLChi by stirring in the acidic polymer solution. Considering that drugs can be loaded into the lumen of Hal by vacuum suction (e.g., aceclofenac [23], curcumin [28]), the drugs entrapped in the lumen were

not expected to affect the mucoadhesive properties of eHal-polycation nanocomposites. Accordingly, mucoadhesive properties evaluation was performed using drug-free samples.

2. Experimental

2.1. Materials

Hal clay ($Al_2Si_2O_5(OH)_4 \cdot 2H_2O$) was purchased from Sigma-Aldrich Chemie GmbH (Germany). Low molecular weight chitosan (50-190 kDa; degree of deacetylation 75-85%) was obtained from Sigma-Aldrich (UK). Methacrylated low molecular weight chitosan (degree of methacrylation 27%) was synthesized according to the method described in [12]. Mucin from the porcine stomach, type II (Sigma-Aldrich Co., USA) was used to evaluate mucoadhesive properties. Deuterated trifluoroacetic acid and deuterated water were acquired from Sigma-Aldrich Chemie GmbH (Germany). Methylthiazolyldiphenyl-tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS) and culture medium (RPMI-1640) were also purchased from Sigma-Aldrich Chemie GmbH (Germany). The human lung fibroblast cell line (MRC5) was obtained from the American Type Culture Collection (Manassas, VA, USA). Ultrapure water from a GenPure apparatus (TKA Wasseranfbereitungssysteme GmbH, Germany) was used for all experiments. All other chemicals and reagents used were of analytical grade.

2.2. Preparation of nanoclay-polymer composites

To increase the surface area and inner lumen diameter, Hal was treated with acetic acid according to a previously described protocol [23]. The final sample was a white solid that was easily crushed to powder with a mortar and pestle, sieved through a 125 μm sieve and labeled as eHal. In addition, eHal was coated with LChi and MeLChi. The preparation of nanocomposites is described in detail in the Supplementary material.

2.3. Characterization of eHal and eHal-polycation composites

2.3.1. Scanning electron microscopy (SEM)

The surface morphology of all samples was examined by scanning electron microscopy (SEM) (JSM-6390LV, JEOL, Japan) at 20 kV. To avoid electrostatic charging during imaging, all samples were coated with a thin conductive gold layer using SCD 005 Cool Sputter Coater (Bal-Tec, Germany) at 30 mA for 100 s before analysis. SEM images were acquired at two different magnifications (30000× and 8000×). ImageJ software, version 1.52a (National Institutes of Health, USA) was used to evaluate particle size. For each sample captured on SEM micrographs, one hundred tubes were randomly selected, and their external diameter and length were measured. The dimensions of the samples were expressed as the mean of length and external diameter.

2.3.2. Fourier transform infrared (FT-IR) spectroscopy

The Nicolet iS10 FT-IR spectrometer (Thermo Fisher Scientific, USA) was used to obtain the attenuated total reflectance FT-IR spectra of the prepared nanocomposites and their individual components. The measurements were performed in a wavelength range between 4000 and 400 cm^{-1} with a resolution of 2 cm^{-1}.

2.3.3. Thermal analysis

Differential scanning calorimetry (DSC) / Thermogravimetry (TG) utilised a STA 449 F5 Jupiter Simultaneous Thermal Analyzer with Proteus® software produced by Netzsch, Germany. Samples were heated at a rate of 10 ºC min^{-1} from 25 to1000 ºC using Pt80Rh20 thermocouples and Pt80Rh20 crucibles with lids. An empty crucible with lid was used as reference. The oven gas was streaming with 70 ml min^{-1} (20 ml min^{-1} protective gas - synthetic air and 50 ml min^{-1} purge gas -

synthetic air). Prior to analyses, samples were kept in a dessicator over a saturated solution of NH_4Cl (a relative humidity of 75%) for 24 h.

