Abstract: Alginate is a biopolymer extracted from brown algae intracellular matrix. It is constituted by two monomers: D-mannuronic (M) and L-guluronic (G). It has many interesting properties, such as low toxicity, biocompatibility and biodegradability. The aim of the present study was to characterize the alginate rich in guluronic acid (AGA) and evaluate its effect in human neutrophils. Analyzing the NMR spectra, it was possible to see a considerable separation for alginate polymer blocks (higher G-block content). The neutrophils were incubated with AGA and stimulated by PMA. Myeloperoxidase (MPO) released by cells was used as marker of neutrophil degranulation. The AGA inhibited the neutrophils degranulation induced by PMA, and it was not observed any cytotoxic effects of this alginate in human neutrophils. This work showed the anti-inflammatory activity, without interfering with the cell viability. The chemical and biological characteristics of AGA support its potential use as functional biomaterial for healthcare.

Key-words: material, alginate, human neutrophils, toxicity, separation.

1. INTRODUCTION
Alginate is a biopolymer extracted from brown algae such as *Laminaria hyperborea* and *Macrocystis pyrifera*. It has two monomers: D-mannuronic (M) and L-guluronic (G) and it can be formed by exclusive blocks of M monomers (M blocks), exclusive blocks of G monomers (G blocks) and both (MG blocks). There is a variation of M/G rate according to the source of the alginate, where the algae were collected, so the same company can produce different alginates.

Sodium alginate, salt formed by the treatment of alginate with sodium carbonate (Na$_2$CO$_3$) it is widely used in many industries as cosmetic, biotechnological, food and pharmaceutical.

According to Pawar and Edgar (2012), a series of chemical and biochemical modification have been developed for alginate, some of them to enhance proprieties and some of them to manipulate its structure so a new propriety is added, such as solubility, antithrombogenicity, reactivity, and affinity with macromolecules.

![Structure of sodium alginate, a) G Block, b) M block and c) MG block.](image)
As shown in the figure 1, mannuronic acid has a linear structure, while guluronic acid has a wavy chain. Due to this conformation, when in contact with divalent charges, as Ca$^{2+}$, the G blocks forms a cation-bridge structure as shown in figure 2. The cation occupies the intermolecular space and then gelation occurs.

![Figure 2: Alginate and calcium in a crosslinking process.](image)

According to Otterlei et al (1991), alginate rich in M blocks is more capable to stimulate the production of cytokines in contact with the human body, such as interleukins 1 and 6. These signaling molecules are produced during immune system reactions, mediating and regulating important organism responses, such as the inflammatory processes. Also according to Otterlei et al (1991), alginate with high G/M rates is about 10 times less capable of stimulate these signaling molecules release. Therefore, cytotoxicity and anti-inflammatory property tests with human neutrophils (one of the main cells of immune system) were performed.

With this in mind, this study aimed to evaluate the alginate as a biomaterial with enhanced anti-inflammatory properties, increasing G/M rate and evaluating cytotoxicity, and anti-inflammatory properties, using neutrophils cells, comparing with the alginate of low G/M rate.

2. MATERIALS AND METHODS

2.1. Materials

Sodium alginate was purchased by Dinâmica (Brazil). Calcium chloride was purchased from Vetec (Brazil). Chloride acid and sodium hydroxide for hydrolysis
and pH control were purchased from Sigma. All reagents were obtained in analytical degree.

For biological evaluation it was used the following materials: Phorbol 12-myristate-13-acetate (PMA), 3,3′,5,5′-Tetramethylbenzidine (TMB), Triton X-100, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and DMSO (dimethyl sulfoxide).

2.1.1. Methods

2.1.2. Alginate separation process: The separation was performed according to Hang e Larsen (1962). 7.5g of alginate were added in 250mL of chloride acid 0.5 M. The solution was agitated for 8 hours at 100°C. The pH was adjusted to 2.85 pH using drops of sodium hydroxide (2M). At this pH the guluronic acid is insoluble, so the precipitation is performed. The solution was filtrated with common paper filter and left in room temperature so it could dry properly.

2.1.3. Nuclear Magnetic Resonance (NMR): NMR spectra were obtained on an apparatus Agilent DD2 of 600 MHz (for H1 nucleus) and One Probe of 5mm internal diameter (1H-19F/15N-31P). The tests were conducted at Laboratório Multisuário de Química De Produtos Naturais (LMQPN) da Embrapa Agroindústria Tropical (EMBRAPA). The samples were prepared dissolving 10mg in 600 μL deuterated water. The spectrum was obtained at 70°C, with 20s between the acquisitions.

2.1.4. Biological Evaluation in human neutrophils: Human leucocyte-rich blood from healthy adults was obtained from HEMOCE (blood bank), Fortaleza, CE, Brazil. Neutrophils were isolated by Lucisano and Mantovani’s method (...). The polymorphonuclear cells were suspended in Hanks balanced salt solution (HBSS) containing 80–90 % neutrophils with viability higher than 95 % established by exclusion with trypan blue assay. Cytotoxicity: MTT assay. Human neutrophils (2.5 x 10^6 cells/mL) were exposed to alginate rich in guluronic acid - AGA(5 – 100 μg/mL) for 30 min, at 37°C, and then incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
(MTT). After 3 h under a 5 % CO₂ atmosphere, the cells were washed with PBS, and 100 µl of dimethyl sulfoxide were added to the medium for solubilization of the formazan product. The absorbance was measured at 550 nm, and a decrease in absorbance indicated a decrease in cell viability.

