

## CHEMICAL MODIFICATION OF ALGINATE WITH L-CYSTEINE TO EXTEND ITS USE IN DRUG DELIVERY SYSTEMS

YUBEXI CORREA MARCANO and MARCOS A. SABINO G.

*Department of Chemistry, B5IDA Group, Simón Bolívar University, AP 89000 Caracas, Venezuela*

✉ *Corresponding author: M. A. Sabino G., msabino@usb.ve*

*It is an honor for us to contribute a small but valuable piece of our work to this special issue dedicated to the 70<sup>th</sup> anniversary of Acad. Bogdan C. Simionescu. Congratulations on this 70<sup>th</sup> anniversary and continued success.*

In this research, alginic acid was chemically modified by coupling it with the amino acid L-cysteine to improve its solubility, biocompatibility and mucoadhesive properties, which allows expanding its use in pharmacological applications. L-Cysteine was grafted in different ratios (2:1, 1:1 and 2:3 molar ratios) and these alginate-L-cysteine conjugates were characterized by Infrared Spectroscopy (ATR-FTIR) and Nuclear Magnetic Resonance (<sup>1</sup>H-NMR). The concentration of the thiol group from the coupled cysteine was determined. The improvement in mucoadhesivity was demonstrated by mechanical tensile studies, and the conjugate with the ratio of 2:1 presented the highest adhesion capacity. From these modified biopolymers, micro/nanoparticles were synthesized by the ionic/microemulsion gelation method (Zn<sup>+2</sup> divalent ion). Using Scanning Electron Microscopy (SEM), the morphology of the obtained micro and nanometric spherical particles was observed. The results indicated that thiolated alginate may have potential for use in drug delivery systems, especially in those where it is required to extend the residence time in the epithelial mucosa.

**Keywords:** alginic acid, L-cysteine, thiolated biopolymer, mucoadhesives, ionic gelation, nanoparticles

### INTRODUCTION

Currently, the development of mucoadhesive polymers is one of the numerous areas of major interest in the field of polymeric biomaterials. This type of polymers could form interfacial interactions or even covalent bonds with mucous membranes (such as the digestive tract), and thus, could prolong the residence time of a drug within that environment, taking advantage of its dosage.<sup>1-3</sup> This can be achieved by incorporating an adhesive molecule either by some type of pharmaceutical formulation or by a functionalization process, to maintain a close contact with the absorption tissue. Both systems would allow the release of the drug near the required site of action, increasing its bioavailability.<sup>4,5</sup> As it is well known, the interest in the release of drugs through carrier materials has increased considerably over the past few years. The reasons are obvious, given that if the carrier has the potential to direct the drug to its target, an optimal pharmacological effect could be obtained and, at the same time, the adverse effects

of the drug could decrease.<sup>6</sup> Polysaccharides, such as alginate, chitosan and hyaluronic acid, are among the natural polymers used for bioadhesion processes and for the synthesis of nanoparticles.<sup>7</sup> A reaction such as thiolation may be a good method of chemical modification for such biopolymers, while alginate is one of the agents that could facilitate the absorption of a biopolymer into this type of mucosa. With these considerations in mind, the general objective of this research was to chemically modify alginic acid with L-cysteine amino acid in order to achieve thiolated gels with improved mucoadhesivity. Also, the study aimed to obtain nanoparticles from the modified alginate and investigate their potential use in the release of drugs, starting from the supposition that the nanoparticles could have better stability and extended residence time in a mucosa, such as the epithelial tissue present in the walls of the intestine.

## EXPERIMENTAL

### Materials

Sodium alginate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), L-cysteine monohydrate hydrochloride, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) (DTNB), and polyvinylpyrrolidone (PVP) were supplied by Sigma-Aldrich. Tween 80 was purchased from Merck, and zinc sulphate heptahydrate ( $ZnSO_4 \cdot 7H_2O$ ) from Lobal Chemie. All the chemicals were of analytical grade.

### Synthesis of the cysteine polymer conjugate: alginate-g-cysteine

The procedure for the synthesis of the alginate graft L-cysteine conjugate (alginate-g-cysteine) was adopted from the work done by Bernkop-Schnurch *et al.*<sup>8</sup> The carboxylic acid group of the biopolymer was activated by the addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) in a 1:2 ratio (EDAC:NHS). The modification process was performed for three proportions of the amino acid L-cysteine, namely, 2:1, 1:1 and 2:3 (EDAC:L-cysteine). The obtained alginate-g-cysteine conjugate (2:1, 1:1 and 2:3) was dialyzed for 48 h in 1 mM HCl/1% NaCl solution. After that time, the samples were frozen and then lyophilized for 24 hours at  $-48^\circ C$  and 0.125 mBar.