2.3.4. X-ray powder diffraction analysis

X-ray powder diffraction (XRD) was performed using the Philips PW-1710 automatic diffractometer (Philips, The Netherlands) with a Cu tube at 40 kV and a current of 30 mA. The instrument was equipped with a graphite monochromator with a curved diffracted beam and a proportional counter filled with Xe. The XRD patterns were recorded in a 2θ range from 4 to 65º with a step of 0.02º and a sampling time of 1 s per step. The divergence and reception gap were set to 1 and 0.1, respectively. All XRD measurements were performed at room temperature in a stationary sample holder. The Joint Committee on Powder Diffraction Standards (JCPDS) database was used for sample identification.

2.3.5. Nuclear magnetic resonance (NMR) spectroscopy

^{1}H-NMR spectra were recorded using a Bruker Avance III spectrometer (Bruker Biospin GmbH, Germany) at 400 MHz. Samples of LChi and MeLChi were prepared by stirring in a solution of deuterated water acidified with 0.1% deuterated trifluoroacetic acid. The solutions were heated to 70-80 °C for 2-3 min and then left at room temperature for half an hour. The ^{1}H-NMR spectra were recorded after the complete dissolution of LChi and MeLChi. The same procedure was used for the composites eHal-LChi and eHal-MeLChi as well as for physical mixtures (prepared by geometric dilution with mortar and pestle) of eHal and LChi, i.e. eHal and MeLChi (in a ratio of 2:0.5). The ^{1}H-NMR spectra were recorded after good dispersion of the above physical mixtures and composite samples.

2.4. Assessment of mucoadhesive properties

To evaluate potential interactions between Hal (and its derivatives, such as eHal and eHal-polycation nanocomposites) with mucin, mucoadhesive studies were performed using three *in vitro* methods.

The first method was based on zeta potential measurements (ζ) of the mixture at 20 °C using a Zetasizer Nano ZS90 (Malvern Instruments, UK). The ζ measurements can provide insight into the mechanism of interaction between Hal (and its derivatives, such as eHal, eHal-polycation nanocomposites), and mucin. The ζ-potential of mucin free dispersions of the samples in deionized water at a certain concentration was used as a reference. Any changes in their ζ-potential values during incubation with mucin could be considered as an indication of their interaction [35]. The pH of mucin dispersion was measured using a HI 2223 pH meter (Hanna Instruments, USA).

For the test, a mucin stock dispersion was prepared by adding mucin powder at a concentration of 0.1% (w/v) to deionized water. The mucin dispersion was ultrasonically homogenized for 30 min at room temperature. Then, the accurately weighed Hal, eHal, eHal-LChi, and eHal-MeLChi (from each 25 mg) were added to 25 mL of aqueous mucin dispersion (0.1% w/v) and shaken for 4 h at 300 rpm and 20 ± 1 °C. The ζ-potential of the mixture was measured at specific time intervals (1 and 4 h) and compared with the ζ-potential of the mucin-free aqueous dispersions under the same conditions. Each measurement was repeated three times at room temperature and the data are presented as mean ± standard deviation (SD).

The mucoadhesiveness of the samples was additionally assessed by measuring their ability to adsorb mucin, using a method previously reported in [36] with a slight modification (Supplementary material, Fig. S1).

The interactions between the samples and mucin were also studied using a Shimadzu Texture Analyzer EZ-LX (Shimadzu Corporation, Japan) operated in a compression mode. A detailed description of the method is provided in the Supplementary material, Fig. S2.

2.5. Cell viability assay

The cytotoxic effect of eHal, eHal-LChi, and eHal-MeLChi against MRC-5 normal human lung fibroblasts was determined by MTT assay. The procedure is described in the Supplementary material.

