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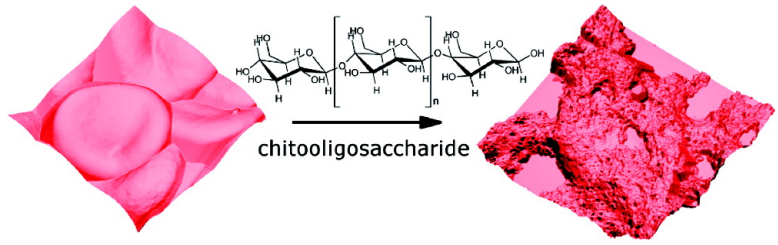

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Articles

Effects of Chitooligosaccharides on Human Red Blood Cell Morphology and Membrane Protein Structure

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Recent studies of chitosan have increased the interest in its conversion to chitooligosaccharides (COSs) because these compounds are water-soluble and have potential use in several biomedical applications. Furthermore, such oligomers may be more advantageous than chitosans because of their much higher absorption profiles at the intestinal level, which permit their facilitated access to systemic circulation and potential distribution throughout the entire human body. In that perspective, it is important to clarify their effect on blood further, namely, on human red blood cells (RBCs). The aim of this work was thus to study the effect of two COS mixtures with different molecular weight (MW) ranges, <3 and <5 kDa, at various concentrations (5.0−0.005 mg/mL) on human RBCs. The interactions of these two mixtures with RBC membrane proteins and with hemoglobin were assessed, and the RBC morphology and surface structure were analyzed by optical microscopy (OM) and atomic force microscopy (AFM). In the presence of either COS mixture, no significant hemolysis was observed; however, at COS concentrations >0.1 mg/mL, changes in membrane binding hemoglobin were observed. Membrane protein changes were also observed with increasing COS concentration, including a reduction in both α- and β-spectrin and in band 3 protein, and the development of three new protein bands: peroxiredoxin 2, calmodulin, and hemoglobin chains. Morphologic evaluation by OM showed that at high concentrations COSs interact with RBCs, leading to RBC adhesion, aggregation, or both. An increase in the roughness of the RBC surface with increasing COS concentration was observed by AFM. Overall, these findings suggest that COS damage to RBCs was dependent on the COS MW and concentration, and significant damage resulted from either a higher MW or a greater concentration (>0.1 mg/mL).

Introduction

Chitin is a natural polymer found in the exoskeleton of crustaceans and insects as well as in the cell walls of certain fungi.[1] Full (or partial) deacetylation of chitin produces chitosan, a linear polysaccharide that is mainly composed of β−1,4−2-deoxy-2-amino-D-glucopyranose and, to a lesser extent, β−1,4−2-deoxy-2-acetamido-D-glucopyranose residues.[2] Evidence has been put forward that chitosan possesses various biological activities, namely, antioxidant,[3] cholesterol-lowering,[4] and antibacterial and antifungal activities,[5−8] besides being useful as an active dietary component for body fat loss.[9,10] These features, combined with its claimed biocompatibility and biodegradability, make chitosan an interesting polymer for several applications in biomedicine and dental medicine as well as in the pharmaceutical, cosmetic, and food industries.[11−15] However, the high MW[16] has limited its practical applications because of its insolubility at pH values >6.3. (Most commercial chitosans have a MW ranging between 100 and 1200 kDa.)[17−20]

Recent studies pertaining to chitosan have focused on its conversion to chitooligosaccharides (COSs) because the latter are not only water-soluble because of their shorter chain lengths (generally, COS MW ⩽ 10 kDa)[19,20] and free amino groups in D-glucosamine units (which confer a positive charge on COS, allowing it to bind to negatively charged surfaces strongly) but also seem to possess versatile functional properties (e.g., antitumor,[21] immunostimulatory,[22] and anti-inflammatory[23,24]) that make them a very promising class of compounds. Furthermore, such oligomers may have more practical applications than

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chitosan because of their much higher absorption profiles at the intestinal level both in vitro and in vivo,[19,25] thus permitting their quick access to the blood flow.