### Determination of thiol group content

The degree of thiolation was determined by the addition of the Ellman reagent (DTNB).<sup>9</sup>

### Determination of mucoadhesive capacity by uniaxial stress test

The test samples were prepared using commercial sheets of unflavored gelatin/collagen. The samples and the test conditions were in accordance with the technical standard method ASTM-D638-10.<sup>10</sup>

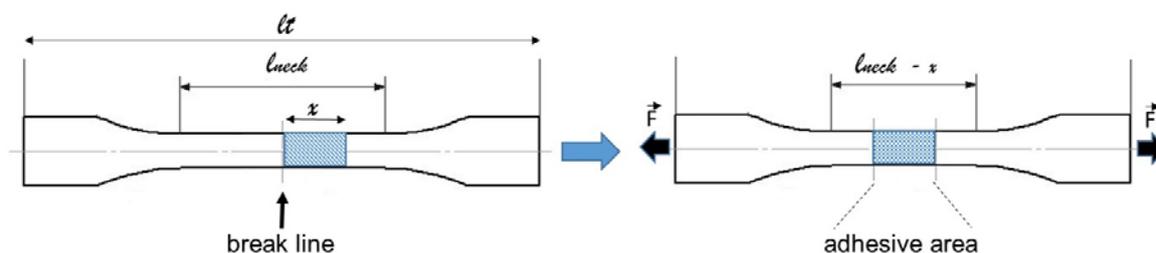


Figure 1: Sample preparation for evaluating mucoadhesive performance

### Preparation of nanoparticles, encapsulation of antibiotic and characterization

By combining the ionic/microemulsion gelation techniques, nanoparticles were prepared, following the protocol used by Sereno *et al.*<sup>11</sup> Three solutions were prepared with the following composition: (1) 1% solution of alginic acid or conjugate (2:1), 0.8% Tween

### Tensile essays

For the tensile tests, type IV dumbbell-shaped specimens were cut in half. In the adhesion area, solutions of alginate-g-cysteine (2:1, 1:1, 2:3) were placed individually to function as adhesive, as shown in Figure 1. As a control adhesive, L-cysteine and alginic acid solutions were also used. The drying time prior to the tensile tests was 24 hours at room temperature.

The test specimens were analyzed in a JILLOYD Instruments universal test machine, model T5003, with flat jaws suitable for this test, using a load cell with a maximum capacity of 100 N. The uniaxial tensile tests were performed in triplicate, at 5 mm/min and at room temperature.

### Characterization by ATR-FTIR spectroscopy

The FTIR-ATR spectra of the dry powder samples were performed on a Thermo Scientific Infrared Spectrophotometer with an ATR accessory, model Nicolet iS5. Scans were taken in the range of  $4000-400\text{ cm}^{-1}$ , with KBr beamsplitter and at a resolution of  $4\text{ cm}^{-1}$  (32 scans).

### Characterization by Proton Nuclear Magnetic Resonance ( $^1H$ -NMR)

For the  $^1H$ -NMR analysis, a JEOL Nuclear Magnetic Resonance Spectrometer of 400 MHz was used. 128 scans were performed at room temperature and  $D_2O$  was used as solvent.

### UV-Visible spectrophotometry

The concentration of L-cysteine grafted onto the alginate was determined using a UV spectrophotometer (Agilent 8453) in the wavelength range of 160-600 nm. This technique was also used to study the drug release process, which will be explained later.

80, 1% trimethoprim/sulfamethoxazole (SMZ/TMP) antibiotic (commercially known as Bactrimel) and 0.001 g of  $NaHCO_3$  in 5 mL of deionized water; (2) solution with 3.50 g of  $ZnSO_4 \cdot 7H_2O$  in 5 mL of deionized water (pH = 1.5 adjusted using HCl); and (3) solution with 0.25 g of PVP in 10 mL of water. After magnetic stirring, the second solution was poured into

the third portion gradually and an ultra-dispersive head (IKA™ T10 Basic Ultra-Turrax™) was used. Then, 35 mL of isopropyl alcohol was added in 5 mL portions. Then, the solution (1) was added dropwise to the conjugate and stirred for 5 minutes; upon completion of the addition, it was left under stirring using an ultradispersor for an additional 30 minutes. Finally, the solution was allowed to settle, to precipitate the obtained particles, the supernatant was removed carefully, centrifuged, dialyzed and the particles were lyophilized.

#### ***In vitro* release of SMZ/TMP from the nanoparticles**

Following the release protocol of the USP XXXI 2008,<sup>12</sup> the release of the encapsulated ion was evaluated by simulating *in vitro* conditions of gastric pH (HCl, pH 1.5) and the pH of the human intestine (phosphate buffer with pH 6.5) (both tests were performed at room temperature, without considering the presence of enzymes).