3. Results and discussion

3.1. Scanning electron microscopy (SEM)

The SEM images of all samples at different magnifications (8000× and 30000×) are shown in Fig. 1. It can be clearly seen that the pristine Hal (Figs. 1a and 1e) has a tubular structure with a smooth and flat surface, which is consistent with the results of Murphy et al. [37]. No significant change in the particle morphology of Hal was observed after etching with acetic acid (Figs. 1b and 1f). This observation is consistent with previous results on the morphology of Hal etched in 0.01 M sulfuric acid [38]. The average characteristic size (external diameter of 50-100 nm and 300-700 nm in length) was maintained for pristine Hal and eHal. Similar results were previously reported for hydrochloric and sulfuric acid-activated Hal, showing that acid treatment selectively affects the inner octahedral layers of Hal, while the external diameter and length of the tubes remain unchanged [25].

The morphology of the composite particles at 30000× magnification (Figs. 1c and 1d) shows that the tubular shape of the nanotubes has been preserved. The tubular structure of the composite particles can also be seen at 8000× magnification (Figs. 1g and 1h). There were no significant

differences in the SEM images of the composites compared to eHal, clearly indicating the presence of the polymer on the nanoclay surface. This can be explained by the low concentration of the polymer on the surface of the etched halloysite, which is not clearly visible in the SEM analysis. Using data from ImageJ (National Institutes of Health, USA), an average external diameter of ≈82 nm was determined for the eHal tubes. In contrast, the eHal-LChi and eHal-MeLChi nanocomposites had external diameters of ≈106 nm and ≈115 nm, respectively. The increase in external diameter supports the adsorption of the polymers on the external surface of the eHal nanotubes.

[Figure 1.] SEM images at 30 000× magnification of (a) pristine Hal; (b) eHal; (c) eHal-LChi; and (d) eHal-MeLChi, and SEM images at 8 000× magnification of e) pristine Hal; (f) eHal; (g) eHal-LChi; and (h) eHal-MeLChi

3.2. Fourier transform infrared (FT-IR) spectroscopy

The functional groups on the surface of the eHal and eHal-polycation nanocomposites were further analyzed by FTIR analysis (Fig. 2). Fig.-2 shows the FT-IR spectra of LChi, MeLChi, and eHal-polycation nanocomposites. The FT-IR spectrum of eHal has been discussed previously [23]. Pure LChi showed a peak at 3354 cm^{-1} representing the stretching of the hydroxyl groups and the symmetric stretching of the amine groups at 2871 cm^{-1} (Fig. 2). The peak observed at 1374 cm^{-1} corresponds to -CH_3 stretching, while the absorption bands at 1026 cm^{-1} and 1150 cm^{-1} can be assigned to C-O stretching. The signal at 1651 cm^{-1} is attributed to the C=O stretching of the amide group. The FTIR spectrum of LChi is consistent with previous reports [39], [40].

According to Fig. 2, the largest differences between LChi and MeLChi were observed in the range (1538-1652 cm^{-1}). The coupling of the methacrylated groups to the LChi backbone led to the

appearance of a characteristic double bond peak in this region, indicating the C=C stretching of the alkenyl group. The absorption band at 1621 cm^{-1} can be assigned to the C=O stretching of the amide in the methacrylate moiety of MeLChi, confirming the chemical modification of LChi. Moreover, the increased intensity of the absorption band at 2921 cm^{-1}, corresponding to the C-H stretching of the alkyl groups, proves the successful methacrylation of LChi, which was also confirmed by DSC, XRD, and ^{1}H-NMR analyses. These results are consistent with previous reports on methacrylated chitosan [12], [41].

[Figure 2.]

Comparing the FT-IR spectral details of LChi and eHal-LChi, it was found that after the interaction of LChi with eHal in the nanocomposites (eHal-LChi), the stretching vibration of the protonated amino group observed in LChi at 1564 cm^{-1} was shifted to 1559 cm^{-1} in the nanocomposites. Similar results have been previously reported for nanocomposites of eHal and medium molecular weight chitosan [23]. In the eHal-MeLChi nanocomposites, the C=C stretching vibration of the alkenyl group was shifted from 1538 cm^{-1} in MeLChi to 1558 cm^{-1} in the composite sample. The shift of these absorption bands confirmed that both polymers were effectively linked to eHal in the prepared nanocomposite samples.