Chitosan toxicity has been described to be dependent on MW and the degree of acetylation as well as on concentration;[26] the absence of cytotoxic effects on the human intestinal caco-2 cell line has been reported for chitosans with MWs of <10 kDa.[25] Previous studies that focused on the effects of chitosans on red blood cells (RBCs) reported damaging consequences such as lysis as well as membrane damage and cell aggregation.[27−29] However, to the best of our knowledge, there are no data in the literature about the impact of COSs on RBCs, the most abundant cells in blood.[30] In this perspective, it is important to clarify the effects of the COS MW and concentration when COSs reach the blood circulation. Human RBCs, as a metabolically simplified model system, are particularly attractive for physical and pathobiological studies. This simplified model has been used to study a great variety of substances or situations, such as oxidative stress,[31−33] lifelong somatic mutations,[34] and cytotoxicity.[35] Although less specialized than many other cell membranes, RBCs have many functions in common with them, such as active and passive transport and the production of ionic and electric gradients;[36,37] therefore, they may be considered to be representative of the plasma membrane in general.

The aim of this work was to study the effect of two COS mixtures with different MW ranges, <3 and <5 kDa, at different concentrations on human RBCs. The interactions of the two COS mixtures with RBC membrane proteins and with hemoglobin (Hb) were evaluated, and RBC morphology and surface structure were further analyzed by optical microscopy (OM) and atomic force microscopy (AFM).

Experimental Procedures

Materials. COS mixtures, characterized by two distinct MW ranges, <3 and <5 kDa, were purchased from Nicechem (Shanghai, China) and used as received. Such COSs had been obtained via enzymatic hydrolysis of chitosan derived from crab shells. Their deacetylation degree was in the range of 80−85%, as indicated by the supplier.

Molecular Weight Determination. The average MW was determined by static light scattering (SLS) with a Zetasizer Nano ZS (Malvern Instruments) at 633 nm. The sample was measured at various concentrations (0.075 to 2.5 mg/mL), and the Rayleigh equation was applied. The refractive index and the differential refractive index increment values of the samples were 1.34[38] and 0.142, respectively.[39]

Preparation of Red Blood Cells. Blood was obtained from healthy volunteers by venipuncture and collected in tubes containing EDTA as anticoagulant. Blood samples were immediately centrifuged at 405*g* for 10 min; plasma and buffy coat were carefully removed and discarded. Red blood cells were washed three times with phosphate-buffered saline (PBS; 125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) at 4 °C and finally resuspended in PBS to obtain an RBC suspension at 10% (v/v) hematocrit.

To assess the effect of both COS mixtures on RBCs, in vitro studies were performed by incubating the RBC suspensions with the COS mixtures under different conditions and evaluating the RBC lysis, the morphology, the linkage of Hb to the membrane, and the membrane protein profile.

Hemolysis and Red Blood Cell Morphological Changes. RBC suspensions were prepared at 10% hematocrit as previously described, and the assays were performed at several COS concentrations: 5.0, 1.0, 0.50, 0.10, 0.050, 0.010, and 0.005 mg/mL. In every set of experiments ($n = 4$), a control with only RBCs in PBS was used; control and sample tests were run in duplicate. The incubation of the RBC suspensions was carried out at 37 °C for 3 h under gentle shaking.

Hemolysis was spectrophotometrically determined according to Ko et al.[40] This was determined by comparing the absorption of a sample at 540 nm (A) with that of a completely hemolysed sample at the same wavelength (B); after the incubation period, a 50 μL aliquot of the RBC suspension was taken, diluted with 20 volumes of saline solution, and centrifuged (1180*g* for 10 min at 4 °C). Absorption (A) of the supernatant was read at 540 nm (maximum Hb absorbance, in accordance with Lambert−Beer law). To yield the absorption (B) of a complete hemolysis, another aliquot of the RBC suspension was treated with 20 volumes of distilled water, and after centrifugation, absorption was measured at the same wavelength. The percentage of hemolysis was then calculated as (A/B) × 100 and is presented in the results as the difference relative to the corresponding control.

For the study of morphological changes, 50 μL aliquots of RBC suspensions were taken from the control and the test tubes at the end of the incubation period and were mounted in a slide with a coverslip; OM evaluation was immediately performed.