Initially, 10 mg of alginic acid and alginate-g-cysteine particles (2:1) were taken. These were immersed into 20 mL HCl (each separately) and 1 mL aliquots (in triplicate) were taken every 20 min for 2 hours. The aliquots were centrifuged and then analyzed by UV-Visible spectroscopy, in order to determine the amount of drug released under these conditions. After that, the investigation was performed in gastric medium. For this, the remaining solutions with the particles in acid medium were centrifuged and decanted, the solid was placed in a phosphate buffer at pH 6.5 and aliquots were also taken in triplicate every 20 min for 2 h. Subsequently, the UV-Visible absorption of the aliquots was evaluated.

For the measurements, a wavelength equal to 257 nm, which was determined as the  $\lambda_{\max}$  for the absorption of this antibiotic, was used.<sup>13</sup>

#### **Particle morphology by Scanning Electron Microscopy (SEM)**

SEM microphotographs were taken using a JEOL scanning electron microscope, model JSM-6390. Before observation, the particles were coated with gold/palladium, using a Balzers-SCD-030 sputter-coater. The voltage used was 15 KV.

## **RESULTS AND DISCUSSION**

### **Synthesis of the alginate-L-cysteine conjugate**

Conjugation of the cysteine molecule to the alginate chain is expected to occur by a reaction with the formation of amide bonds between the amino group of the amino acid and a carboxylic acid group of the biopolymer, as shown in Figure 2.

### **Characterization by FTIR-ATR spectroscopy**

Comparing the FTIR-ATR spectrum of alginic acid with the spectra of the three modified alginates with different ratios (Fig. 3) reveals a number of peaks that are indicative of the modification. It can be seen that the band corresponding to the C=O vibration (carbonyl from amide I) is located at approximately 1646  $\text{cm}^{-1}$ . The amide band II is a combination of the C=O stretching and N-H bending, which occurs in the range of 1570-1515  $\text{cm}^{-1}$  for secondary amides. In the spectra of the modified alginate, the amide band II is observed at a wave number around 1557  $\text{cm}^{-1}$ . The vibration of the C-N bond occurs near 1407  $\text{cm}^{-1}$  due to the existence of a partial  $\pi$  bond between carbon and nitrogen for resonance.<sup>14,15</sup> Meanwhile, for alginic acid, the carbonyl band is observed at 1718  $\text{cm}^{-1}$ , for the free L-cysteine – at 1747  $\text{cm}^{-1}$  and for the amino acid – at 1426  $\text{cm}^{-1}$ . The band corresponding to the S-H stretching vibration is characteristically weak and is usually undetectable in IR spectra, and the band corresponding to hydrogen bonding for S-H is much weaker than those for O-H and N-H.<sup>15</sup>

### **Characterization by Proton Nuclear Magnetic Resonance (<sup>1</sup>H-NMR)**

In the spectrum of modified alginate (Fig. 4.A), several peaks, which were not present in that of the unmodified alginate, occur in the region of 2.8-5.0 ppm (Fig. 4.B), indicating that a functionalization reaction occurred. For a classical <sup>1</sup>H-NMR spectrum of the L-cysteine amino acid,<sup>9</sup> about three peaks can be distinguished, one of which is due to the resonance of the proton *Ha* ( $\delta = 4.8$  ppm). This proton in the amino acid is in an environment that has a high electronic density, because it is adjacent to the NH group and is neighboring the SH, which causes the proton *Ha* to have a displacement to lower fields. In this case, for alginate modification, the proton *Ha* is observed between  $\delta = 4.5$ -5.0 ppm. The peak located around  $\delta = 2.9$  ppm corresponds to the protons *Hb*, which are adjacent to the SH group. Some of the signals observed can be attributed to possible residues of the EDC and NHS coupling compounds, despite the purification process to which the (purified) alginate was subjected. This may justify the presence of signals in the region between 1-2.9 ppm.<sup>16</sup>