3.3. Thermal analysis

At DSC curve (Fig. 3), the dehydration of eHal is described by the endothermic peaks at 77 and 148 ºC related to the removal of the physically adsorbed water and the interlayer water during the transformation from Hal-10 A˚ to Hal-7 A˚, followed by the high intensity endothermic peak centered at and 505 ºC originated from dehydroxylation of the structural aluminol groups.

Exothermic peak at 996 ºC of eHal is attributed to the formation of γ-Al_2O_3 [42]. From TG curve, the total mass loss of eHal is 17.15 % up to 1000 °C, which emphasizes its inherent stability as an inorganic material (Fig. 4).

For the LChi, in the interval between 20 and 200 °C, at DSC curve, endothermic peak at 75 °C is visible attributed to the water evaporation, while in the temperature region from 200 to 650 °C exothermic peaks at 299, 323 and 510 °C originated from the degradation of polymer due to the rupture the anhydrous glycoside ring and deamination process (Fig. 3a) [43]. The DSC curve of eHal-LChi nanocomposite shows slightly visible changes in the endothermic and exothermic peaks compared to pure LChi, only in the temperature region from 20 to 650 °C, the mass loss of eHal increased from 13.66 % after modification with LChi to 14.87 % indicating the chitosan functionalization on the surface of eHal (Fig. 4) [44].

[Figure 3.]

At DSC curve of MeLChi, the attached methacrylated groups to the LChi are confirmed with the change of the intensities and with the shift of the characteristic exothermic peaks of LChi. For MeLChi, exothermic peaks are visible at 237, 314 and 492 °C. After modification of eHal with MeLChi, changes in the thermal behavior of composite eHal-MeLChi are more visible. The endothermic peaks of eHal visible at 77 and 148 °C were shifted toward higher temperatures (85 and 154 °C) and were more pronounced in the eHal-MeLChi composites (Fig. 3b). The mass loss in the temperature region from 20-200 °C increased from 2.96 % for eHal to 5.70 % for eHal-MeLChi composite (Fig. 4). In the temperature region 200-650 °C, at DSC curve of nanocomposite, high intensity exothermic peak originated from MeLChi appeared at 315 °C, while mass loss increased from 13.66 % (eHal) to 25.10 % (eHal-MeLChi). Also, the intensity of the

dehydroxilation peak of eHal decreased after modification with MeLChi probably due to the overlapping with the exothermic peak of MeLChi and peak is slightly shifted toward lower temperature (from 505 °C for eHal to 499 °C for nanocomposite). These results are another confirmation of the successful modification of eHal with MeLChi.

[Figure 4.]

3.4. X-ray powder diffraction method (XRD)

The effect of polymer functionalization on the crystalline structure of eHal was analyzed using XRD. The XRD patterns of eHal showed diffraction patterns at $2\theta = 12.02°$, 19.96°, 24.72°, 35.98°, 38.4°, 54.98°, and 62.5° (Fig. 5). All observed diffraction peaks are indexed with the diffraction of the halloysite powder (JCPDS 29-1487). These results agree well with our previous report that the crystalline structure of pristine Hal was not affected by acetic acid treatment. After functionalization with LChi and MeLChi, the reflections of eHal remain unchanged, indicating that functionalization with these polymers does not affect the structure of eHal. This is in accordance with the observation that LChi and MeLChi adsorb only on the external surface of eHal nanotubes.