Degranulation assay: measured of myeloperoxidase release by cells. Boyum (...), the cells (2.5 × 10⁶ cells/mL) were incubated with alginate rich in guluronic acid (3, 6, 12,5 , 25, 50 and 100 µg/mL) or HBSS for 15 min, 37°C. Human neutrophils were stimulated by the addition of PMA (0.1 µg/mL) for 15 min at 37 °C. The reaction was stopped by cooling and the suspension was centrifuged for 10 min. at 2000 × g at 4°C. Aliquots (50 µl) of the supernatants were added to phosphate buffered saline [PBS (100 µl)], phosphate buffer (50 µl, pH 7.0) and H₂O₂ (0.012 %). After 5 min. at 37 °C, 3,3',5,5'- tetramethylbenzidine (1.5 mM, 20 µl) was added, and the reaction was stopped with 30 µL of sodium acetate (1.5 M, pH 3.0). The absorbance was determined using a spectrophotometer reader, set at 620 nm.

Statistical analysis: The results are expressed as mean ± S.E.M. The statistical significance of differences between groups was determined by One-Way ANOVA, followed by Tukey for multiple comparisons as a post hoc test. The significance level was set at P < 0.05.

3. RESULTS

3.1. Nuclear Magnetic Resonance (NMR)

Analyzing the spectrum for both alginates it was possible to see many changes in the conformation chain after the modification. The pecks were larger before the modification, showing that the hydrolysis broke the chain, and therefore, the steric hindrance was minimized. Analyzing the 13C spectrum, the picks of the M monomer were significantly lower in the modified alginate. For quantitative analysis, the pecks were derived and the proportion was found, as shown in the following picture.
Before the separation, the alginate was 51.62% constituted of M and 48.38% of G, giving a rate of G/M = 0.94. After separation, the NMR showed that the modified alginate had 22.69% of M and 77.31% of G, therefore the rate was 3.4.

3.2. Biological evaluation in human neutrophils

The toxic effect of alginate before and after separation were investigated through MTT test, which is a tetrazolium salt that can be reduced to formazan by the mitochondrial dehydrogenase, enzyme active only in living cells.

As shown in Figure 4, the addition of calcium alginate (normal alginate) (10, 50 e 100 µg/mL) in human neutrophils did not reduce significantly the cells viability (89.5 ± 1.7; 90.6 ± 2.3; 86.3 ± 1.2 %) when related to control group (cells viability: 94.3 ± 2.3 %), while triton X-100 0.2% (cytotoxicity drug) descreased in about 90 % the cell viability.

The treatment of the human neutrophils with AGA at concentrations ranging from 10 to 100 µg/mL (115.9 ± 6.3 %; 111.0 ± 4.8 %; 105.5 ± 5.9 %, respectively) also did not reduce significantly the viability of cells (control group/DMSO 0.1%: 100
On the other hand, Triton x-100 (28.16 ± 2.201%) as the reference drug, significantly decreased the viability of cells when related to control group. The control group (DMSO 0.1%) did not alter significantly when related to HBSS group (culture medium).

![Graph](image)

**Figure 4:** Evaluation of alginate before and after separation toxicity measured by MTT test in human neutrophils. Data from two to six samples. Control: Vehicle (water), TX: Triton X-100 (0.2%). * vs. HBSS: Hanks’ solution (untreated cells). Results represent means ± EPM. (p < 0.05; ANOVA and Tukey’s post hoc test).

Myeloperoxidase was used as marker of neutrophil degranulation and its release by human neutrophil was determined by spectrophotometry.

Alginate before the separation (10, 50 and 100 µg/mL) demonstrated a good effect in inhibiting the myeloperoxidase enzyme delivery by the neutrophils, induced by PMA, resulting in a maximum inhibition of 52.33% compared to the standard drug, the indomethacin, that was capable of inhibit the degranulation in 73.35%.

The addition of AGA (10, 50 and 100 µg/mL) to neutrophils before PMA exposure significantly (p<0.05) reduced MPO release with an average inhibition of 30.02 ± 2.5; 59.3 ± 1.4; 63.3 ± 1.0 %, respectively. While indomethacin, non steroidal anti-inflammatory, reduced in 76.1 ± 1.8 % the MPO release.
Figure 6: Effects of alginate before and after separation on the release of human neutrophil myeloperoxidase (MPO) stimulated by phorbol myristate acetate - PMA. Freshly isolated cells (5 × 10⁶) were pre-incubated with indicated concentrations of Alginate prior to the addition of PMA (0.1 µg/mL). Control: Vehicle (water), Indo: Indomethacin (100 µM, standard drug). * vs. HBSS: Hanks’ solution (untreated cells). Results represent means ± EPM. (p < 0.05; ANOVA and Tukey’s post hoc test).

The modified alginate showed a better viability in the neutrophils, which means less toxicity and aggression to the human cells. The AGA showed even a better viability than the control, while Alg had a small decrease.

4. CONCLUSION

Analyzing the NMR spectra, it was possible to see a considerable separation for alginate polymer blocks, thus obtaining a material with a higher G-block content. This alginate rich in guluronic acid inhibit neutrophil pro-inflammatory responses, including degranulation (measured by MPO release), without affect the viability of cells. Therefore, this work shows a better alginate to be used in healing dress or as
biomaterial for healthcare, where there is almost no inflammatory response and the human cells can grow properly.

REFERENCES