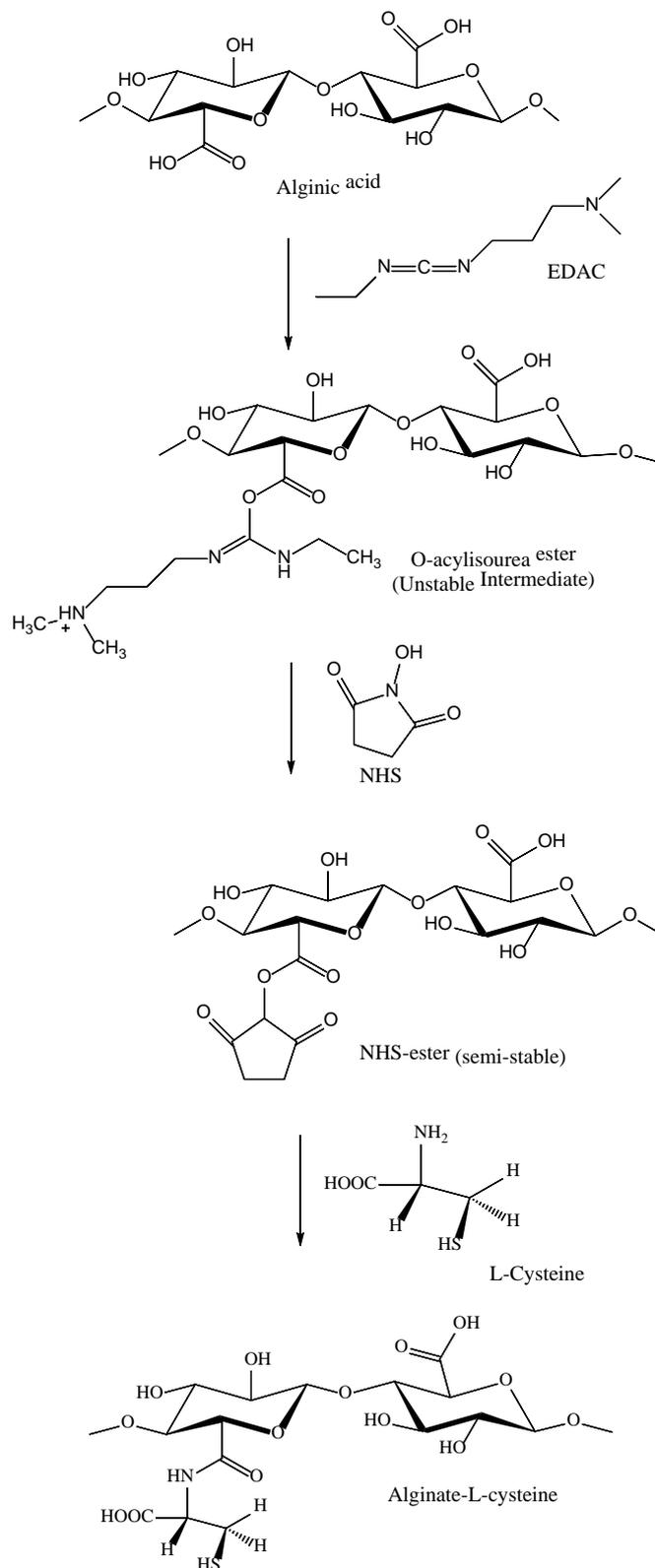


Figure 2: Schematic illustration of alginate-cysteine conjugation through amide bonding between the primary amino group of L-cysteine and carboxylic acid group of alginate mediated by EDAC

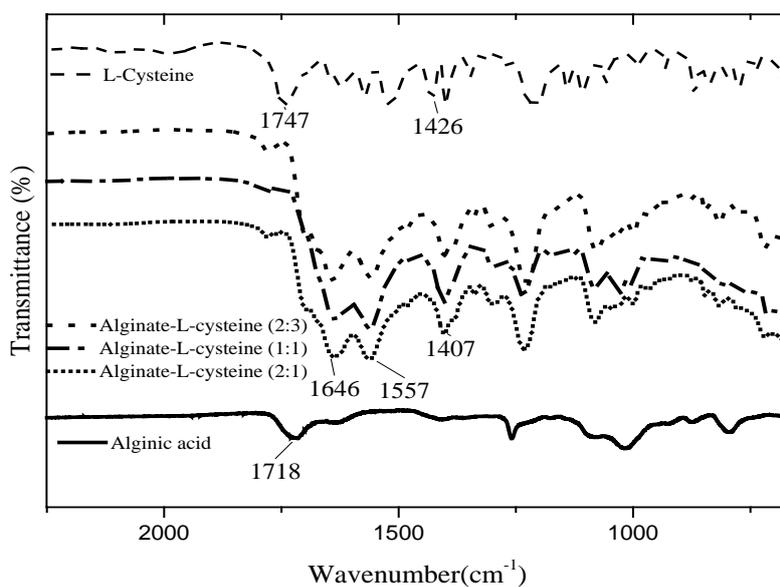


Figure 3: FTIR spectra of alginate-L-cysteine (2:1), (1:1), (2:3), compared to those of alginate acid and L-cysteine