In addition, the XRD patterns were used to study the effects of methacrylation on the crystalline structure of LChi. Two intense crystalline peaks (2θ) at 12.06° and 20.28° were observed in pure chitosan previously reported in [45]. The change in the XRD pattern of MeLChi, characterized by a broadening of the peak at 20.28° due to the destruction of the intermolecular hydrogen bonds, indicates successful methacrylation of LChi. Kolawole et al. [12] reported lower crystallinity of methacrylated chitosan (degree of methacrylation, 34.3%) compared to pure chitosan, with broadening of the peak around 20° and disappearance of the peak around 10°. In this study, the

281 characteristic peak at 12.06° did not disappear, which can be explained by the lower degree of
282 methacrylation of chitosan (27%).

283 **[Figure 5.]**

284 3.5. Nuclear magnetic resonance (NMR) spectroscopy

285 The ^{1}H-NMR spectra of LCh and MeLChi are shown in Fig. 6. The ^{1}H-NMR spectrum of LChi
286 (Fig.6a) shows a peak at 1.98 ppm attributable to methyl groups and peaks at 3.1-3.81 ppm
287 attributable to hydrogen atoms of the glucosamine ring. In the ^{1}H-NMR spectrum of MeLChi (Fig.
288 6b), new peaks appeared at 5.60 and 5.99 ppm, which were due to the presence of hydrogen atoms
289 of the methylene group. These peaks indicated the successful methacrylation of chitosan. The
290 physical mixtures of eHal and LChi (Fig. 6c), and eHal and MeLChi (Fig. 6d) showed the same
291 peaks characteristic for LChi and MeLChi, respectively. The presence of hydrogen atoms was not
292 detected in the ^{1}H-NMR spectra of eHal-LChi and eHal-MeLChi nanocomposites (Figs. 6e and6f).
293 The peak of the methyl group, which is not involved in the interaction of LChi and MeLChi with
294 eHal, was preserved, confirming that the interaction between the polymers and eHal occurred.

295 **[Figure 6.]**

296 3.6. Assessment of mucoadhesive properties

297 The mucoadhesive properties of the Hal, eHal, eHal-LChi, and eHal-MeLChi nanocomposites
298 were evaluated by measuring the changes in their ζ-potential upon interaction with negatively
299 charged mucin; the results are shown in Fig. 7. The ζ-potential value of the mucin particles was -
300 12.2 mV, confirming their negative charge due to the presence of sialic acid. In several studies,
301 the negative ζ-potential of mucin was reported to be about -10 mV [46], [47]. This slight difference
302 in surface charge can be explained by the different conductivity and pH of water used for

preparation of mucin dispersion, as well as by the different concentration and pH of the mucin dispersion.

Pristine Hal and eHal nanotubes initially exhibited a negative ζ-potential due to the negatively charged silanol groups located mainly on their external surface [48]. After incubation with mucin at 20 ± 1 °C for 1 h, ζ-potentials of pristine Hal and eHal increased from (-25.9 ± 0.9) mV to (-14.2 ± 0.8) mV and from (-28.3 ± 1.1) mV to (-13.1 ± 0.8) mV, respectively. The values obtained were close to the ζ-potential of pure mucin (-12.2 mV), indicating that the surface of the complexes formed between mucin and nanotubes has a similar charge density to mucin. The interaction arises from hydrogen bonds between the hydroxyl groups of mucin and the silanol groups of nanotubes and from hydrogen bonds between the thiol groups of mucin and the silanol groups of nanotubes. With increasing incubation time with mucin, the ζ-potential of the nanotubes slightly decreased.