Red Blood Cell Membrane Studies. RBC suspensions (10%) were incubated at 37 °C for 3 h under gentle shaking. Afterward, RBCs were washed in saline solution and immediately subject to hypotonic lyses according to Dodge et al.[41] The obtained membranes were washed in Dodge buffer, to which phenylmethylsulfonyl fluoride was added in the first two washes as a protease inhibitor, with a final concentration of 0.1 mM. The protein concentration of the RBC membrane suspensions was determined by Bradford's method.[42]

Membrane-bound hemoglobin (MBH) was spectrophotometrically measured after protein dissociation of membrane components with Triton X-100 (5% in Dodge buffer) at 415 nm.[43] We corrected the absorbance at this wavelength by subtracting the absorbance of the background at 700 nm; these values were coupled with the membrane protein concentrations for the calculation of the MBH percentage. To clarify the nature of the Hb, spectral scans were performed (370−630 nm).[44,45]

Membrane suspensions of RBCs were treated with a solubilization buffer, heat denatured, and submitted to electrophoresis (8 or 6 μg protein/lane). The electrophoresis was carried out on a discontinuous polyacrylamide system in the presence of sodium dodecylsulfate (SDS-PAGE) using a 5−15% linear polyacrylamide gradient gel and a 3.5−17% exponential polyacrylamide gradient gel, according to Laemmli and Fairbanks methods, respectively.[46,47] All samples were run in duplicate for each set of experiments.

Mass Spectrometry Protein Analysis. Because some unusual protein bands were observed after RBC incubation with COS mixtures, they were subject to mass spectrometry. Protein bands of approximately 24, 17, and 16 kDa observed in SDS-PAGE gels (apparently due to the interaction of COSs at higher concentrations with RBCs) were excised from those gels, washed three times with 25 mM ammonium bicarbonate/acetonitrile (ACN, 50%) and once with ACN, and dried in a SpeedVac; 25 μL of a 10 μg/mL sequence grade modified porcine trypsin (Promega) in 25 mM ammonium bicarbonate was added to the dried gel pieces, and the samples were incubated overnight at 37 °C. Extraction of tryptic peptides was performed by the addition of 10% formic acid (FA) and 50% ACN three times, which were lyophilized in a SpeedVac. Tryptic peptides were resuspended in 10 μL of a CAN/FA (50:0.1%) solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in ACN/FA (50:0.1%); 0.5 μL aliquots were spotted on the MALDI sample target plate. Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems) in the positive ion reflector mode. Spectra were obtained in the mass range of 800−4500 Da with ca. 1500 laser shots. For each sample spot, a data-dependent acquisition method was created to select the most intense peaks, excluding from the matrix trypsin autolysis or acrylamide peaks, for subsequent MS/MS data acquisition. Trypsin autolysis peaks were used for internal calibration of the mass spectra, allowing a routine mass accuracy of better than 20 ppm. Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal Mascot (Matrix Science)

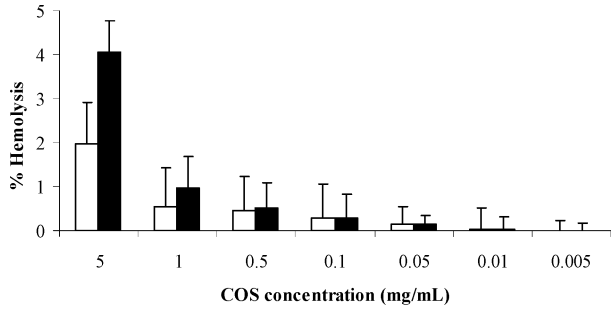

Figure 1. Relative hemolysis (average ± standard deviation) of RBC suspensions incubated for 3 h at 37 °C at various concentrations of COS mixtures: (□) <3 and (■) <5 kDa.

software to search the peptide mass fingerprints and MS/MS data. Searches were performed against the NCBI nonredundant protein database.

Atomic Force Microscopy Measurements. After 3 h of incubation at 37 °C under gentle shaking, RBC suspensions were diluted with PBS to obtain a final suspension at 0.1% (v/v) hematocrit. After RBC fixation by the addition of glutaraldeyde 0.25% (v/v), 50 μL of each suspension was applied to a clean glass surface and air dried. The RBCs were then gently rinsed with deionized water to remove salt crystals and air dried again before analysis.

All AFM studies were carried out with a Veeco Multimode IVa atomic force microscope (Veeco, Santa Barbara, CA) equipped with a j-type scanner (ca. 100 × 100 × 5 μm scan range). Cell surface studies were carried out in tapping mode by the use of silicon cantilevers with a resonant frequency of ~300 kHz (AppNano, Santa Clara, CA). Several independently produced samples were analyzed, and several areas were studied on each sample. In addition to the large images showing the morphology of several cells reproduced here, higher-resolution images were collected of individual cells. These images were used to collect data on the roughness of the plasma membrane. As previously carried out by Girasol et al.,[48] a number of small areas on the cell surface were analyzed to ensure that overall cell morphology did not skew the results. The data shown here are the medians of all measurements for each treatment condition. Gwyddion 2.9 software was used for height data display.