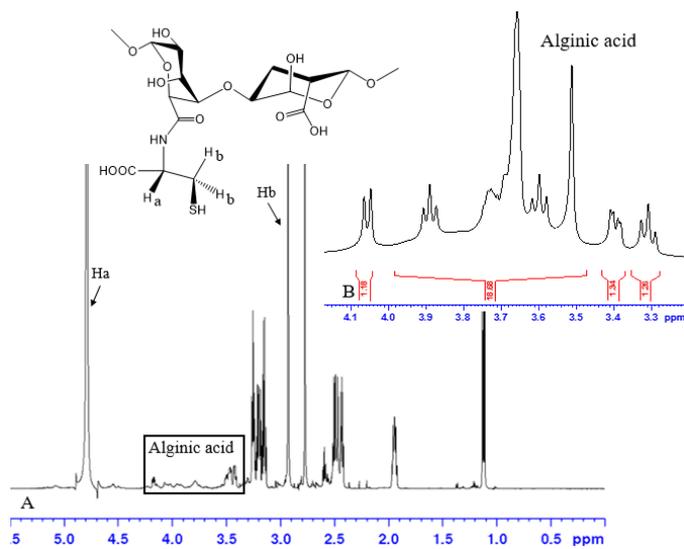


Figure 4: <sup>1</sup>H-NMR spectra of (A) alginate acid (B) alginate-L-cysteine (2:3) solution in D<sub>2</sub>O



Figure 5: Formation of TNB<sup>2-</sup> ion in a solution of the alginate-L-cysteine conjugate (2:3) (yellow), and unmodified alginate solution (transparent)

### Determination of the presence and concentration of thiol group in the conjugate

The modification due to the coupling of the cysteine to the alginate to obtain the three conjugates (2:1; 1:1 and 2:3) was determined by the reaction with the Ellman reagent. When 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was added to each of the alginate-g-cysteine proportions, a yellow solution was obtained as a consequence of  $\text{TNB}^{2-}$  ion formation, as observed in Figure 5.

The presence of the thiol group indicates that sulfur is present in the sample, but does not provide information about the state of the sulfur (as a reactive sulfhydryl group or if it is oxidized).

This phenomenon has already been reported for chitosan-TBA and for other thiolate compounds.<sup>17,18</sup> In addition, the thiol group is very susceptible to oxidation upon exposure to atmospheric oxygen.<sup>19</sup> In this type of reaction, it is possible that disulfide-like bonds or cross-links (-S-S-) are created, but this structure does not necessarily represent a disadvantage in the development of drug delivery systems. In contrast, these systems may be very convenient for controlling the release of drugs, by controlling the viscosity of these gels, and also for the dimensional stability of the particles.<sup>20,21</sup>

According to the literature, the degree of modification is related to the increase of the adhesive property. It is for that reason that it is important to determine not only the presence of the group, but also the concentration reached by this modification. In some research studies, the carbodiimide-mediated thiolation reaction (without the use of NHS) has been reported to have immobilized thiol groups in the range of 230 to 340  $\mu\text{mol/g}$  of the polymer.<sup>22,23</sup> Table 1 below shows the concentration of the cysteine present in the alginate-g-cysteine conjugate. The results are consistent with the amino acid ratios used for the modification. However, the results do not necessarily imply an improvement in the mucoadhesive response, as further demonstrated by the mechanical tests.

### Tensile tests for measuring mucoadhesivity

Table 2 summarizes the results of stress at break ( $\sigma$ ) and strain at break ( $\epsilon$ ) of the gelatin/collagen specimens tested. The sample where the alginate solution was used as adhesive showed the lowest breaking strength of all the tested specimens, despite it having the

highest deformation at break. However, the sample with the modified alginate acid acquired similar results, for both stress and strain at break, to those obtained when the amino acid was used as adhesive. In the case of the conjugate (2:3), where the greatest number of grafted amino acid groups were obtained, a low resistance was achieved, which could be explained considering that there is a critical concentration that favors mucoadhesivity. From this value, other interactions would be favored, or it is possible that cross-linking reactions are favored, which justifies the achieved stiffness resulting in low deformation at break.

The adhesive property of alginate acid can be attributed to the presence of -OH groups, which can form hydrogen bonds with the amino acid residues present in the gelatin/collagen films. On the contrary, in the modified polymer, it is due to the disulfide bond (-S-S-) formation between the -SH groups of the modified polymer and the -SH groups of the cysteine residues present in the gelatin/collagen film.