The ζ-potential measurements are also used to evaluate polymer adsorption on the eHal surface. The ζ-potentials for eHal-LChi and eHal-MeLChi nanocomposites were +25.9 ± 0.9 mV and +24.4 ± 0.8 mV, respectively, indicating successful binding of the cationic polymers to the external surface of the eHal nanotubes. In aqueous mucin dispersion (pH≈4.5), the sialic acid residues of mucin (pKa 2.6) are present in ionized form, indicating an overall negative charge of the glycoprotein [49]. At the same pH, the amine groups of chitosan are protonated, and given the strength of electrostatic interactions, positively charged species of the nanocomposite particles may interact with mucin. Indeed, the positive charge of eHal-LChi and eHal-MeLChi nanocomposites decreased significantly from +25.9 ± 0.9 mV to -13.3 ± 0.5 mV and from +24.4 ± 0.8 mV to -12.6 ± 0.4 mV, respectively, after 1 h incubation with mucin. This change in the surface charge of the nanocomposite particles strongly suggests that their interaction with mucin is electrostatic in nature. The complexes formed between mucin and nanocomposites exhibited a

lower ζ-potential value compared to mucin itself, which can be explained by the different conformation of adsorbed mucin [50], [51].

[Figure 7.]

The mucoadhesive properties of the samples were further determined by measuring their ability to adsorb mucin. The results of the mucoadhesion measurements followed the trend eHal-MeLChi > eHal-LChi > eHal > Hal. As shown in Fig. 8, pristine Hal exhibited the lowest mucoadhesive potential: 49 ± 0.7 % of the initially added mucin was adsorbed onto the nanotubes after 1 h of incubation. These results are consistent with a previous study showing that Hal has mucoadhesive potential [21]. Acetic acid treatment increased the specific surface area of nanotubes from 51.6 m^2/g to 57.2 m^2/g [23] for Hal and eHal, respectively. Due to the larger surface area, eHal was able to adsorb a higher percentage of mucin than the pristine Hal, with 54 ± 0.7 % of mucin adsorbed on the eHal surface after 1 h of incubation. For all samples tested, most of the mucin was bound to the nanotubes within the first hour of incubation and did not differ according to the duration of incubation. This could be due to the saturation of the nanotube surface by mucin chains.

After functionalization of eHal with LChi, the amount of mucin adsorbed on the composite surface increased to 66.8 ± 0.8 % after 1h of incubation. This was expected since chitosan itself has good mucoadhesive properties [52], [53]. The ~~strong~~ binding of eHal-LChi nanocomposites to mucin can be attributed to electrostatic interactions between the positively charged amines of LChi and the negatively charged sialic acid residue of mucin, as well as hydrogen bonds between the hydroxyl groups of LChi and the hydroxyl groups of mucin. Similar behavior was reported in [21], where the functionalization of pristine Hal with chitosan had a positive effect on mucoadhesive properties.

[Figure 8.]

The introduction of methacrylate groups into MeLChi provides an opportunity for formation of covalent bonds with mucin in addition to the previously mentioned interactions (Fig. 9). The interactions between the alkenyl groups of MeLChi and the thiol groups of mucin are likely responsible for a greater percentage of adsorbed mucin by eHal-MeLChi nanocomposites (77.7 ± 0.9 %) compared to eHal-LChi nanocomposites (66.8 ± 0.8 %) after 1 h of incubation. These results were expected based on the findings of Kolawole et al. [12], who reported better mucoadhesivity of methacrylated chitosan compared with pure chitosan.

[Figure 9.]

The mucoadhesiveness of the samples was additionally evaluated with a texture analyzer. Table 1 shows the Fadh values of all tested samples after detachment from the mucin compact.

Table 1. Values of Fadh of sample compacts to the porcine mucin compact (mean ± SD; n = 4).

Samples	Fadh (N)
Hal	0.78 ± 0.06
eHal	0.98 ± 0.08
eHal-LChi	1.35 ± 0.08
eHal-MeLChi	1.59 ± 0.07

In the present study, the eHal-MeLChi nanocomposite compacts showed the strongest adhesive properties to the mucin compacts, whereas the halloysite compacts showed the weakest interaction with mucin. Pristine Hal showed limited interactions with mucin due to its negative surface charge, which inhibits adhesion to negatively charged mucin. This study showed that Fadh, which is required for the detachment of the eHal compact from the mucin compact, was greater than the

Hal compact, with values of 0.98 ± 0.08 N (eHal) and 0.78 ± 0.06 N (Hal), respectively. The better mucoadhesive properties of eHal compared with the pristine nanotubes could be explained by the larger specific surface area of eHal, which contributes to a stronger interaction with mucin.