Statistical Analyses. Mean values and standard deviations were accordingly calculated from the obtained experimental data. Because several of the studied variables presented a non-Gaussian distribution, those data are presented as median (interquartile range) values. For statistical analysis, the Statistical Package for Social Sciences (SPSS) version 15.0 for Windows was used. To evaluate the differences between groups, the nonparametric Kruskal−Wallis H test was used. When statistical significance was achieved, single comparisons (two groups) were made by the use of the Mann−Whitney U test. A p value of <0.05 was considered to be statistically significant.

Results

The MW values obtained with SLS for the two samples were 1.2 and 5.3 kDa for <3 and <5 kDa COSs, respectively.

In the presence of either COS mixture, no appreciable hemolysis was observed at any of the concentrations tested (Figure 1). Only very small, statistically insignificant increases in hemolysis were observed as COS concentrations increased from 0.10 to 1 mg/mL. The hemolysis increase was statistically significant at only the highest concentration (5 mg/mL); this increase was greater for <5 kDa COS. Even so, the highest percentage of hemolysis did not exceed 5% for the <5 kDa COS mixture at the highest concentration studied and did not exceed 3% for the <3 kDa mixture.

Figure 2 illustrates the morphology of RBCs after exposure to COS mixtures at several concentrations. At COS concentrations of <0.1 mg/mL, no changes in RBC morphology were observed, though a few echinocytes were observed in both control and test samples (crenated RBCs with many blunt spicules or knobs), which could be due to the long incubation period or the proximity to the glass surface;[49,50] however, at higher concentrations, COSs seemed to interact with RBCs at concentrations of ⩾0.10 and >0.10 mg/mL for <5 and <3 kDa COS, respectively. At the aforementioned concentrations, both COS mixtures promoted RBC adhesion, aggregation reactions, or both, which seemed to be enhanced by increasing COS concentrations. At 5.0 mg/mL, almost all RBCs in suspension aggregated (or adhered to COS) for both COSs. No clusters were seen for any of the mixtures at 0.050 mg/mL. Once again, the <5 kDa COS mixture appeared to induce slightly more damage. Damaged RBCs have been associated with an increase in MBH because of the development of oxidative stress within the RBC.[51] As Hb denatures (due to oxidative stress), it binds to the cytoplasmic pole of the erythrocyte transmembrane protein band 3, hence promoting its aggregation. An increase in MBH was observed as the tested concentration of either mixture was increased (Table 1); the amount of MBH was considerably higher than that of the control in the assays performed with 5.0 and 1.0 mg/mL of either mixture, and the MBH values were always higher for <5 kDa COS than for <3 kDa COS. In fact, MBH at 5.0 mg/mL was ca. 60 and 103 times (<3 and <5 kDa, respectively) greater than that observed with the control. For concentrations of 0.10 mg/mL and below, MBH values were similar to, or even lower than that of the control.

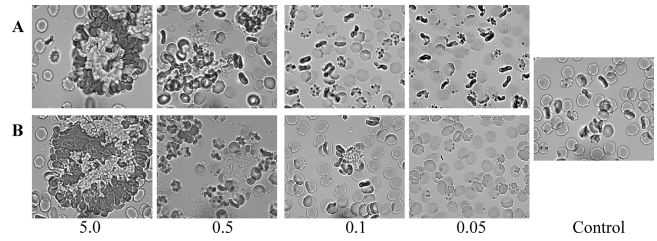

Figure 2. OM assessment of RBC morphology incubated for 3 h at 37 °C in the presence of COS mixtures (A) <3 and (B) <5 kDa at various concentrations (mg/mL).

Table 1. Percentage of Membrane-Bound Protein (Median Interquartile Range) after Incubation of RBCs for 3 h at 37 °C in the Presence of Various Concentrations of COS Mixtures

	MBH ($\times 10^{-4}$%)	
COS (mg/mL)	<3 kDa	<5 kDa
5.0	3351.2 (3227.4−3449.8)	5547.3 (5337.7−5755.5)
1.0	423.5 (400.9−455.3)	465.4 (433.3−538.9)
0.50	126.6 (113.9−132.4)	136.7 (120.1−162.5)
0.10	39.3 (35.8−44.8)[a]	42.3 (36.8−46.8)[a]
0.050	31.5 (25.8−39.4)[a]	46.5 (43.4−51.9)[a]
0.010	36.0 (33.7−42.9)[a]	34.3 (34.2−37.7)[a]
0.005	24.9 (20.2−29.1)[a]	37.2 (33.3−38.4)[a]
control	53.0 (47.7−59.2)[a]	53.0 (47.7−59.2)[a]

[a] Statistically not different (p > 0.05).