Bernkop-Schnürch *et al.*<sup>7</sup> performed tensile studies on alginate acid and an alginate/L-cysteine blend on porcine mucosa, and their results revealed that there is a strong influence of the coupling of L-cysteine on the mucoadhesive properties of the polymer, due to the immobilization of the thiol group. On the other hand, Guggi *et al.*<sup>24</sup> also determined the mucoadhesive capacity of microparticles of thiolated sodium alginate to the buccal mucosa of chicken, and found that the thiolated microparticles had greater retention when they were spread on the buccal mucosa, compared to the unmodified alginate microparticles. Both authors attributed the superiority of the mucoadhesive property of the modified polymer to the formation of disulfide bonds with the mucous membranes, which is a much stronger interaction than the hydrogen bonds formed by the unmodified alginate.<sup>7,24</sup>

### Encapsulation of a drug in the particles obtained

After evaluating the degree of adhesion, micro/nanoparticles of the conjugate were synthesized, using essentially the ratio of 2:1, which exhibited the best mucoadhesive behavior (according to Table 1). The technique used was ionic gelation/ microemulsification, as reported by O. Sereno *et al.*<sup>11</sup>

Table 1  
Concentration of L-cysteine amino acid present in the alginate-g-cysteine conjugate

Sample	[Thiol] $\mu\text{mol}/(\text{g-alginate})$
Alginate-L-cysteine 2:1	1939
Alginate-L-cysteine 1:1	3826
Alginate-L-cysteine 2:3	5068

Table 2  
Adhesive properties of conjugates

Sample/adhesive	$\sigma_r$ (Mpa)	$\epsilon_r$ (%)
L-cysteine adhesive	$6.0 \pm 2.0$	$1.5 \pm 0.4$
Alginic acid adhesive	$2.0 \pm 1.0$	$7.0 \pm 5.0$
Alginate-L-cysteine 2:1 adhesive	$6.6 \pm 2.0$	$1.5 \pm 0.1$
Alginate-L-cysteine 1:1 adhesive	$6.0 \pm 3.0$	$1.2 \pm 0.2$
Alginate-L-cysteine 2:3 adhesive	$2.4 \pm 0.3$	$0.6 \pm 0.4$

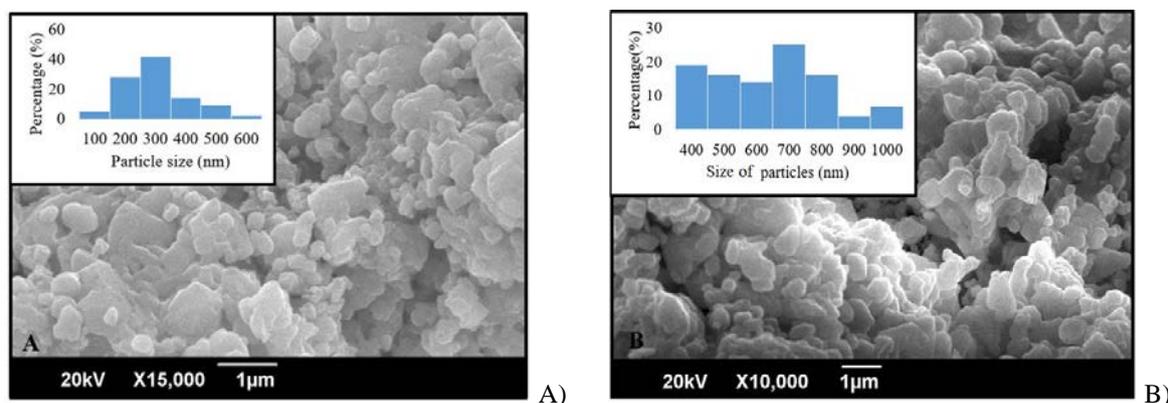


Figure 6: Micrographs of (A) alginic acid/ $\text{Zn}^{2+}$  particles with the drug and (B) alginate-L-cysteine (2:1) conjugate/ $\text{Zn}^{2+}$  particles with the SMZ/TMP drug

The drug that was used for the encapsulation process was sulfamethoxazole/trimethoprim (SMZ/TMP), which is an antibiotic used in the treatment of urinary, respiratory, gastrointestinal and bacterial infections.

Figure 6 shows: (A) the particles of alginic acid/ $\text{Zn}^{2+}$ ; and (B) the alginate-g-cysteine (2:1)  $\text{Zn}^{2+}$  conjugate loaded with the drug SMZ/TMP, respectively. SEM micrographs, for both cases, exhibit particle agglomerates with spherical morphology, with sizes in the nanometer scale. In the case of alginic acid/ $\text{Zn}^{2+}$ , particles with sizes in the order of 200-300 nm are predominant, whereas the particles of the alginate-g-cysteine/ $\text{Zn}^{2+}$ /drug conjugate have a slightly greater size, of about 400-700 nm. The difference in size between the particles obtained with the alginic acid and the conjugate is due to the fact that, in the case of the conjugate, the presence of

the cysteine branch could generate an increase in the molecular volume, which, with the encapsulation of the drug, would lead to an increase in the final size of these particles. A similar difference in size has also been reported in the research of Das *et al.*,<sup>25</sup> where the synthesized alginate microparticles loaded with a utero-inhibiting drug (Isoxsuprine) were larger than the alginate particles without the drug.