In addition, the force required to break the mucoadhesive bonds between eHal-LChi and mucin compacts was 1.35 ± 0.08 N, greater than for eHal compacts. This suggests that noncovalent modification of eHal with LChi significantly improves mucoadhesive properties. Such results are expected due to the presence of positively charged amino groups in the structure of LChi, which can interact electrostatically with negatively charged mucin. Furthermore, this study revealed significant differences in the detachment force of eHal-LChi and eHal-MeLChi nanocomposite compacts from mucin compacts. The better mucoadhesive behavior of the eHal-MeLChi nanocomposites, with a mean detachment force (1.59 ± 0.07) N is probably due to the presence of methacrylate groups that form covalent bonds with the thiols of mucin. These results support our hypothesis that LChi and MeLChi have a marked effect on the mucoadhesiveness of eHal, and among the tested samples, eHal-MeLChi stands out as the composite with the best mucoadhesive properties.

3.7. Cell viability assay

The cytotoxic effect of eHal, eHal-LChi, and eHal-MeLChi nanocomposites on MRC-5 cells was determined by the MTT assay; the results are shown in Table S1 (Supplementary material). According to the standard ISO 10993-5 [54], a cell viability more than 70% is acceptable for a biocompatible nanocarrier (ISO 10993-5:2009). The results showed that eHal and eHal-polycation nanocomposites were biocompatible as the resulting percentage of viability in MRC-5 cells was more than 70% within investigated concentration range, even after prolonged exposure. The cell survival rate of eHal-LChi (90.7 ± 3%) and eHal-MeLChi (96.3 ± 3%) nanocomposites at a

concentration 1000 μg/mL, indicated that functionalization with LChi and MeLChi did not affect cytocompatibility of eHal. Comparable values of cell survival rates of eHal-LChi and eHal-MeLChi confirmed that methacrylation of LChi does not affect cytocompatibility. This result was expected since Zanon et al. [55] showed that methacrylation did not affect the good biocompatibility of chitosan when tumor cell lines (H1299 and A549 GFP) were studied. These data suggest good biocompatibility of the new eHal-polycation composites under the cell culture conditions.

4. Conclusion

Selectively etched halloysite and corresponding composites with polycationic polymers (low molecular weight chitosan (LChi) and its methacrylated derivative (MeLChi)) were studied. The successful functionalization of eHal with the polycationic polymers was confirmed by ζ-potential measurements, SEM, FT-IR, DSC, XRD, and ^{1}H-NMR. The results of *in vitro* mucoadhesion studies showed that the mucoadhesive properties of eHal were improved by functionalization with LChi and MeLChi. Overall, the eHal-MeLChi nanocomposite exhibited remarkable mucin-binding properties, which were attributed to the presence of methacrylate groups that interact with thiol groups of mucin. The enhanced mucoadhesive properties of the eHal-MeLChi nanocomposites were confirmed by ζ-potential measurements, mucin adsorption capacity, and mucoadhesion studies using a texture analyzer. The MRC5 cell cytotoxicity assay showed excellent cytocompatibility of the halloysite-polycation composites, even after prolonged exposure over 72 hours, while the cell survival rate for the eHal-MeLChi nanocomposite at a concentration of 1000 μg/ml was more than 90% and the IC50 values were above 1000 μg/ml.

These results suggest that modification of selectively etched halloysite with MeLChi may be a promising approach to improve the functional properties of these nanocarriers. The improved

mucosal adhesion of halloysite-chitosan nanocomposites and good biocompatibility make them promising materials for drug delivery application.

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Declaration of conflicting interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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