The increase in MBH at COS concentrations of >0.1 mg/mL (i.e., 0.5, 1.0, and 5.0 mg/mL) led us to investigate the nature of Hb that was linked to the RBC membrane. We observed, by performing spectral scans of cell lysates in the presence of COS mixtures (after 3 h of incubation at 37 °C), that there were no changes in the oxy-Hb peaks (540 and 578 nm; data not shown) or in its concentration (data not shown) when compared with the control assay (i.e., RBCs without COSs); neither were changes seen in the met-Hb peak area (630 nm).

As the concentration of COS increased between 0.1 and 1.0 mg/mL, a small reduction in α- and β-spectrin and band 3

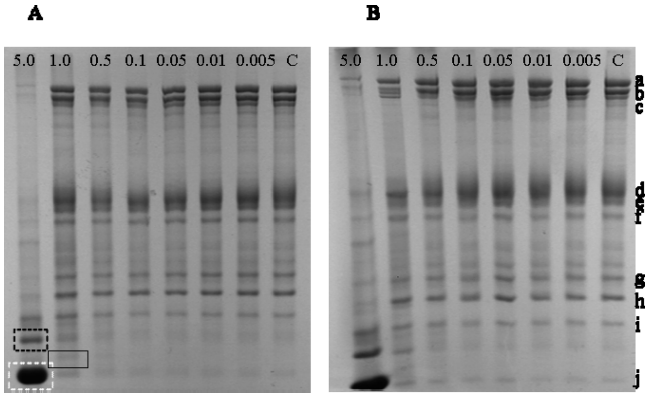

Figure 3. SDS-PAGE (exponential gradient gel) of RBC membrane proteins incubated for 3 h at 37 °C in the presence of COS mixtures (A) <3 and (B) <5 kDa at various concentrations (mg/mL): (a) α-spectrin (240 kDa), (b) β-spectrin (220 kDa), (c) ankyrin (210 kDa), (d) band 3 (90−100 kDa), (e) protein 4.1 (80 kDa), (f) protein 4.2 (72 kDa), (g) actin (43 kDa), (h) glyceraldehyde-3-phosphate dehydrogenase (35 kDa), (i) stomatin/tropomiosin (27 kDa), and (j) globin chains (16 kDa). C: control.

Table 2. Overview of Proteins Identified by MALDI-TOF MS and MS/MS

protein	Swiss-Prot accession no.	no. peptides matched	sequence coverage (%)
peroxiredoxin 2	P32119	9	37
hemoglobin α chain	P68871	13	81
hemoglobin β chain	P69905	7	54
calmodulin	P62158	11	38

protein was observed in the case of the 5 kDa COS, but the treatment with 3 kDa COS at these concentrations had no noticeable effect. However, all three protein bands decreased greatly in intensity when the concentration of either COS was increased from 1.0 to 5.0 mg/mL. The development of unusual protein bands with MWs between 16 and 29 kDa was also detected, with a concentration dependence mirroring that of the disappearance of those three membrane proteins. Mass spectrometry analysis identified the new bands (≥99.998% certainty) as peroxiredoxin 2 (black dashed square in Figure 3), Hb chains (white dashed square in Figure 3), and calmodulin (solid black square in Figure 3) (Table 2). The intensity of the peroxiredoxin 2 band, an important protein in antioxidant RBC defense and in redox cell signaling,[52] increased in the presence of COS for both assays with <3 and <5 kDa COSs; the same was observed for the Hb band. This increase in Hb chains at the highest COS concentrations is in agreement with the results obtained in the MBH analysis. For calmodulin, a calcium regulatory protein,[53] its band also appeared with treatment with either COS at concentrations of 1.0 and 5.0 mg/mL and, to a lesser extent, 0.5 mg/mL.