In addition to the observation of particle morphology, the efficiency of drug encapsulation (%EE) in the particles was also investigated in this research. For this, the respective SMZ/TMP calibration curve was constructed using the UV technique. For the alginic acid particles, 78% of the drug was encapsulated, while for the particles obtained with alginic acid-g-cysteine, an encapsulation efficiency of 85% was obtained. In previous studies, unmodified calcium alginate

microspheres were used to encapsulate insulin through an extrusion process,<sup>26</sup> and an encapsulation efficiency of 65% was obtained. By internal gelation, between 65 and 79% of insulin was encapsulated, using calcium ion as a cross-

linking agent.<sup>27</sup> Thus, this method seems to be viable for the encapsulation of drugs, judging by the high percentage of encapsulation that can be achieved.

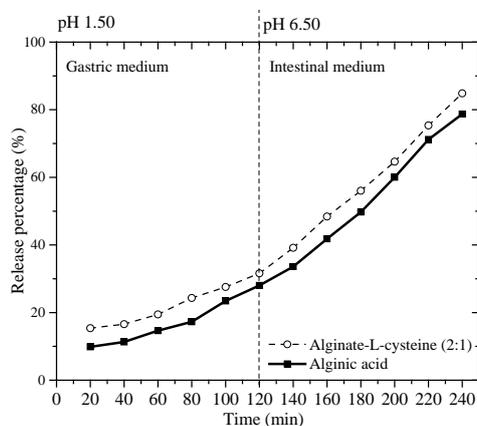


Figure 7: *In vitro* drug release from micro/nanoparticles in simulated gastric and intestinal medium without enzymes: (-■-) alginic acid and (-○-) alginate-g-cysteine (2:1), both of them containing the SMZ/TMP drug

#### ***In vitro* release of SMZ/TMP in simulated gastrointestinal medium**

Finally, antibiotic release tests were performed *in vitro* in simulated gastrointestinal media (see Fig. 7). Under the simulated gastric pH, the SMZ/TMP drug loaded into the alginic acid particles was released with a maximum amount of approximately 28%, whereas the alginate-g-cysteine particles exhibited a release of  $\approx 30\%$  for the same duration. When the same particles were subjected to a medium with intestinal pH, they appeared to undergo partial swelling or dissolution, thereby allowing almost the total release of the SMZ/TMP drug. The release percentages for alginic acid and alginate-L-cysteine conjugate (2:1) were of 80% and 85%, respectively.

Theoretically, alginate is contracted at low pH (gastric environment) and the encapsulated drug could not be released.<sup>30</sup> According to studies, the amount of drug released from the particles in the initial stage may be due to traces of the drug adhering to the surface of the particles, or to the drug dispersed more towards the surface of the particles.<sup>28</sup> When the pH was raised to 6.50, both alginic acid and cysteine are deprotonated. However, in the buffer solution used as release medium,  $\text{Na}^+$  and  $\text{HPO}_4^{2-}$  ions are present. In this case, the alginic acid can be converted into a soluble salt of sodium, and plus the  $\text{Zn}^{+2}$  ions can be sequestered by the  $\text{HPO}_4^{2-}$  ions, which would

destabilize the “egg box” conformation,<sup>29</sup> generally adopted by this biopolymer. This could explain the swelling of the particles and the slight differences in the drug release process. In spite of these slight differences, it should be remembered that the conjugate possesses a greater adhesion power to mucous membranes (as in the intestinal epithelial tissue), as opposed to unmodified alginic acid. Thus, it could be inferred that this drug could be absorbed more efficiently through the walls of the digestive tract.

#### **CONCLUSION**

The L-cysteine amino acid was chemically grafted to alginic acid to form a new biomaterial alginate-g-cysteine. Immobilization of the amino acid resulted in a substantial improvement in the mucoadhesive property of the modified alginic acid. Also, from this new biomaterial, it was possible to obtain particles in the nanometric scale by means of a combined ionic gelation/microemulsification technique. Highly satisfying results were achieved with regard to the efficiency of encapsulation and release of a commercial antibiotic. The results demonstrate a high potential of this thiolated biopolymer to be used as a vehicle for drug delivery systems, as it exhibits improved stability and extended residence time in a mucosa, such as the epithelial tissue of the human intestine.

**ACKNOWLEDGMENT:** The authors would like to thank the polymer group (GPUSB-1) for the mechanical tests, the Laboratory E-USB for their support in the use of the Scanning Electron Microscope, the Laboratory of Photochemistry and Photobiology (USB) for the FTIR-ATR analyses. Also, the contribution of the Venezuelan Institute of Scientific Research (IVIC) by performing the <sup>1</sup>H-NMR analyses is gratefully acknowledged.