The morphological studies of the control using AFM showed the typically biconcave RBCs of 7.0 to 8.0 μm in diameter with smooth surfaces (Figure 4A). As shown with OM, a few cells also had the echinocyte conformation. Images of RBC samples incubated with <3 kDa COS at 0.05, 0.5, and 5.0 mg/mL are depicted in Figure 4B−D, respectively. The samples incubated with 0.05 mg/mL showed only a few differences when compared with the control RBC; however, in the 0.5 mg/mL image, a small overall increase in RBC surface roughness was noticeable, but no gross morphological changes (i.e., size and shape) could be observed. At 5.0 mg/mL of COS, the RBCs seemed to be completely covered by a COS layer that made the visualization of the cell surface impossible. To clarify the roughness seen in the RBC images, we measured the surface roughness from high-resolution images. Using smaller 2 μm images of the center of the RBCs, the roughness was analyzed as described above; the results are presented in Table 3. Roughness values (Rrms) consistent with the aforementioned images were attained; namely, an increase in Rrms was observed with increasing concentration. The lower Rrms value for <5 kDa COS at 0.05 mg/mL (relative to the control) was the exception to this trend; the images of the RBC membrane in this case had a morphology that was rather different from all other cases because the membrane had many holes on its surface (see Figure 5). Surface differences are more evident when the topographic profiles attained for both mixtures (Figure 6) are observed. The results

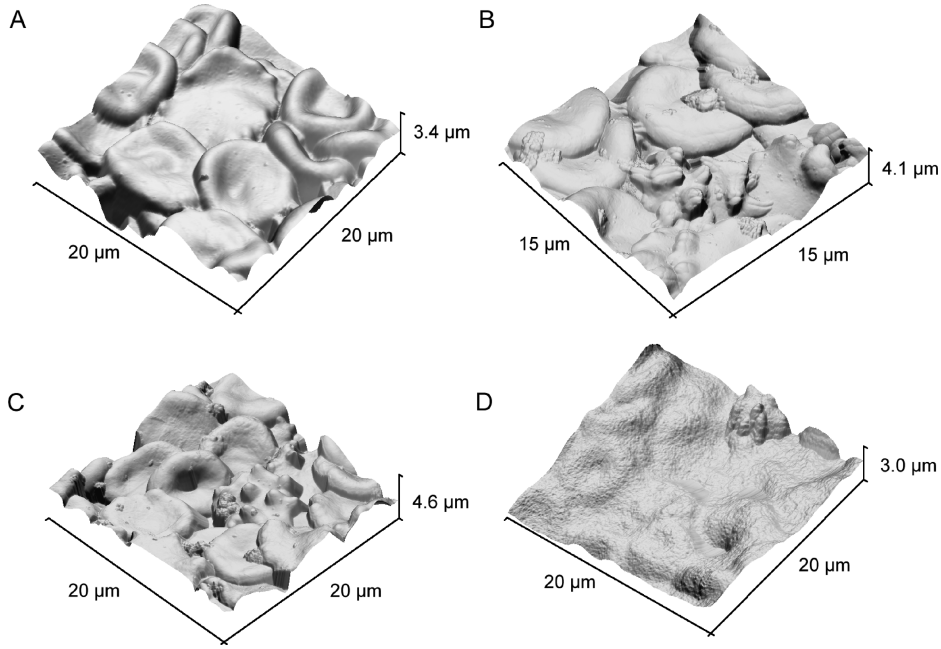

Figure 4. AFM assessment of RBC surface structure incubated for 3 h at 37 °C in the presence of COS (<3 kDa): (A) control, (B) 0.05, (C) 0.5, and (D) 5 mg/mL.

Table 3. Roughness (Median Interquartile Range) of RBC Surface (2 μm) Incubated for 3 h at 37 °C in the Presence of Various Concentrations of COS Mixtures

	Rrms (nm)	
COS (mg/mL)	<3 kDa	<5 kDa
5.0	8.7 (7.6−10.6)	16.6 (13.2−17.4)
0.5	2.8 (2.8−3.2)	2.23 (2.2−2.5)
0.05	1.6 (1.4−2.0)[a]	1.19 (1.0−1.4)[a]
control	1.9 (1.0−2.2)[a]	1.9 (1.0−2.2)[a]

[a] Statistically not different ($p > 0.05$).

show an increase in roughness of the surface topography that is proportional to the concentration of COS used for either mixture. A large difference between <3 and <5 kDa COS was observed at 5.0 mg/mL, whereas at lower concentrations (i.e., 1.0 and 0.5 mg/mL) the profiles were closer to those of the control RBC.

Discussion

In the presence of COSs, no significant hemolysis was observed at any of the concentrations or MWs studied. (See Figure 1.) However, as previously stated, a trend to higher hemolysis rates at higher COS concentrations was observed. The optical images (Figure 2) corroborate the hemolysis study: the RBC density was approximately the same for all concentrations and the control, and there were no hemolysed cells (ghosts). Furthermore, the trend of a higher impact at higher COS concentrations and MWs is apparent by the increasing size of the aggregates with increasing COS concentration and the fact that the <5 kDa COS caused aggregates at 0.1 mg/mL, whereas for <3 kDa COS there were no aggregates at this concentration. The development of RBC/COS clusters may have a major hemorheological impact. Red blood cell adhesion to COS without simultaneous significant hemolysis suggests that some disturbances may occur at the RBC membrane or at the cytoplasmatic level.

It is known that RBCs have a poor biosynthesis capacity and therefore suffer and accumulate the damages that occur along their lifespans.[54] These lesions, namely, reduction in sialic acid (responsible for the negative charge of the RBC membrane), exteriorization of acidic membrane phospholipids (phosphatidylethanolamine and phosphatidylserine), and Hb oxidation and linkage to the membrane, are indicators of RBC damage and aging.[51,55] When the Hb membrane linkage was studied after incubation with COS mixtures at increasing concentrations, high MBH values (Table 1) were observed at 0.5 and 1.0 and especially at 5.0 mg/mL for both COSs. (Once again, <5 kDa COS caused a greater effect.) This indicated a possible oxidative stress induced by both compounds on RBCs during incubation. Further analysis of the nature of that Hb bound to the membrane by examination of the attained spectra showed, however, that most of the Hb was not in the oxidized form. These results suggest that the Hb linked to the membrane is not mainly due to an oxidation process but is probably due to the presence of high concentrations of COS on the RBC surface (as visualized by AFM) and the development of attraction forces (due to the strong positive charge of COS and its iron-binding capacity), which may eventually lead to the formation of Hb−chitosan complexes.[56,57]

In agreement with the aforementioned results, no changes in RBC membrane protein composition were observed for either COS mixture at concentrations of <0.1 mg/mL. (See Figure 3.) In the case of the 5 kDa COS mixture, a slight reduction in α- and β-spectrin and band 3 concentrations was found between 0.1 and 1.0 mg/mL; this effect occurred to a greater extent at 5 mg/mL for both COS mixtures. The reduction in spectrin, a cytoskeletal membrane protein,[58] and band 3, the major transmembrane protein linking the cytoskeleton to the lipidic bilayer and an anion exchange protein,[59] may underlay a membrane structure disturbance as well as metabolic and electrolytic disturbances within the cell. Actually, the development of two new bands, one identified as peroxiredoxin 2, which has been associated with the development of oxidative stress within the cell,[60] and the other identified as calmodulin, is probably also related to a disturbance in RBC metabolism. The development of a third band of Hb chains agrees with our membrane studies that show an increase in MBH at higher COS concentrations for both mixtures. It seems reasonable to assume that a direct interaction of COSs on RBC constituents occurs (with a potential impact on RBC metabolism) because at concentrations above 0.1 mg/mL, the membrane surface was almost covered by a COS layer (Figure 4C,D) that was not observed at the lowest concentrations tested.

AFM images (Figure 4) helped to elucidate the impact of COSs on the RBC surface. This impact was most obvious at 5.0 mg/mL because the COSs completely covered the RBCs. This result is likely related to other data obtained in this work such as the high level of MBH or the drastic reduction in spectrin and band 3 proteins. In fact, as seen in OM images at this concentration, many RBCs lost their typically biconcave shape possibly because of the great attraction forces that such a high concentration of positively charged COS exerts on them. At lower concentrations, the smoothness of the RBC surfaces increased, suggesting that at concentrations of <0.5 mg/mL, the adhesion of COSs to the RBC surface can be considered to be null. The surface profiles plus the roughness results support the aforementioned AFM images: at 5.0 mg/mL, the RBC surfaces exhibit extremely rough surfaces (for both mixtures) because of the formation of a COS layer, which is shown by the profiles as well as Rrms values that are much higher than those obtained at lower concentrations. In the case of <5 kDa COS, the AFM images showed the appearance of several new features. These differences were the large globular features seen in Figure 5B at the highest COS concentration and holes on the surface (Figure 5A) at the lowest concentration, which may be related to interference with the cytoskeleton of the RBC. It is interesting that at 0.05 mg/mL and in the case of <5 kDa, a decrease in membrane roughness occurred. A similarly small but significant decrease in roughness when compared with the control RBCs was reported by Girasole et al.[48] in the case of cells with spherocytosis. Taking in consideration the fact that spherocytosis may be due to spectrin and band 3 deficiencies,[61] this may be related to the loss of spectrin upon treatment with <5 kDa COS. If this was the case, then a greater number of holes would be present at higher concentrations (e.g., at 5 mg/mL, see protein profile in Figure 3); unfortunately, the coating of the cell by COS prevented this observation by AFM.