## REFERENCES

- <sup>1</sup> S. Duggan, W. Cummins, O. O'Donovan, H. Hughes and E. Owens, *Eur. J. Pharm. Sci.*, **100**, 64 (2017).
- <sup>2</sup> S. B. de Souza Ferreira, T. D. Moço, F. B. Borghi-Pangoni, M. V. Junqueira and M. L. Bruschi, *J. Mech. Behav. Biomed. Mater.*, **55**, 164 (2015).
- <sup>3</sup> K. Netsomboon and A. Bernkop-Schnürch, *Eur. J. Pharm. Biopharm.*, **98**, 76 (2016).
- <sup>4</sup> H. Hägerström, K. Edsman and M. Stromme, *J. Pharm. Sci.*, **92**, 1869 (2003).
- <sup>5</sup> J. Woodley, *Clin. Pharmacokinet.*, **40**, 77 (2001).
- <sup>6</sup> G. Tiwari, R. Tiwari, B. Sriwastawa, L. Bhati, S. Pandey *et al.*, *Int. J. Pharm. Investig.*, **2**, 2 (2012).
- <sup>7</sup> A. Bernkop-Schnürch, S. Scholler and R. G. Biebel, *J. Control. Release*, **66**, 39 (2000).
- <sup>8</sup> A. Bernkop-Schnürch, C. E. Kast and M. F. Richter, *J. Control. Release*, **71**, 277 (2001).
- <sup>9</sup> G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
- <sup>10</sup> ASTM D638, Standard Test Method for Tensile Properties of Plastics, ASTM International, West Conshohocken, PA, 2010.
- <sup>11</sup> O. Sereno, S. Ortiz, F. Dantas, J. V. Silva and M. A. Sabino, *Acta Microsc.*, **22**, 311 (2013).
- <sup>12</sup> US Pharmacopeia XXXI, US Pharmacopeial Convention, Rockville, MD, 2008.
- <sup>13</sup> S. P. Vijaya, E. J. Jebaseelan and N. Sundaraganesan, *Spectrochim. Acta, Part A Molecular.*, **118**, 1 (2014).
- <sup>14</sup> Y. Zhao, S. Gao, S. Zhao, Y. Li, L. Cheng *et al.*, *Mater. Sci. Eng. C*, **32**, 2153 (2012).
- <sup>15</sup> A. Barth, *Biochim. Biophys. Acta, Bioenerg.*, **1767**, 1073 (2007).
- <sup>16</sup> M. Davidovich-Pinhas, O. Harari and H. Bianco-Peled, *J. Control. Release*, **136**, 38 (2009).
- <sup>17</sup> A. H. Krauland, D. Guggi and A. Bernkop-Schnürch, *Int. J. Pharm.*, **307**, 270 (2006).
- <sup>18</sup> R. Nogueira, M. Lammerhofel, N. Maier and M. W. Lindner, *Anal. Chem. Acta*, **533**, 179 (2005).
- <sup>19</sup> I. O. Egwim and H. J. Gruber, *Anal. Biochem.*, **288**, 188 (2001).
- <sup>20</sup> A. Bernkop-Schnürch, A. H. Krauland, V. M. Leitner and T. Palmberger, *Eur. J. Pharm. Biopharm.*, **58**, 253 (2004).
- <sup>21</sup> M. Greindl and A. Bernkop-Schnürch, *Pharm. Res.*, **23**, 2183 (2006).
- <sup>22</sup> A. Greimel, M. Werle and A. Bernkop-Schnürch, *J. Pharm. Pharmacol.*, **59**, 1191 (2007).
- <sup>23</sup> A. B. Jindal, M. N. Wasnik and H. A. Nair, *Indian J. Pharm. Sci.*, **72**, 766 (2010).
- <sup>24</sup> D. Guggi, M. Marschütz and A. Bernkop-Schnürch, *Int. J. Pharm.*, **274**, 97 (2004).
- <sup>25</sup> B. Das, S. Dutta, A. K. Nayak and U. Nanda, *Int. J. Biol. Macromol.*, **70**, 506 (2014).
- <sup>26</sup> C. Gray and J. Dowsett, *Biotechnol. Bioeng.*, **31**, 607 (1988).
- <sup>27</sup> C. M. Silva, A. J. Ribeiro, I. V. Figueiredo, A. R. Gonçalves and F. Veiga, *Int. J. Pharm.*, **311**, 1 (2006).
- <sup>28</sup> S.-C. Chen, Y.-C. Wu, F.-L. Mi, Y.-H. Lin, L.-C. Yu *et al.*, *J. Control. Release*, **96**, 285 (2004).
- <sup>29</sup> G. T. Grant, E. R. Morris, D. A. Rees, P. J. C. Smith and D. Thom, *FEBS Lett.*, **32**, 195 (1973).