By combining the results of hemolysis with those of RBC membrane protein analysis and with those provided by AFM, we may state that at COS concentrations of ≤0.1 mg/mL, no significant damage occurs in RBCs. Our results also indicate that COS damage to RBCs is dependent on the MW and concentration: a higher MW or a higher concentration leads to greater damage of RBCs. However, further studies are needed, namely, bioavailability studies, to attempt to establish safe COS concentrations in blood.

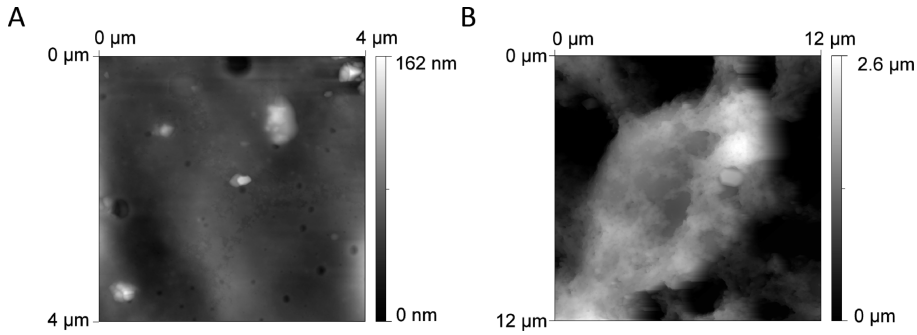

Figure 5. Tapping mode image of RBC incubated for 3 h at 37 °C in the presence of COS (<5 kDa): (A) 0.05 and (B) 5 mg/mL.

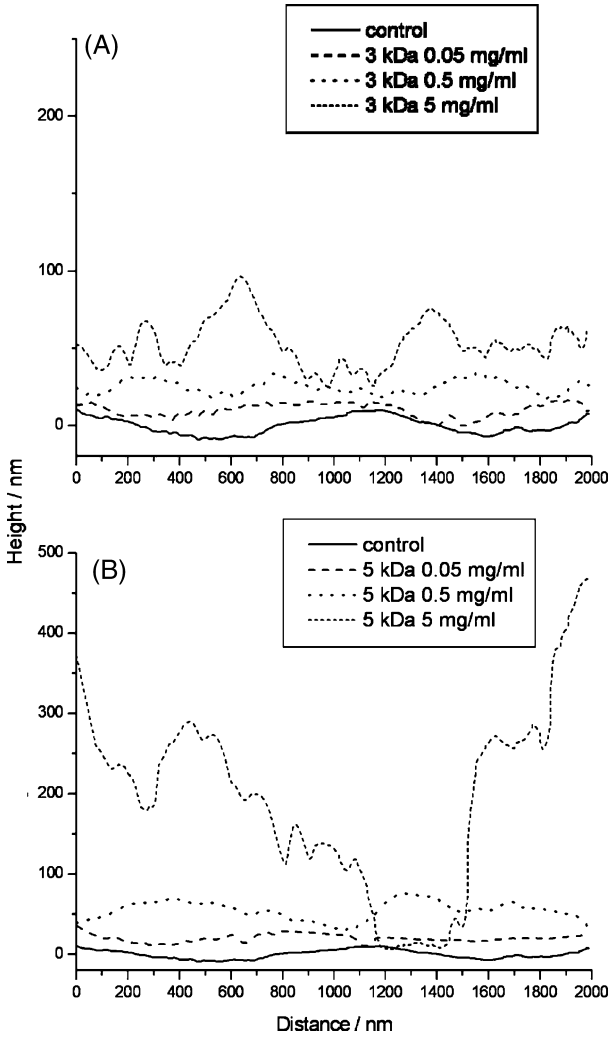

Figure 6. Topographic profiles of RBC surface incubated for 3 h at 37 °C in the presence of various concentrations of COS mixtures with (A) <3 and (B) <5 kDa.

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